

Original Contribution

Using Gene Transcription to Assess Ecological and Anthropological Stressors in Brown Bears

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Abstract: Increasingly, population- and ecosystem-level health assessments are performed using sophisticated molecular tools. Advances in molecular technology enable the identification of synergistic effects of multiple stressors on the individual physiology of different species. Brown bears (*Ursus arctos*) are an apex predator; thus, they are ideal candidates for detecting potentially ecosystem-level systemic perturbations using molecular-based tools. We used gene transcription to analyze 130 brown bear samples from three National Parks and Preserves in Alaska. Although the populations we studied are apparently stable in abundance and exist within protected and intact environments, differences in transcript profiles were noted. The most prevalent differences were among locations. The transcript patterns among groups reflect the influence of environmental factors, such as nutritional status, disease, and xenobiotic exposure. However, these profiles also likely represent baselines for each unique environment by which future measures can be made to identify early indication of population-level changes due to, for example, increasing Arctic temperatures. Some of those environmental changes are predicted to be potentially positive for brown bears, but other effects such as the manifestation of disease or indirect effects of oceanic acidification may produce negative impacts.

Keywords: Brown bear, *Ursus arctos*, Gene transcription

INTRODUCTION

The use of sophisticated molecular tools is changing the nature of population- and ecosystem-level health assessments. Although cells and populations are vastly different scales of measure, the earliest observable signs of health impairment are altered levels of gene transcripts, evident prior to clinical manifestation (McLoughlin et al. 2006).

Thus, gene transcription provides an early warning of potentially compromised health as well as indication of environmental stressors affecting individuals, populations, and possibly ecosystems (McLoughlin et al. 2006; Bowen et al. 2012; Miles et al. 2012; Bowen et al. 2015). Advances in molecular technology enable the identification of synergistic effects of multiple stressors on the individual physiology of different species (i.e., lingering oil and nutritional deficits in sea otters, direct and indirect effects of climate change on polar bears) (Bowen et al. 2012; Miles et al. 2012; Bowen et al. 2015). As such, the analysis of the

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transcription component of gene expression, by which information from the DNA template of a particular gene is transcribed into messenger RNA (mRNA), can be particularly useful in identifying the mechanisms of subtle but potentially deleterious effects of disturbances on wildlife. The amount of mRNA transcribed is physiologically driven by the extent of intrinsic and extrinsic stimuli such as infectious agents, toxins, toxic exposure, trauma, or neoplasia.

The genes we chose to use in this study can be grouped into functional categories that reflect the influence of environmental factors, such as nutritional status, disease, and xenobiotic exposure. Varying ranges of geometric means of transcript levels can be interpreted in several ways. Genes with larger ranges may be subject to greater environmental variation in a particular system than genes with smaller ranges. However, the physiology of the genes themselves may play a part in that some gene transcripts are more transient than others (Xu and Cao 2010; Moll et al. 2011).

Population-level investigations using gene transcription are generally conducted in response to an apparent event (i.e., chemical spill, population decline) (Bowen et al. 2012; Dubansky et al. 2013; Bowen et al. 2015). Difficulties with this approach are multifaceted. Although gene transcription analyses can provide advanced identification of potential ecological and immunologic drivers, the post-event timing of the investigation may prohibit clear insight into the ecological pathways involved. During a population-level crisis or event, organisms are clearly at the latter stages of a succession of physiological, biochemical, or immunologic events triggered by one or more causative incidents. In addition, missing from the post-event analysis is the ability to assess apparent perturbations in real time, prior to clinical manifestations. We may gain considerably in our ability to understand why species are at risk if we take a different approach—using a model of baseline and long-term monitoring of sensitive molecular parameters (e.g., gene transcription patterns) to continually assess populations for subtle yet significant changes. This would also allow for the population to act as its own “baseline” or “reference.” Finding suitable (i.e., disease- and stimulus-free) free-ranging or captive animals to act as reference animals can be problematic (Bowen et al. 2015). Additionally, a model of continual assessment can help refine the direction of further research efforts in a timely manner.

Brown bears (*Ursus arctos*) are an apex predator; thus, they are ideal candidates for detecting potentially ecosys-

tem-level systemic perturbations using transcript-based tools. Our study provided the opportunity to understand gene transcription patterns among distinct brown bear populations found in three National Parks in Alaska: Gates of the Arctic National Park and Preserve (Gates), Lake Clark National Park and Preserve (Lake Clark), and Katmai National Park and Preserve (Katmai). Our goals included: (1) the first application of gene transcription to brown bears; (2) establishing baselines for these populations; as well as (3) comparisons among these populations to highlight conditions or constraints that were capable of producing a deleterious effect in one or more of the Parks.

MATERIALS AND METHODS

Study Areas

All samples were obtained opportunistically for a study with an original intent other than gene transcription analysis. We conducted our studies in Gates (2014–2016), Katmai (2015–2016), and Lake Clark (2015–2016) (Fig. 1). Gates is an interior arctic ecosystem characterized by the mountainous terrain of the Brooks Range, spruce forests (*Picea* spp.), and lowland riparian areas (Wilson et al. 2014). Primary food resources include moose (*Alces alces*), caribou (*Rangifer tarandus*), Dall’s sheep (*Ovis dalli*), small mammals, berries and herbaceous vegetation, as well as runs of chum salmon (*Oncorhynchus keta*) and Chinook salmon (*O. tshawytscha*) from mid-July to early September (Hilderbrand et al., *in review*). In Katmai, our study focused on the coastal portion of the park, bounded by the Aleutian Range to the west and the Gulf of Alaska to the east. Brown bears in Katmai consume marine mammals, marine invertebrates, salmon [May–October; chum salmon, Chinook salmon, sockeye salmon (*O. nerka*), and coho salmon (*O. kisutch*)], flounder (*Platichthys stellatus*), sedge (*Carex* spp.), goose tongue (*Plantago maritima*), and berries (Hilderbrand et al., *in review*). Our Lake Clark study area includes the Chigmit Mountains, lowland spruce forests, riparian areas, and subarctic tundra (Stanek et al. 2017). Lake Clark brown bears primarily eat moose, caribou, Dall’s sheep, small mammals, berries, and Bristol Bay salmon (May–October) Mangipane et al. *in press*. Salmon are more abundant and widely distributed in Katmai and Lake Clark than in Gates. Further, more salmon species are present in Lake Clark and Katmai than Gates (Alaska Department of Fish and Game Anadromous Waters Cata-

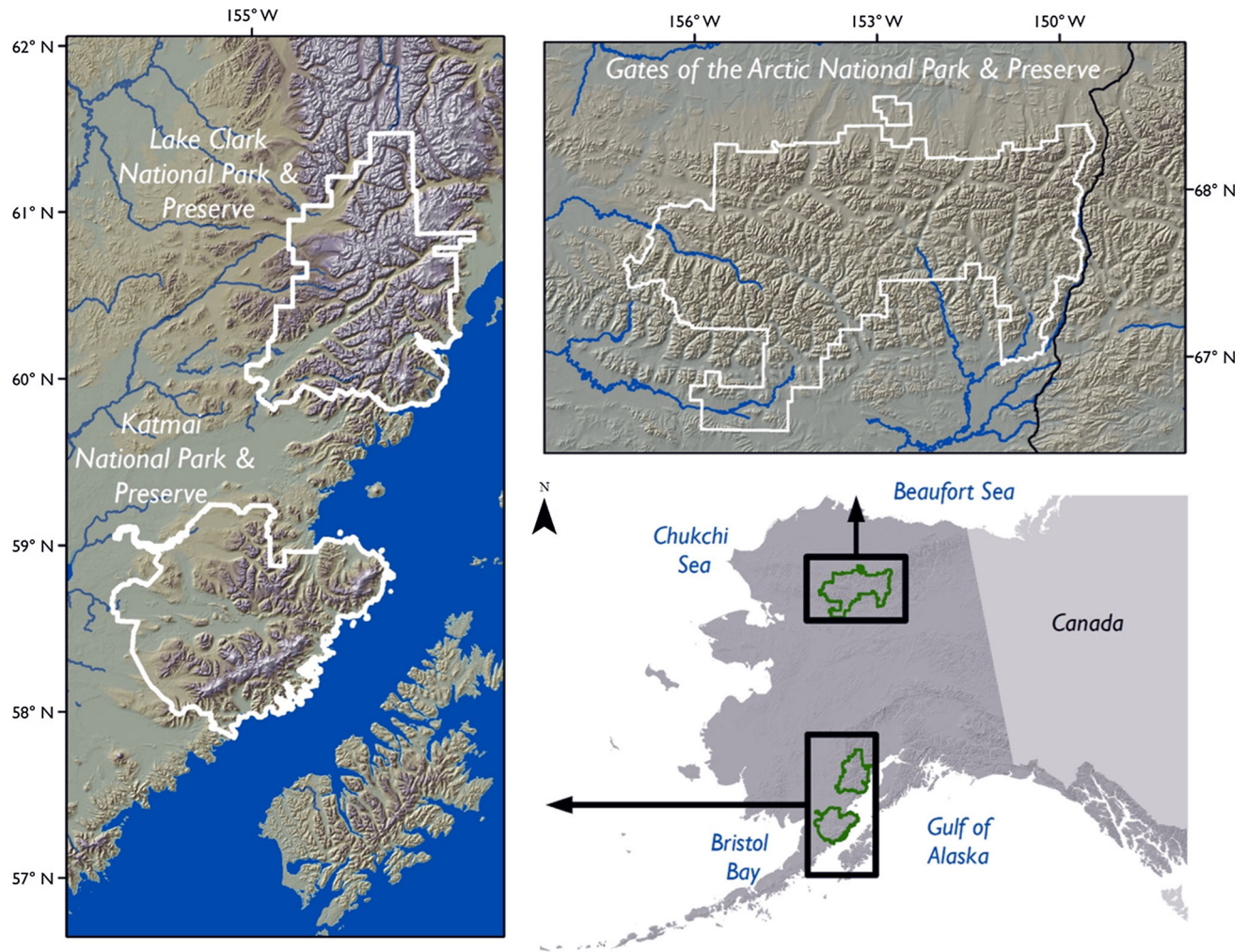


Figure 1. Map of sampling locations in Alaska including Gates National Park and Preserve, Lake Clark National Park and Preserve, and Katmai National Park and Preserve.

log <https://www.adfg.alaska.gov/sf/SARR/AWC/Catalog/>). In terms of anthropogenic stressors, Gates and Lake Clark are both adjacent to two large but undeveloped ore deposits and the Katmai coast was oiled during the 1989 Exxon Valdez grounding (Sellers and Miller 1999).

Animals

A total of 130 brown bears were sampled in Gates, Lake Clark, and Katmai between 2014 and 2016 (Table 1). Bears were anesthetized via darting from a helicopter using a 1:1 mixture of tiletamine hydrochloride and zolazepam hydrochloride (Telazol, Fort Dodge Laboratories, Fort Dodge, Iowa, USA) at a dose rate of 10–12 mg/kg (Taylor et al. 1989). Sex for each bear was recorded, and bear age was estimated based on tooth wear, with an accuracy of 0.26 ± 2.74 (1 SD) years (Hilderbrand et al., in review). Each female bear was classified at the

time of capture as either alone, with cubs of the year, with yearlings, or with older cubs. Our capture and handling procedures were approved by the Institutional Animal Care and Use Committees of the National Park Service (AKR_KATM_Hilderbrand_BrownBear_2014, AKR_LACL_Mangipane_BrownBear_2014, AKR_GAAR_Gustine_GrizzlyBear_2014), and the U.S. Geological Survey, Alaska Science Center (2014-01, 2015-04, 2015-06).

Blood Collection and RNA Extraction

A 2.5 ml sample from each brown bear was drawn directly into a PAXgene blood RNA collection tube (PreAnalytiX, Switzerland) from the cephalic vein and then frozen at -20°C until extraction of RNA. Rapid RNA degradation and induced transcription of certain genes after blood extraction have led to the development of methodologies

Table 1. Number of Brown Bears Sampled in Gates of the Arctic National Park and Preserve (Gates), Lake Clark National Park and Preserve (Lake Clark), and Katmai National Park and Preserve (Katmai), Separated by Year, Season, and Sex.

Location	Year	Season	Sex	N	# Bears with multiple samples
Gates	2015	Spring	M	6	11
			F	18	
	2016	Spring	M	8	
			F	13	
Lake Clark	2014	Fall	M	4	7
			F	16	
	2015	Fall	M	2	
			F	4	
		Spring	M	9	
			F	6	
	2016	Spring	M	6	
			F	7	
Katmai	2015	Fall	M	0	7
			F	3	
		Spring	M	1	
			F	8	
		Summer	M	0	
			F	6	
	2016	Spring	M	1	
			F	12	

for preserving the RNA transcription profile immediately after blood is drawn. The PAXgene tube contains a blend of RNA stabilizing reagents that protect RNA molecules from degradation by RNases and prevents further induction of gene transcription. Without this stabilization, copy numbers of individual mRNA species in whole blood can change more than 1000-fold during storage and transport. The RNA from blood in PAXgene tubes was isolated according to manufacturer's standard protocols, which included an on-column DNase treatment to remove contaminating gDNA (silica-based microspin technology), and the extracted RNA was stored at -80°C until analysis. All RNA was quantified using a Qubit 2.0 Fluorometer.

cDNA Synthesis

A standard cDNA synthesis was performed on 2 g of RNA template from each animal. Reaction conditions included 4 units reverse transcriptase (Omniscript, Qiagen, Valencia, CA), 1 M random hexamers, 0.5 mM each dNTP, and 10 units RNase inhibitor, in RT buffer (Qiagen, Valencia, CA). Reactions were incubated for 60 min at 37°C , followed by

an enzyme inactivation step of 5 min at 93°C , and then stored at -20°C until further analysis.

Targeted Genes

The 14 genes targeted in our study represent multiple physiological systems that play a role in immuno-modulation, inflammation, cell protection, tumor suppression, cellular stress response, xenobiotic metabolizing enzymes, and antioxidant enzymes. These genes can be modified by biological, physical, or anthropogenic impacts and consequently provide information on the general type of stressors present in a given environment (Table 2).

PCR Primers

Degenerate primers were designed based upon multi-species alignments (GenBank) as previously described (Bowen et al. 2007). Briefly, degenerate primer pairs developed for the brown bear were utilized on cDNA from three randomly selected brown bear samples. Degenerate primer pairs were designed to amplify 14 genes of interest and one ribosomal

Table 2. Documented Function of 14 Genes Identified in Free-Ranging Brown Bears Sampled at Gates of the Arctic National Park and Preserve (Gates), Lake Clark National Park and Preserve (Lake Clark), and Katmai National Park and Preserve (Katmai).

Gene	Gene function
CaM	Calmodulin (CaM) is a small acidic Ca^{2+} -binding protein that activates various Ca^{2+} -dependent enzyme reactions, thereby modulating a wide range of cellular events, including metabolism control, muscle contraction, exocytosis of hormones and neurotransmitters, and cell division and differentiation (Chen et al. 2012)
MHC DRB	A component of the major histocompatibility complex, the DRB class II gene is responsible for the binding and presentation of processed antigen to T_H lymphocytes, thereby facilitating the initiation of an immune response (Goldsby et al. 2003; Bowen et al. 2006). Up-regulation of MHC genes has been positively correlated with parasite load (Wegner et al. 2006), whereas down-regulation of MHC has been associated with contaminant exposure (Dong et al. 1997)
Interleukin 1 beta (IL1B)	Inflammatory mediator (Parham 2015)
HSP70	The heat-shock protein 70 is produced in response to infection, inflammation, exercise, exposure of the cell to toxins (ethanol, arsenic, trace metals, and ultraviolet light, among many others), starvation, hypoxia (oxygen deprivation), thermal or other stress (Iwama et al. 1999; Tsan and Gao 2004). Heat-shock proteins also act as molecular chaperones (De Maio 1999)
AHR	The aryl hydrocarbon receptor responds to polycyclic aromatic hydrocarbons, polyhalogenated hydrocarbons, dibenzofurans, and dioxin (Oesch-Bartlomowicz and Oesch 2005). AHR signaling can modulate T-regulatory (T_REG) (immune-suppressive) or T-helper type 17 ($\text{T}_\text{H}17$) (pro-inflammatory) immunologic activity (Quintana et al. 2008; Veldhoen et al. 2008)
CCR3	The chemokine receptor 3 binds at least seven different chemokines and is expressed on eosinophils, mast cells (MC), and a subset of Th cells (Th2) that generate cytokines implicated in mucosal immune responses (Gurish et al. 2002; Kringel et al. 2006). Up-regulation of CCR3 occurs in the presence of parasites (Gurish et al. 2002; Kringel et al. 2006)
TGF-beta (TGFb)	Transforming growth factor beta. Immunosuppressive cytokine (Parham 2015)
COX2	Cyclooxygenase-2 catalyzes the production of prostaglandins that are responsible for promoting inflammation (Goldsby et al. 2003). Cox2 is responsible for the conversion of arachidonic acid to prostaglandin H ₂ , a lipoprotein critical to the promotion of inflammation (Harris et al. 2002). Up-regulation of Cox2 is indicative of cellular or tissue damage and an associated inflammatory response
THRa	Increases in thyroid hormone receptor transcription could indicate exposure to organic compounds including PCBs and associated potential health effects such as developmental abnormalities and neurotoxicity (Tabuchi et al. 2006). Hormone-activated transcription factors bind DNA in the absence of hormone, usually leading to transcriptional repression (Tsai and O'Malley 1994)
Tbet	A Th1-specific T box transcription factor that controls the expression of the hallmark Th1 cytokine, IFN gamma (Parham 2015)
Gata3	A Th2-specific transcription factor controls transcription of cytokines Il-4, 5, and 13 (Parham 2015)
CD69	Earliest inducible cell surface glycoprotein acquired during lymphoid activation. Involved in lymphocyte proliferation (Parham 2015)
Mx1	The Mx1 gene responds to viral infection, characterized by the induction and secretion of cytokines that mediate an antiviral state (Kibenge et al. 2005; Tumpey et al. 2007)
IL17	Interleukin 17 is a pro-inflammatory cytokine that responds to invasion by extracellular pathogens and induces destruction of the pathogen's cellular matrix. Excessive production of IL17 has been implicated in autoimmune disease (Parham 2015)
S9	Ribosomal subunit S9. Reference Gene

Amplification efficiencies of all primer pairs were between 90 and 105%.

housekeeping gene (Bowen et al. 2007). PCR amplifications using these primers were performed on 20 ng of each cDNA sample in 50 µl volumes containing 20–60 pmol of each primer, 40 mM Tris-KOH (pH 8.3), 15 mM KOAc, 3.5 mM Mg (OAc)₂, 3.75 µg/ml bovine serum albumin (BSA), 0.005% Tween-20, 0.005% Nonidet-P40, 200 µM each dNTP, and 5U of Advantage 2 Taq polymerase (Clontech, Palo Alto, CA). The PCR was performed on an MJ Research PTC-200 thermal cycler (MJ Research, Watertown, MA) and consisted of 1 cycle at 94 °C for 3 min, and then 40 cycles at 94 °C for 30 s, at 60 °C for 30 s, and 72 °C for 2 min, with a final extension step of 72 °C for 10 min. The products of these reactions were electrophoresed on 1.5% agarose gels and resulting bands visualized by ethidium bromide staining. Definitive bands representing PCR products of a predicted base pair size of the targeted gene were excised from the gel and extracted and purified using a commercially available nucleic acid-binding resin (Qiaex II Gel extraction kit, Qia-gen, Valencia, CA).

Nucleotide sequences of isolated fragments were determined by dideoxy nucleotide methodology using an automated sequencer (Model 373; Applied Biosystems, Foster City, CA). Nucleotide sequences of the PCR products were analyzed using Align and Contig sequence alignment soft-

ware programs (Vector NTI; Informax Inc, North Bethesda, MD) and compared to known sequences using the NCBI BLAST program (Altschul et al. 1990), and the IMGT/HLA database (Robinson et al. 2001). Primer pairs appropriate for real-time PCR were designed based on the elucidated brown bear sequences for each gene (Table 3).

Real-Time PCR

Real-time PCR systems for the individual, brown bear-specific reference or housekeeping gene (S9) and genes of interest were run in separate wells. Briefly, 1 l of cDNA was added to a mix containing 12.5 l of QuantiTect Fast SYBR Green Master Mix [5 mM Mg²⁺] (Qiagen, Valencia, CA), 0.5 µl each of forward and reverse sequence specific primers, and 10.5 µl of RNase-free water; total reaction mixture was 25 µl. The reaction mixture cDNA samples for each gene of interest and the S9 gene were loaded into MicroAmp Fast Optical 96-well reaction plates in duplicate and sealed with optical sealing tape (Applied Biosystems, Foster City, CA). Reaction mixtures containing water, but no cDNA, were used as negative controls; thus, approximately three individual brown bear samples were run per plate.

Table 3. Brown Bear-Specific Quantitative Real-Time Polymerase Chain Reaction Primers Used in the Analysis of free-Ranging Brown Bears in Gates of the Arctic National Park and Preserve (Gates), Lake Clark National Park and Preserve (Lake Clark), and Katmai National Park and Preserve (Katmai).

Gene	Forward Primer	Sequence (5' 3')	Reverse Primer	Sequence (5' 3')
<i>AHR</i>	Urar spp. AHR F	ATGGAAAGGAGCTTCGTGTG	Urar spp. AHR R	AGGTGGAAGTATTGATCCATCT
<i>HSP70</i>	Urar spp. HSP70 F	GCACCTTCGACGTGTCCAT	Urar spp. HSP70 R	CACGAAGTGGTTCCACCAGC
<i>IL1B</i>	Urar spp. IL1B F	GAATCTGTACCTGTCCTGTG	Urar spp. IL1B R	GTCTTGTGAAGACAAATCGCT
<i>MX1</i>	Urar spp. MX1 F	GCTGGCTTACCGTCAGGAG	Urar spp. MX1 R	CTGCAGCAGCTGCAGCATG
<i>CD69</i>	Urar spp. CD69 F	TCAGATGATTGGATTGGATAC	Urar spp. CD69 R	TCAGAATCAATGAACGCAAGAG
<i>CaM</i>	Urar spp. CaM F	ACAGTGAAGAAGAAATTCGCGA	Urar spp. CaM R	CATCTACTTCTTCATCTGTTAGT
<i>COX2</i>	Urar spp. COX2 F	CAGTCTCTTAACGAGTATCGC	Urar spp. COX2 R	GCTCCATGGCATCAATATCAC
<i>CCR3</i>	Urar spp. CCR3 F	GTCTTGCTCTGCCTCTGCT	Urar spp. CCR3 R	ATGAGCCGGATGGCCTTGTA
<i>MHC DRB</i>	Urar spp. DRB F	CACCAACGGCACGGAGCG	Urar spp. DRB R	TGGTTGAAGTACTCAGCGATG
<i>IL-17</i>	Urar spp. IL-17 F	ACTCCAGAAGGCCCTCAGA	Urar spp. IL-17 R	TACAGTCCCTTCAGCACTGA
<i>IFNG</i>	Urar spp. IFNG F	GACACCATCAAGGAGGACAT	Urar spp. IFNG R	TCGCTTTGCGCTGAACCTG
<i>THRa</i>	Urar spp. THRa F	ATGATCCGATCACTGCAGCA	Urar spp. THRa R	GTGACTGGCCGATGTCATC
<i>TGFb</i>	Urar spp. TGFb F	CGAGCCTGAGGCGGACTA	Urar spp. TGFb R	GCACTGCTTCTCGGAGCTC
<i>Tbet</i>	Urar spp. Tbet F	CACCATGTCCTACTACCGAG	Urar spp. Tbet R	TCTGAAGCTCCAGGACCAG
<i>GATA3</i>	Urar spp. GATA3 F	CACTCCAGCCCGCACCTC	Urar spp. GATA3 R	GCACTCCTTCTCGTCCTGC
<i>S9</i>	Urar spp. S9 F	GAGTTGAAGCTGATCGGCGA	Urar spp. S9 R	GTCCTTCTCGTCCAGCGTC

See Table 1 for interpretation of gene abbreviations.

Amplifications were conducted on a Step One Plus Real-time Thermal Cycler (Applied Biosystems, Foster City, CA) using Step One Software V. 2.2.2. Reaction conditions were as follows: 95 °C for 20 s, 40 cycles of 95 °C for 3 s, and 60 °C for 30 s. The melt curve consists of 95 °C for 15 s, 60 °C for 1 min, 0.3 °C per second temperature increase, and then 95 °C for 15 s. Reaction specificity was monitored by melting curve analysis using a final data acquisition phase during a step and hold process of a 0.3 °C per second temperature increase from 60 °C to 95 °C. Cycle threshold crossing values (C_T) for the genes of interest were normalized to the S9 housekeeping gene.

Statistical Analyses

Mixed effects statistical models were used to simultaneously estimate and account for multiple influences on gene transcription levels, which make them appropriate for the multiple uncontrolled factors that occurred as a result of opportunistic samples acquired for this study. Parameter estimates for all model effects were calculated using the *lme4* package in R 2.8.1 (R Development Core Team 2012).

RESULTS

Geometric Means Tables

Elevated geometric mean transcript values (as indicated by low C_T values) were apparent at Gates for five genes, Lake Clark for seven genes, and at Katmai for four genes (Tables 4, 5). The largest ranges of geometric means across genes (most variable transcription) were identified for IFN- γ , IL17, DRB, and MX1, while the smallest ranges occurred for AHR, CD69, and Gata3. The largest ranges of geometric means within a park were identified for Gates in 10 out of 15 genes.

Elevated geometric mean transcript values (as indicated by low C_T values) were apparent in females without cubs for two genes, females with cubs for one gene, and in males for 12 genes 271 (Table 4). The largest ranges of geometric means across genes (most variable transcription) were identified for IFN- γ , Tbet, Gata3, and MX1, while the smallest ranges occurred for CaM and IL1 β . The largest ranges of geometric means within a group were identified for females with cubs in 12 out of 15 genes (Table 5).

Table 4. Geometric Mean ($\pm$ 95% Confidence Interval) Normalized (to the S9 Housekeeping Gene in Each Animal) Cycle Threshold (C_T) Transcription Values for Targeted Genes (See Table 1) in Brown Bears, Gates of the Arctic National Park and Preserve (Gates), Lake Clark National Park and Preserve (Lake Clark), and Katmai National Park and Preserve (Katmai; 2014–2016).

	<i>CaM</i>	<i>Cox2</i>	<i>IFN-γ</i>	<i>AHR</i>	<i>HSP70</i>	<i>IL17</i>	<i>IL1B</i>	<i>CD69</i>	<i>DRB</i>	<i>MX1</i>	<i>CCR3</i>	<i>THRa</i>	<i>TGFb</i>	<i>Tbet</i>	<i>Gata3</i>
All															
Geo mean	1.95	7.82	12.14	5.40	5.23	16.81	4.96	8.69	5.72	14.20	6.58	8.02	4.72	6.35	7.34
95% LCL	1.77	7.58	11.64	5.20	4.71	16.04	4.77	8.47	5.34	13.80	6.35	7.82	4.54	6.04	7.00
95% UCL	2.14	8.08	12.67	5.60	5.81	17.61	5.15	8.92	6.13	14.61	6.80	8.23	4.91	6.67	8.39
Lake Clark															
Geo mean	1.81	7.73	10.98	5.33	5.14	18.22	4.49	8.12	5.90	13.85	6.56	7.89	4.41	5.62	7.06
95% LCL	1.52	7.43	10.58	5.12	4.32	16.65	4.19	7.93	5.39	13.52	6.28	7.72	4.24	5.28	6.83
95% UCL	2.15	8.05	11.41	5.55	6.13	19.93	4.82	8.32	6.45	14.11	6.85	8.07	4.59	5.98	7.25
Gates															
Geo mean	2.23	8.02	13.63	5.16	4.82	15.15	5.45	9.10	5.01	15.03	6.51	7.83	4.89	7.36	7.78
95% LCL	1.98	7.72	12.51	4.91	3.97	14.53	5.18	8.86	4.39	14.43	6.20	7.57	4.64	6.79	7.53
95% UCL	2.51	8.33	14.86	5.43	5.86	15.79	5.73	9.34	5.71	15.64	6.82	8.10	5.15	7.98	8.03
Katmai															
Geo mean	1.81	7.38	11.88	5.57	6.11	16.67	5.12	8.79	6.70	13.34	6.39	8.20	4.75	6.02	7.89
95% LCL	1.45	7.02	11.07	5.36	5.20	15.27	4.79	8.53	5.83	12.27	6.16	8.02	4.54	5.73	7.64
95% UCL	2.25	7.75	12.75	5.78	7.18	18.19	5.47	9.06	7.71	14.51	6.63	8.38	4.97	6.33	8.14

Note that the smaller the mean value, the higher the level of transcription. Italics denote significant difference among groups.

Table 5. Geometric Mean ($\pm$ 95% Confidence Interval) Normalized (to the S9 Housekeeping Gene in Each Animal) Cycle Threshold (C_T) Transcription Values for Targeted Genes (See Table 1) in Gates of the Arctic National Park and Preserve (Gates) Brown Bears: Females Without Cubs (F0), Females with Cubs (F1), and Males (M).

		CaM	<i>Cox2</i>	IFN- γ	<i>AHR</i>	HSP70	IL17	IL1B	<i>CD69</i>	DRB	<i>MX1</i>	CCR3	<i>THRa</i>	TGFb	<i>Tbet</i>	Gata3
F0	Geo mean	2.36	8.04	13.71	5.25	5.15	15.46	5.53	8.97	5.38	14.98	6.37	8.00	4.97	7.82	7.94
F0	95% LCL	2.06	7.62	12.34	4.96	4.17	14.48	5.17	8.71	4.51	14.51	6.01	7.73	4.70	7.19	7.70
F0	95% UCL	2.70	8.50	15.23	5.57	6.34	16.50	5.92	9.25	6.43	15.46	6.76	8.27	5.25	8.50	8.19
F1	Geo mean	2.30	10.41	17.29	6.91	5.76	16.36	5.37	11.32	4.66	18.98	8.19	9.86	6.48	9.95	4.92
F1	95% LCL	1.78	6.00	10.97	3.20	4.53	11.82	4.81	6.83	2.86	12.98	4.09	5.56	2.87	5.30	1.46
F1	95% UCL	2.96	18.08	27.25	14.95	7.33	22.66	5.99	18.74	7.58	27.74	16.37	17.51	14.62	18.70	16.63
M	Geo mean	2.00	7.83	12.88	5.00	3.90	14.81	5.31	9.26	4.38	14.37	6.83	7.50	4.79	6.39	7.45
M	95% LCL	1.46	7.30	10.81	4.42	2.32	13.99	4.75	8.66	3.43	13.64	6.19	6.83	4.22	5.29	6.85
M	95% UCL	2.73	8.40	15.36	5.65	6.56	15.68	5.92	9.91	5.59	15.15	7.54	8.24	5.44	7.71	8.10

Note that the smaller the mean value, the higher the level of transcription. Italics denote significant difference among groups.

Table 6. Mixed effects Statistical Models (Corrected for Multiple Tests) were Used to Simultaneously Estimate and Account for Multiple Influences on Gene Transcription Levels.

	CaM	<i>Cox2</i>	IFN- γ	<i>AHR</i>	HSP70	IL17	IL1B	<i>CD69</i>	DRB	<i>MX1</i>	CCR3	<i>THRa</i>	TGFb	<i>Tbet</i>	Gata3
Location	0.02	0.03	0.00			0.00	0.02	0.02	0.02	0.00				0.00	
Sex		0.03					0.03			0.05					
Season			0.01	0.01	0.03										
Year	0.00	0.03	0.03		0.02		0.00				0.01		0.01		

We used linear mixed effects models to analyze the influence of location, sex, season, and year on each gene transcription level. Parameter estimates for all model effects were calculated using the lme4 package in R 2.8.1 (R Development Core Team 2012). Significance was determined at $P \leq 0.05$.

Table 7. Mixed Effects Statistical Models (Corrected for Multiple Tests) were Used to Simultaneously Estimate and Account for Multiple Influences on Gene Transcription Levels.

	CaM	<i>Cox2</i>	IFN- γ	<i>AHR</i>	HSP70	IL17	IL1B	<i>CD69</i>	DRB	<i>MX1</i>	CCR3	<i>THRa</i>	TGFb	<i>Tbet</i>	Gata3
Breeding status		0.03		0.05				0.04		0.00		0.04		0.02	
Year	0.01				0.02		0.02		0.04						

We used linear mixed effects models to analyze the influence of breeding status (females without cubs, females with cubs, males) and year on each gene transcription level. Parameter estimates for all model effects were calculated using the lme4 package in R 2.8.1 (R Development Core Team 2012). Significance was determined at $P \leq 0.05$.

Mixed Effects Models

Mixed effects model analysis of all brown bears sampled identified effects of location (the Parks), sex, season, year on transcript levels of 15 genes (Table 6). Nine of the 15 genes analyzed (CaM, COX2, IFN- γ , IL17, IL1B, CD69, DRB, MX1, and Tbet) differed significantly among locations. Seven genes (CaM, COX2, IFN- γ , HSP70, IL1B,

CCR3, and TGFb) differed by sample year. The genes COX2, IL1B, and MX1 differed between sexes, whereas IFN- γ , AHR, and HSP70 differed among seasons.

Mixed effects model analysis of Gates bears in three different sex/reproductive classes (females with or without cubs and males) indicated significant effects associated with year and reproductive state. Significance of sample year

aligned with four genes (CaM, HSP70, IL1B, and DRB), whereas significance of reproductive status aligned with six genes (COX2, AHR, CD69, MX1, THRa, and Tbet). Transcript values of 11 of the 15 genes were lowest in females with cubs (Table 7).

Mixed effects model analysis of Lake Clark brown bears showed influences of sex (COX2, CD69, DRB), season (CaM, IFNG, AHR, IL1B, CD69, MX1, CCR3, THRa, TGFb, Tbet, Gata3), and year (CaM, COX2, AHR, CD69, CCR3, THRa, TGFb, Gata3) on transcript profiles. Mixed effects model analysis of Katmai brown bears was also assessed; sex (THRa), season (AHR, HSP70), and year (IL17, CCR3) influenced transcription of a few genes.

DISCUSSION

Our study represents the first analyses of gene transcript profiles in multiple brown bear populations. Transcript levels differed among years, seasons, sexes, and locations. The most prevalent differences were among locations (i.e., Gates, Lake Clark, Katmai). The transcript differences among locations probably reflect the influence of environmental factors, such as nutritional status, disease, and possibly xenobiotic exposure.

Seven of the nine genes exhibiting significantly different transcription among locations had lower transcription in Gates bears (CaM, Cox2, IFNg, IL1B, CD69, MX1, and Tbet). Low levels of transcripts have been associated with food limitation or nutritional deficiency in sea otters (Bowen et al. 2016) as well as in mice experiencing a nutritional deficit (Saucillo et al. 2014). Alternatively, low levels of transcription may be the result of unbalanced physiological resource allocation. For example, immune defenses exist to impede infections, but other ecological demands can supersede this, causing immune defenses to be compromised (Martin et al. 2010). Continued transcription of genes responsible for immunologic function can be physiologically costly and often require the reallocation of nutrients and energy from one portion of an individual's resource budget to other metabolic functions (Graham et al. 2010). In situations where physiological resources are limited (i.e., nutritional deficit), reallocation of resources may not be possible, leaving the individual susceptible to pathogens and other stressors (Graham et al. 2010; Martin et al. 2010). Brown bears in Gates are the most nutritionally limited of the three study populations and are characterized by lower structural size and body fat reserves than bears

from Lake Clark or Katmai (Hilderbrand et al., *in review*). Our results add another line of evidence that Gates bears are the most physiologically challenged of the bears we studied.

Not surprisingly, transcriptional differences among bears within populations were evident. In Gates, females with cubs had an overall pattern of lower transcription in comparison with other bears from Gates. Studies have shown that females with cubs have decreased body and lean mass than females without cubs, possibly explaining the pattern observed (Hilderbrand et al. 2000). Based on these results and supporting evidence from Hilderbrand et al. (*in review b*), we posit that female brown bears with cubs in Gates are right at the margin of meeting their energetic demands.

Transcriptional similarities across different groups sampled are often just as noteworthy as differences (Bowen et al. 2016). For example, although one might anticipate transcript profiles in Katmai bears to be consistent with lingering oil from the *Exxon Valdez* oil spill in 1989 transcript profiles (i.e., higher transcribed levels of AHR, COX2, HSP70, IL1B) (Bowen et al. 2012), these differences were not found. In this case, although lingering oil may persist along the Katmai coast, it does not appear to be affecting bears strongly enough to be detected by gene transcription analyses.

However, without an established reference range, or inclusion of environmental or other biological variables, interpretation of these differences must be made with caution. While significant differences of particular genes were identified among locations, all of the transcript values measured may be within "normal" boundaries. Monitoring populations prior to the onset of clinical manifestation would negate in part the requirement for a "reference" data set (Bowen et al. 2015). Proactive longitudinal studies on wildlife species at risk could provide baseline data sets upon which apparent perturbations in real time could be assessed.

Establishment of a normal reference range for gene transcript panels is critical for use in wildlife and ecosystem health monitoring (Mancia et al. 2008). Although the populations we studied are apparently stable and exist within a relatively pristine environment (Hilderbrand et al. 2013), use of the transcription profiles measured as baseline as opposed to reference should be made with caution as determination of "normal" in any animal, particularly wildlife, is problematic. This determination is usually a relative one, with the findings in one individual or popu-

lation being compared and contrasted with reference standards developed from similar populations (McLoughlin 2006). However, longitudinal studies on these populations with comparisons to these initial results should be made to identify early indications of populations-level changes. For example, increasing Arctic temperatures may create more available forage like deciduous plants, salmon, and moose (Welch et al. 1998; Elmendorf et al. 2012; Joly et al. 2012; Van der Wal and Stien 2014). These changes are predicted to have potentially positive effects (e.g., Nielsen et al. 2013) on bear populations, but other effects such as the manifestation of disease or cascading effects of oceanic acidification (i.e., decreased forage availability) may produce negative impacts (Burek et al. 2008).

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AUTHOR CONTRIBUTIONS

All authors contributed equally to the project.

COMPLIANCE WITH ETHICAL STANDARDS

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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