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Enhanced biological processes associated with alopecia in polar bears (*Ursus maritimus*)

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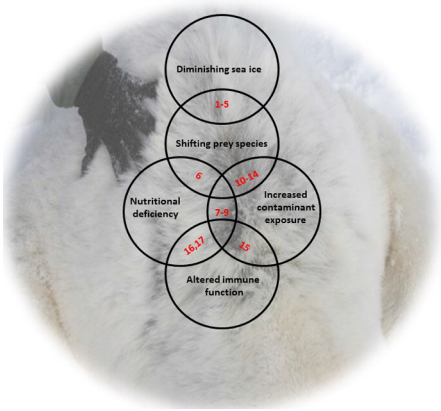
HIGHLIGHTS

- We investigate potential causes of alopecia in polar bears.
- Transcriptomics show enhanced immune and stress responses and viral defense.
- Alternative method of investigation can serve as early warning indicator.
- Advanced technology allow for holistic system of surveillance and investigation.

GRAPHICAL ABSTRACT



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ABSTRACT

Populations of wildlife species worldwide experience incidents of mass morbidity and mortality. Primary or secondary drivers of these events may escape classical detection methods for identifying microbial insults, toxin exposure, or additional stressors. In 2012, 28% of polar bears sampled in a study in the southern Beaufort Sea region of Alaska had varying degrees of alopecia that was concomitant with reduced body condition. Concurrently, elevated numbers of sick or dead ringed seals were detected in the southern Beaufort, Chukchi, and Bering seas in 2012, resulting in the declaration of an unusual mortality event (UME) by the National Oceanic and Atmospheric Administration (NOAA). The primary and possible ancillary causative stressors of these events are unknown, and related physiological changes within individual animals have been undetectable using classical diagnostic methods. Here we present an emerging technology as a potentially guiding investigative approach aimed at elucidating the circumstances responsible for the susceptibility of certain polar bears to observed conditions. Using transcriptomic analysis we identified enhanced biological processes including immune response, viral defense, and response to stress in polar bears with alopecia. Our results support an alternative mechanism of investigation into the causative agents that, when used proactively, could serve as an early indicator for populations and species at risk. We suggest that current or classical methods for investigation into events of unusual morbidity and mortality can be costly, sometimes unfocused, and often inconclusive. Advances in technology allow for

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implementation of a holistic system of surveillance and investigation that could provide early warning of health concerns in wildlife species important to humans.

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1. Introduction

Polar bears (*Ursus maritimus*) are a flagship, sentinel species of the Arctic with a distribution limited to regions where sea ice provides adequate access to marine mammal prey, and a substrate for maternity dens and efficient locomotion. However, the spatial extent of Arctic sea ice during the autumn ice minimum period has declined by over 12% per decade based on analysis of satellite imagery since 1979 (Stroeve et al., 2012), with higher rates of decline greatest in marginal seas like the southern Beaufort (Stroeve et al., 2014). These dramatic changes to the sea ice ecosystem have been implicated in reduced body condition, growth, survival, and abundance of polar bears in the southern Beaufort Sea and elsewhere due, in part, to difficulty in accessing prey (Regehr et al., 2007, 2010; Rode et al., 2010). Consequently, the long-term persistence of polar bears is threatened by rising global temperatures and the resulting cascading effects, which have been projected to induce regional declines in abundance by ~2050 (Amstrup et al., 2008) unless greenhouse gas emissions are mitigated (Amstrup et al., 2010).

Indirect effects of climate change pose added risk to polar bears. Increased body burdens of legacy contaminants in polar bears have been linked to climate change via altered trophic interactions. In the western Hudson Bay region of the Canadian Arctic, changes in polar bear foraging ecology have led to an increase in the proportions of open-water seal species (e.g., harp [*Phoca groenlandica*] and harbor [*Phoca vitulina*] seals) represented in diets, along with a concomitant increase in the tissue concentrations of several chlorinated and brominated contaminants (McKinney et al., 2009). Similar changes have occurred in East Greenland where harp seals and hooded seals (*Cystophora cristata*) have become more common and ringed seals (*Pusa hispida*) more scarce as polar bear prey (McKinney et al., 2013). Studies also have found associations between high levels of legacy chlorinated contaminants in certain polar bear subpopulations and biomarkers of toxic effects on endocrine, immune, and reproductive function (Bernhoft et al., 2000; Polischuk et al., 2002; Lie et al., 2004, 2005; Letcher et al., 2010; Sonne, 2010; Bechshøft et al., 2012; Dietz et al., 2015; Jenssen et al., 2015).

In 2012, southern Beaufort Sea polar bears were observed with varying degrees of alopecia, a syndrome characterized by patchy hair thinning and loss and occasional epidermal lesions, often distributed irregularly over the body and associated with viral, bacterial, fungal, and parasitic agents (e.g., Lynch et al., 2011) (Fig. 1). In fact, 28% of polar bears captured in the southern Beaufort Sea in 2012 were observed with varying degrees of alopecia (Atwood et al., 2015), as well as reduced body condition for adult females captured during this period (Bowen et al., 2015). The occurrence of alopecia in polar bears was preceded by the occurrence of a similar condition in ringed seals, which resulted in the declaration of an unusual mortality event by the National Oceanic and Atmospheric Administration (NOAA) (2011); http://alaskafisheries.noaa.gov/protectedresources/seals/ice/diseased/factsheet112211.pdf.

Evaluation of the health status of polar bears is based on a combination of the animal's history (e.g., movement, reproductive status), physical examination, and clinical pathology data. Health diagnostics of polar bears have historically relied on the evaluation of select serum biomarkers and the identification of contaminant or pathogen burden within specific tissues as an indicator of a level of insult. However, the application of traditional diagnostic approaches to identify the environmental and/or pathogen-related etiologies for alopecia in polar bears proved unsuccessful (Atwood et al., 2015), highlighting the need for an alternative approach for assessing health, one that measures the influence of external factors on the welfare of the individual, provides evidence of physiological reaction, and extends the scope of interpretation.

Gene-based health diagnostics provide opportunity for an alternate, whole-system or holistic assessment of health not only in individuals or populations but potentially in ecosystems (Acevedo-Whitehouse and Duffus, 2009). Until recently, study of only a small number of genes has been feasible, based on a priori selection of a candidate suite of genes (Bowen et al., 2007, 2012, 2015; Miles et al., 2012). However, recent advances in gene sequencing technology allow for the elucidation of an unprecedented breadth of gene pathways. Gene transcription is the process by which information from the DNA template of a particular gene is transcribed into messenger RNA (mRNA) and eventually translated into a function protein. The amount of a particular gene that is transcribed is physiologically dictated by a number of intrinsic and extrinsic factors, including stimuli such as infectious agents, toxin exposure, trauma, or neoplasia. The earliest observable signs of health impairment are altered levels of gene transcripts that are evident prior to clinical manifestation (McLoughlin et al., 2006). Broad scale identification of gene transcription patterns can provide mechanistic proxies of health (Pedersen and Babayan, 2011). Identifying causal links between exposure to stressors, gene transcript patterns, and individual and population health become possible.

Herein we describe the use of deep sequencing technology to investigate the etiology of a high incidence of alopecia in polar bears. Deep sequencing indicates that the total number of reads is many times larger than the length of the sequence under study, in effect increasing the accuracy of the read. Identification of genes and gene pathways associated with altered physiologic manifestations could provide insight into the causes of specific disease states as well as provide the basis for development of a system for early identification of compromised health and related environmental stressors in free-ranging animals and ecosystems.

2. Materials and methods

2.1. Polar bears

Blood samples from nineteen free-ranging polar bears in the southern Beaufort Sea of Alaska were obtained during April, 2012. Nine polar

Fig. 1. Example of alopecia (e.g., thinning of hair on underside of neck) in southern Beaufort Sea adult male polar bear (*Ursus maritimus*) captured on March 28, 2012 in Alaska, USA.

bears were identified with alopecia syndrome (3 adults, 5 subadults, 1 2-year old cub; 4 males, 5 females). Ten polar bears were identified as clinically normal (10 adults; 6 females, 4 males). Polar bears were immobilized from a helicopter using Telazol® (tiletamine hydrochloride a zolazepam hydrochloride; Zoetis, Florham Park, NJ)-filled projectile syringes fired from a dart gun. Captured bears were ear-tagged and tattooed with a unique identification number, body weight was determined using a spring or dynamometer scale, and body condition was determined by palpation following methods described in Stirling et al. (2008). Age was established using various methods including visual aging (cubs-of-the-year; Ramsay and Stirling, 1988), capture history, and via analysis of cementum annuli from an extracted vestigial premolar (Calvert and Ramsay, 1998).

2.2. Blood collection and RNA extraction

A 2.5 ml sample from each polar bear was drawn directly into a PAXgene™ blood RNA collection tube (PreAnalytiX, Switzerland) from either the jugular or popliteal veins and then frozen at −20 °C until extraction of RNA. Rapid RNA degradation and induced transcription of certain genes after blood draws has led to the development of methodologies 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 prevent induction of gene transcription. Without this stabilization, copy numbers of individual mRNA species in whole blood can change dramatically 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 stored at −80 °C until analysis.

2.3. Transcriptome analysis

Tissue samples were used for individually barcoded directional RNASeq library preparations. The barcoded libraries were pooled and run on four lanes of Illumina HiSeq2500 to generate an estimated 30 million paired end reads (length 100 × 100) per sample. Read quality control was performed using tools developed in house by the University of California Davis Genome Center's Bioinformatics Core: the Bioconductor package Qrqc was used to calculate basic read quality metrics, Scythe and Sickle were used for Illumina adapter trimming and base quality trimming (https://github.com/ucdavis-bioinformatics). The trimmed reads were aligned to an existing polar bear draft genome and two related genomes (giant panda [*Ailuropoda melanoleuca*] and domestic ferret [*Mustela putorius furo*]) using Tophat2 (http://tophat.cbcb.umd.edu/; Kim et al., 2013). The alignment rate to the polar bear draft genome was the highest (>65%) compared to alignment to either of the other two genomes (<60%). Therefore, giant panda and domestic ferret genomes were not used for any further analysis.

Although the alignment rate to the polar bear draft genome was higher than to other genomes, the alignment rate varied significantly among samples from ~65% to ~95%. The completeness of the draft genome was then checked using the Core Eukaryotic Genes Mapping Approach (CEGMA) (Parra et al., 2007). Out of 248 core genes, only 212 are present in the draft genome. Furthermore, an assessment using BLAST (Basic Local Alignment Search Tool) of 12 genes that were studied by PCR against the draft genome resulted in partial hits for 5 genes and no hit for one gene. In order to obtain a reasonable quality of transcriptome, extra sequencing was performed on a larger quantity of template material (i.e., pooled tissue samples) and run on one HiSeq2500 lane, and these reads were pooled with all other RNA-Seq data in this study. In order to improve assembly quality, all reads were trimmed more aggressively — i.e. by setting Sickle's Q-value threshold to eliminate 3′-tails with average Q-value below 30, and by discarding trimmed reads shorter than 50 bp long. All trimmed reads were first aligned to a custom carnivora ribosomal RNA (rRNA) database obtained from National Center for Biotechnology Information (Bethesda, MD) to remove any potential rRNA contamination. The assembly was carried out using Trinity (Grabherr et al., 2011) In silico normalization to a maximum coverage of 30× was done, based on recommendations at the time of this study by Trinity authors. Trinity was then used to assemble normalized reads, using default parameters. Functional annotation of the gene models was accomplished by an exhaustive annotation process using Blast2GO (http://www.blast2go.com; Conesa et al., 2005; Götz et al., 2008).

Reads from individual tissue samples were aligned to the assembled transcriptome using BWA-mem with default parameters (http://bio-bwa.sourceforge.net/; Li and Durbin, 2010); BWA is a software package for mapping low-divergence, unspliced sequences. Alignment rates for all samples were >90%. A custom Python script (sam2counts; https://github.com/ucdavis-bioinformatics) was used to generate a table of raw transcript counts for each sample, which was then summed to obtain counts at the gene level. Subsequently, normalization of data and testing for differential transcription were done using edgeR, an R-based package for empirical analysis of digital gene transcription data (http://www.bioconductor.org/packages/release/bioc/html/edgeR.html; Robinson et al., 2010). Genes with false discovery rate values lower than 0.05 (which predicts 5% false positives in the set satisfying that cutoff) were considered to be significantly differentially expressed. Diagnostic plots were produced to determine if transcription among each sample group, based on Euclidean distance, was distinct from that of other groups. Gene Ontology (GO) enrichment in the list of differentially expressed genes were generated using a hypergeometric test implemented in GOstats (Falcon and Gentleman, 2007). P-value output by the Hypergeometric tests were adjusted for multiple comparisons using the method of Benjamini and Hochberg (1995). GO terms that had adjusted P-values <0.05 were considered to be significantly enriched. GO terms were further analyzed using ReVIGO, a web server that summarizes long lists of GO terms by finding a representative subset of the terms. A simple clustering algorithm that relies on semantic similarity measures was used to summarize GO terms. ReVIGO then visualizes the non-redundant GO term set to assist in interpretation (Supek et al., 2011).

We used hierarchical cluster analysis and subsequent heat map generation to refine and identify those genes most highly transcribed based on variance transformed data.

3. Results and discussion

Approximately 9000 genes were identified that were differentially transcribed between alopecia symptomatic and asymptomatic polar bears. Using hierarchical cluster analysis and heat map generation, we identified the 500 most highly transcribed genes (in all polar bears) that could be categorized to definitive biological processes (Fig. 2).

These analyses showed a remarkable demarcation between affected and unaffected individuals. As validation of this technique for differentiating between symptomatic and asymptomatic animals, one potentially affected polar bear was mistakenly clustered with the control animals in the first analysis. Due to the outlying nature of results, reexamination of the data on this animal revealed that it had been miscategorized. What we thought was hair loss attributed to alopecia was actually recorded as hair imbedded in ice in and around the ear that was removed by scratching activity of the bear.

A large number (405) of biological processes were significantly enriched in affected polar bears relative to unaffected animals (Fig. 3). An enrichment of a multitude of processes associated with intracellular pathogens was evident in affected polar bears, including genes involved in major histocompatibility complex (MHC) class I-associated antigen processing and presentation, toll-like receptors 7 and 8, NFκβ and interferons α and γ. Processes associated with inflammation and immune system activation were enriched in affected polar bears including T

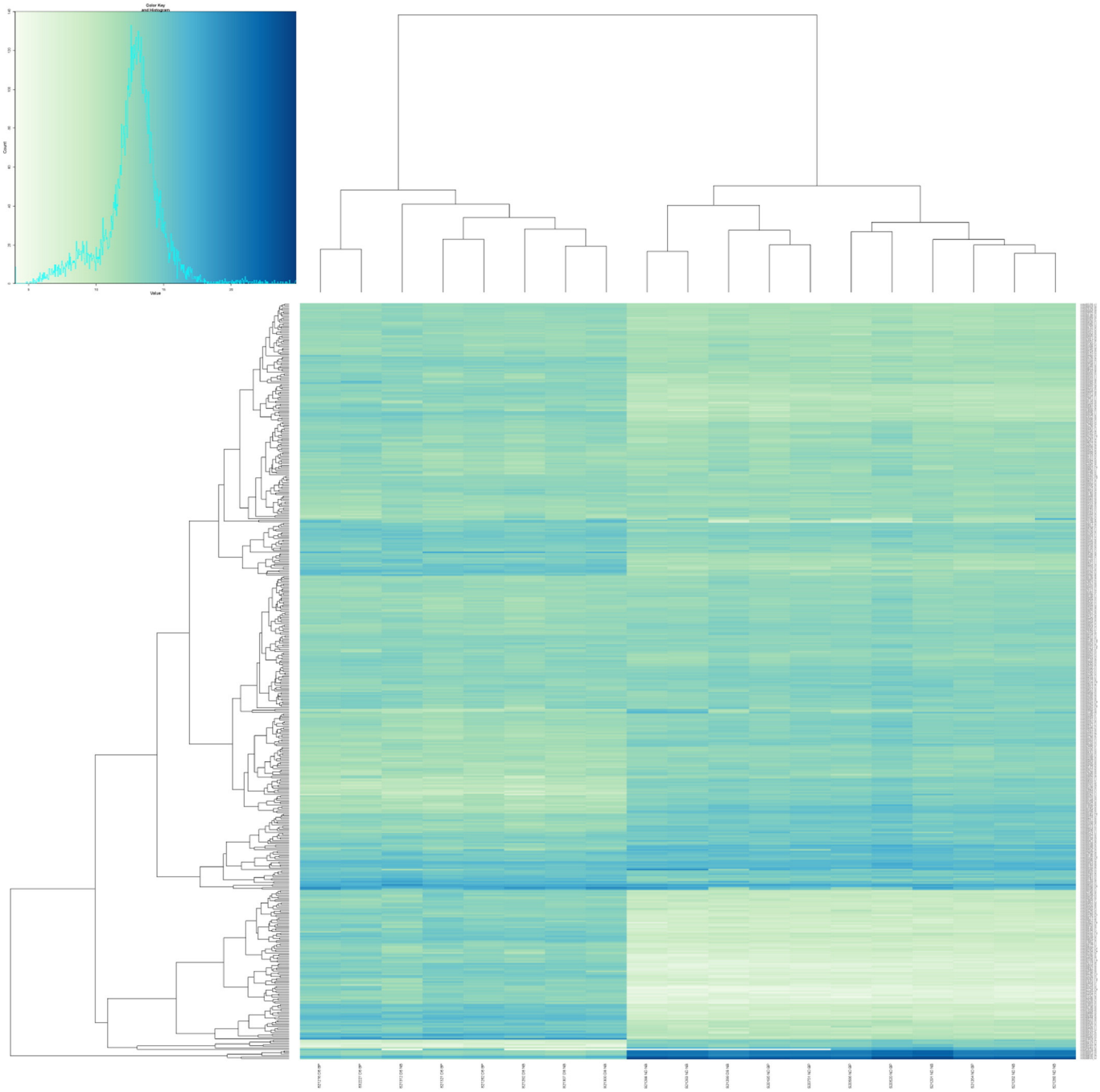

Fig. 2. Heat map showing the 500 most highly transcribed genes based on variance stabilization transformed data. Each row corresponds to an individual gene. Each column represents one of the 19 polar bears investigated in this study. Transcription signal intensity is denoted by color. The tree structures to the left and on the top demonstrate similarities between genes and samples (i.e., the smaller the distance between tree branches, the greater the similarity), respectively, according to the hierarchical cluster analysis results.

lymphocyte activation functions, as well as transcription activity of response regulation to stress genes. DNA damage response-associated gene transcripts were altered in affected polar bears and included enrichment of methylation processes, histone trimethylation, double strand DNA repair, and p53 mediated DNA damage response. In addition, enrichment of aromatic compound catabolic processes and altered fatty acid biosynthesis regulation was identified in polar bears exhibiting signs of alopecia and decreased body condition.

These results led us to the formation of system-specific conceptual drivers of ecology and immunology (Fig. 4).

A proportion of the polar bear population in the southern Beaufort region of Alaska has switched from a diet comprised primarily of ringed seals to a more flexible foraging strategy that includes shore-based scavenging during summer and fall on marine mammal carcasses resulting from human hunters (Rogers et al., 2015). Increased exposure frequency to certain prey items, and associated behaviors such as foraging on shore (Schliebe et al., 2008), may expose the polar bears to pathogens not previously encountered, possibly challenging typical immune response. Alternate marine-based prey items may carry increased contaminant burdens (Macdonald et al., 2005; Thiemann et al., 2008; McKinney et al., 2009, 2013; Carrie et al., 2010) further impacting immunologic response(s) (Vos et al., 2000). While foraging on terrestrial-based prey and vegetation would likely reduce contaminant burdens (Ramsay and Hobson, 1991; Gormezano and Rockwell,

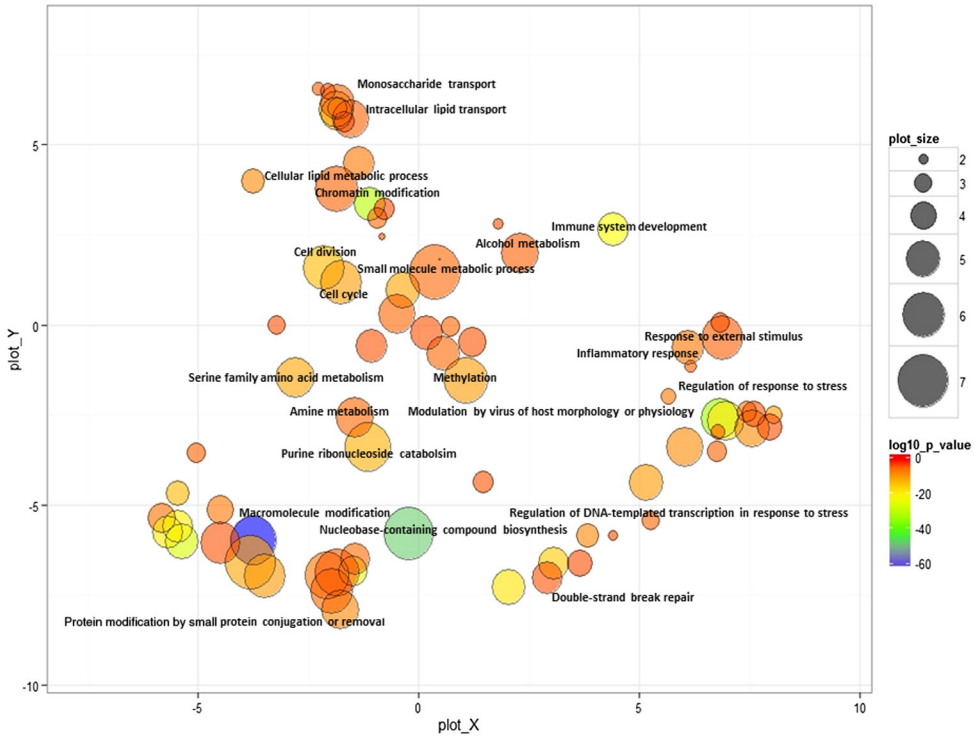

Fig. 3. Biological processes enriched in polar bears exhibiting signs of alopecia. ReVIGO scatterplot of GO categories enriched in alopecic polar bears (GO over-representation analysis (ORA), Benjamini-Hochberg corrected $P < 0.05$), with blue circles denoting higher enrichment, circle size denoting the frequency of the GO term in the underlying database (bubbles of more general terms are larger) and semantically similar (simRel) GO terms closer to each other. Labels indicate processes particularly relevant to interpretation.

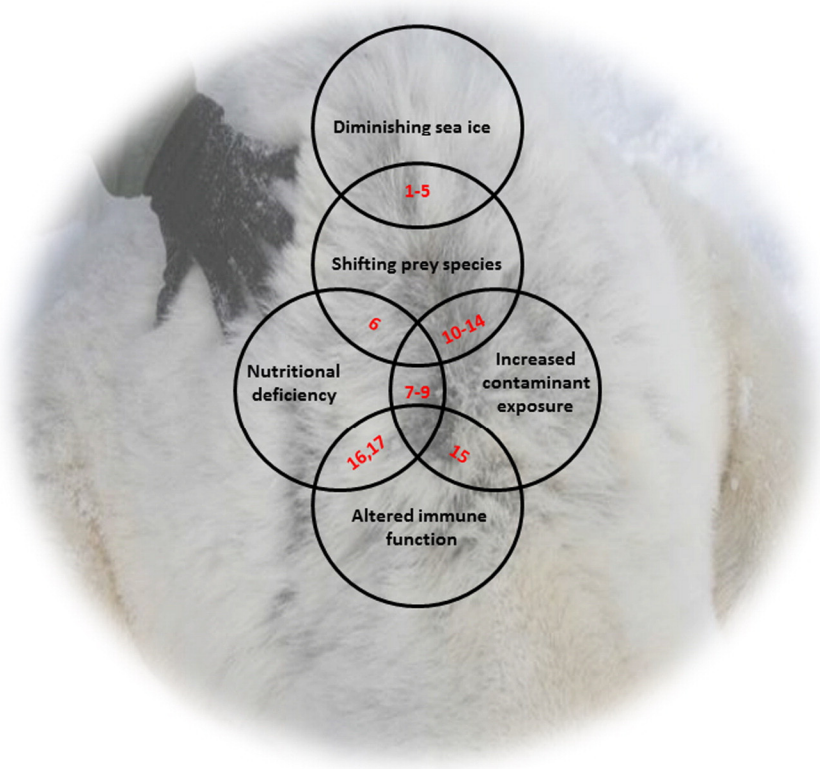

Fig. 4. Conceptual model of the potential climate change-driven factors affecting immune function. Areas of overlap indicate probable causative links. Numbers indicate references: 1) Durner et al., 2009, 2) Edwards et al., 2011, 3) Molnar et al., 2011, 4) Stirling and Derocher, 2012, 5) Derocher et al., 2013, 6) Cherry et al., 2009, 7) Polischuk et al., 2002, 8) Christensen et al., 2007, 9) Helgason et al., 2013, 10) Thiemann et al., 2008, 11) McKinney et al., 2009, 12) McKinney et al., 2013, 13) Macdonald et al., 2005, 14) Carrie et al., 2010, 15) Vos et al., 2000, 16) Acevedo-Whitehouse and Duffus, 2009, and 17) Saucillo et al., 2014.

2013a, 2013b; Iles et al., 2013), it is unlikely to offset nutritional deficiency resulting from lost foraging opportunities on lipid-rich marine mammal prey (Rode et al., 2015). Perhaps simultaneously, increased resource allocation to facilitate extended swimming or foraging bouts due to diminishing sea ice could affect immune function or efficiency (Saucillo et al., 2014). Moreover, nutritional deficiency could mobilize contaminants stored in blubber, further taxing immune function (Letcher et al., 2010) and facilitating disease susceptibility.

Continued transcription of genes responsible for immunologic function, including detoxification, can be physiologically costly (Graham et al., 2010). Perhaps the largest cost is the reallocation of nutrients and energy from one portion of an individual's resource budget to other metabolic functions. Mitigation of stressors imposes demands on animals above those normally required to sustain life and may result in reduction of fitness evidenced by decreased reproductive capability, increased susceptibility to disease, or disadvantageous behavioral changes (Graham et al., 2010; Martin et al., 2010).

3.1. Conclusions

Our results lead to two potentially important realities pertaining to investigations into unusual morbidity and/or mortality events: 1) investigations using standard and widely accepted diagnostics may not be sufficiently sensitive to provide clues as to the causative agents, and 2) although advanced technologies provide far better resolution of potential ecological and immunological drivers, the post-event timing of the investigation may prohibit clear insight into the ecological pathways involved. At the time of death, or disease state, an organism is clearly at the latter stages of a succession of physiological, biochemical, and immunological events triggered by one or more causative incidents. The number of enriched pathways identified in polar bears with alopecia syndrome is a prime example of this: by the time clinical manifestation has occurred, ample time has elapsed in a physiological system already operating sub-optimally for additional conditions to manifest. Even with the dramatic advances in technology experienced over the past decade, the current paradigm of investigations 'after the fact' will not suffice to properly understand causative effects. Historically, large scale investigations into populations and ecosystems have been driven by catastrophic events (i.e., unusual mortality events). These have relied heavily on information obtained using routine clinical diagnostics and pathology from sick or deceased animals. We suggest a different approach, i.e. 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. We can use the current approach to refine the direction of further research efforts; in this case, we could, with some degree of confidence, point at microbial, nutritional, and toxin exposure as components contributing to the alopecia syndrome. In the future, we may gain considerably in our ability to understand why species are at risk if we take a proactive approach of monitoring populations prior to the onset of clinical manifestation. Such a proactive approach would negate in part the requirement for a "healthy control" data set to identify outliers in the population in question; use of control data sets derived from wild animals in captivity should always be interpreted with caution. Proactive longitudinal studies on wildlife species at risk could provide baseline data sets upon which apparent perturbations in real time could be assessed.

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