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Lorin A. Neuman-Lee
Arkansas State University

Patricia A. Terletzky
Utah State University

Todd C. Atwood
U.S. Geological Survey

Eric M. Gese
USDA/APHIS/WS National Wildlife Research Center, eric.gese@usu.edu

Geoffrey D. Smith
Dixie State University

See next page for additional authors

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Authors

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Lorin A. Neuman-Lee¹ | Patricia A. Terletzky² | Todd C. Atwood³ | Eric M. Gese^{2,4} | Geoffrey D. Smith⁵ | Sydney Greenfield⁶ | John Pettit⁶ | Susannah S. French⁶ 

¹Department of Biology, Arkansas State University, Jonesboro, Arkansas

²Department of Wildland Resources, Utah State University, Logan, Utah

³U.S. Geological Survey, Alaska Science Center, Anchorage, Alaska

⁴U.S. Department of Agriculture, Wildlife Services, National Wildlife Research Center, Logan, Utah

⁵Department of Biological Sciences, Dixie State University, St. George, Utah

⁶Department of Biology, Utah State University, Logan, Utah

Correspondence

Susannah S. French, Department of Biology, Utah State University, 5305 Old Main Hill, Logan, UT, 84322–5305.

Email: susannah.french@usu.edu

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Abstract

Assessing the health and condition of animals in their natural environment can be problematic. Many physiological metrics, including immunity, are highly influenced by specific context and recent events to which researchers may be unaware. Thus, using a multifaceted physiological approach and a context-specific analysis encompassing multiple time scales can be highly informative. Ecoimmunological tools in particular can provide important indications to the health of animals in the wild. We collected blood and hair samples from free-ranging polar bears (*Ursus maritimus*) in the southern Beaufort Sea and examined the influence of sex, age, and reproductive status on metrics of immunity, stress, and body condition during 2013–2015. We examined metrics of innate immunity (bactericidal ability and lysis) and stress (hair cortisol, reactive oxygen species, and oxidative barrier), in relation to indices of body condition considered to be short term (urea to creatinine ratio; UC ratio) and long term (storage energy and body mass index). We found the factors of sex, age, and reproductive status of the bear were critical for interpreting different physiological metrics. Additionally, the metrics of body condition were important predictors for stress indicators. Finally, many of these metrics differed between years, illustrating the need to examine populations on a longer time scale. Taken together, this study demonstrates the complex relationship between multiple facets of physiology and how interpretation requires us to examine individuals within a specific context.

1 | INTRODUCTION

As the field of conservation physiology grows, so does our knowledge and understanding of how complex biological systems are within the environment (Madliger & Love, 2016, Nussey et al., 2014, Wikelski & Cooke, 2006). Often physiological responses change due to habitat, weather, sex, age, life history, or even social status (Cavigelli & Chaudhry, 2012, French, DeNardo, & Moore, 2007, Palacios, Sparkman, & Bronikowski, 2011). Ecoimmunology, the examination of the relationships between an immune response and various ecological parameters, presents a valuable addition to conservation research and management (Lee & Klasing, 2004). Not only is the immune system responsive to physiological stress (Dhabhar, 2009), it can indicate the current health of an animal if properly measured and interpreted

(Beechler, Broughton, Bell, Ezenwa, & Jolles, 2012, Budischak, Jolles, & Ezenwa, 2012, Demas, Zysling, Beechler, Muehlenbein, & French, 2011, Hawley, & Altizer, 2011).

Immune function is a critical physiological metric to examine in wild animals due to its ability to provide information on the health and possible survivability of an individual (Lochmiller & Deerenberg, 2000, Matson, Cohen, Klasing, Ricklefs, & Scheuerlein, 2006, Sheldon, & Verhulst, 1996). The immune defense systems (humoral, cell-mediated, and innate systems) work in concert and can be examined separately or together. The innate immune system, however, is critical to examine because it is the first defensive system against foreign bodies and antigens, and includes the complement system, inflammatory responses, and natural killer cells. Innate immunity is, in itself, very complex and has multiple mechanisms to fight off antigens (Demas et al., 2011,

Janeway, Travers, Walport, & Shlomchik, 2001). Briefly, when an antigen is detected by the innate immune system, common constituents of the antigen's surface activate macrophages. These macrophages release cytokines and chemokines into the blood stream that result in inflammation and attracting other immune cells to the site of infection. In many cases, the complement system, which is made up of plasma proteins, is activated and augments the action of other immune components (Janeway et al., 2001). Because of the complexity of this system, trade-offs, or shifting resources from one system to another, within the innate immune system are common and allow the organism to utilize a dynamic approach when combating disease (Beechler et al., 2012, Neuman-Lee & French, 2014).

Innate immune function can be measured using functional assays, such as bactericidal assays, which assess the ability of the serum of the animal to kill foreign bacteria (French & Neuman-Lee, 2012). Also commonly employed is a lysis assay, which examines the complement system of innate immunity by measuring the ability of the serum to lyse a foreign antigen (Greives, McGlothlin, Jawor, Demas, & Ketterson, 2006, Matson, Ricklefs, & Klasing, 2005). Finally, measuring reactive oxygen species (ROS), which are formed as a non-specific immune defense (Babior, 1978, McCord, 2000) and through aerobic respiration (Turrens, 2003), provides insight into the cellular function of an individual (Beaulieu & Costantini, 2014). In conjunction with measuring ROS, determining the concentration of a natural antioxidant barrier, which can mitigate the deleterious effects of ROS (McCord, 2000, McCord & Fridovich, 1969, Uttara, Singh, Zamboni, & Mahajan, 2009), is critical. If ROS production outpaces antioxidants, a state of oxidative stress results (Schafer & Buettner, 2001).

The innate immune system is not only influenced by trade-offs within itself, but also external factors (Lee, 2006). Demographic factors such as sex, age, and reproductive status can all dramatically influence both the immune capabilities of the animal as well as individual components of the immune system. Specifically, females across the taxonomic spectrum tend to have higher immune function than males (Nalbandian & Kovats, 2005). Often juveniles are still developing their immune system while adults tend to have a relatively more robust immune system (Arriero, Majewska, & Martin, 2013, Brandenburg et al., 1997). Reproduction has a complex relationship with immunity, with females often showing a suppression of immune function during pregnancy (Druckmann & Druckmann, 2005, Jackson et al., 2011).

Immunocompetence is often affected by an elevation of glucocorticoids (GCs), such as cortisol (Dhabhar, 2009). Animals undergoing chronic increases in GCs often display decreased immune function (Brooks & Mateo, 2013, French, McLemore, Vernon, Johnston, & Moore, 2007, Neuman-Lee et al., 2015). Conversely, animals can often experience immunoenhancement during periods of acute elevation of GCs (Dhabhar, 2009, Martin, 2009). An increase in GCs is associated with the activation of the hypothalamic–pituitary–adrenal axis, which is often associated with adverse or “stressful” conditions. However, increases in GCs functionally serve to mobilize energy and can be associated with other permissive, stimulatory, suppressive, and preparative actions (McEwen, 2007, Sapolsky, Romero, & Munck, 2000, Wingfield, 2005). Finally, the same animal may have different responses under different conditions, such as resource availability and/or differences

in exposure to antigens (Beldomenico et al., 2008, Nelson & Demas, 1996). Measuring chronic GC levels in the wild can be accomplished using minimally invasive techniques such as sampling hair (Cattet et al., 2014, Macbeth, Cattet, Obbard, Middel, & Janz, 2012, McCormick & Romero, 2017). This technique measures deposition of GC levels, such as cortisol, over a time scale of weeks and months (Meyer & Novak, 2012), reflecting the time period when animals are growing a new coat. In polar bears (*Ursus maritimus*), this occurs seasonally in the spring and summer, indicating that the measured levels of cortisol represent the total cortisol deposition during that time.

Polar bears are specialist carnivores ranging widely over the ice-covered waters of the Arctic Ocean. The habitat generally preferred by polar bears is sea ice over the highly productive shallow waters of the continental shelf, where prey (primarily ringed seals [*Pusa hispida*] and bearded seals [*Erignathus barbatus*]) are most abundant (Durner et al., 2009, Mauritzen et al., 2003). However, over the last four decades, climate-induced warming has triggered dramatic changes to the Arctic marine ecosystem primarily via declines in the extent and availability of sea ice in summer and fall (Serreze & Barry, 2011, Stroeve, Markus, Boisvert, Miller, & Barrett, 2014). The southern Beaufort Sea along the northern coast of Alaska and the Yukon and Northwest territories of Canada has experienced some of the most dramatic reductions in the spatio-temporal availability of sea ice in summer (Stern & Laidre, 2016, Stroeve et al., 2014) which, in turn, have been linked to declines in polar bear body condition, physical stature, reproduction, survival, and abundance (Bromaghin et al., 2015, Regehr, Hunter, Caswell, Amstrup, & Stirling, 2010, Rode, Amstrup & Regehr, 2010). The loss of sea ice habitat in summer has led southern Beaufort Sea bears to become more reliant on terrestrial habitats (Atwood et al., 2016, Pongracz & Derocher, 2016) where they face an increased risk of exposure to anthropogenic activities, such as petroleum exploration and extraction. Because polar bears are apex predators and bioaccumulate contaminants, such as mercury and persistent organic pollutants in their tissues (Cardona-Marek, Knott, Meyer, & O'Hara, 2009, Kannan, Yun, & Evans, 2005), increased exposure to industrial activities and effluent has the potential to adversely impact population health and behavior (Amstrup, Durner, Johnson, & McDonald, 2006, Bowen et al., 2015, Atwood et al., 2016).

Polar bears were listed as “vulnerable” on the IUCN Red List of Threatened Species in 2006 and “threatened” under the U.S. Endangered Species Act (USFWS, 2008) due to observed and projected declines in sea ice habitat and expected adverse effects on population persistence. Because of their conservation status, it is critical that managers have methods to assess changes in polar bear health. To address this, we sought to measure multiple forms of innate immunity and physiology (bactericidal ability, lysis, formation of ROS, formation of an antioxidant barrier, short-term and long-term energy stores) in polar bears across different contexts (sex, age, reproductive status, and stress state, as indicated by an elevation in cortisol) over 3 years.

Because we were testing multiple factors for various physiological variables, we present our specific predictions and rationale in Table 1. Briefly, we predicted that female polar bears would have increased immune capabilities relative to males, but females with cubs would have lower immunocompetence. We also predicted younger

TABLE 1 Predicted changes in physiological variables when examining the factors of sex, age class, and reproductive status

Physiological Variable	Sex (Male vs. Female)	Age (Sub-Adults vs. Adults)	Reproductive Status (Females with Cubs vs. Females without Cubs)
Innate immunity (bactericidal ability and lysis)	Higher in females (Nalbandian & Kovats, 2005)	Higher in adults (Arriero et al., 2013, Brandenburg et al., 1997)	Lower in females with cubs (Druckmann & Druckmann, 2005, Jackson et al., 2011)
Oxidative stress	Lower in females (Behl et al., 1997, Lagranha et al., 2010, Sawada et al., 1998)	Higher in adults (Finkel & Holbrook, 2000)	Higher in females with cubs (Al-Gubory et al., 2010, Alonso-Alvarez et al., 2004, Wang et al., 2001)
Urea to creatinine ratio (UC ratio)	Lower in females (Cherry et al., 2009)	Higher in adults (Cherry et al., 2009)	Higher in females with cubs (Cherry et al., 2009)
Storage energy and BMI (body condition)	Higher in females (Molnar et al., 2009)	Lower in adults (Molnar et al., 2009)	Lower in females with cubs (Molnar et al., 2009)
Hair cortisol	Higher in females (Bechshoft et al., 2015)	Higher in adults (Bechshoft et al., 2015)	Higher in females with cubs (Sapolsky et al., 2009)

animals would have a less robust immune response relative to adult animals. We predicted that individuals with increased levels of cortisol in their hair would have decreased immune function, with the understanding that the two metrics are on different tissues (hair vs. blood) and thus different time scales and may not be related. We further predicted that oxidative stress would vary by sex and reproductive status, and ROS would be correlated with innate immune metrics. Finally, we did not expect differences between years given that conditions in the environment remained stable (TCA, personal observation).

2 | METHODS/MATERIALS

2.1 | Study area

The study area consisted of the southern Beaufort Sea (latitude 71.160, longitude 148.14) along the Alaskan coastline for approximately 160 km. The study area ranged from Demarcation Point (140° west) at the US–Canada border in the east to Point Barrow (156° west). The Beaufort Sea is characterized by a narrow continental shelf that gives way to some of the deepest water of the Arctic Ocean (Jakobsson et al., 2008). The steep increase in bathymetry beyond the shelf break is accompanied by a declining gradient of biological productivity (Dunton, Goodall, Schonberg, Grebmeier, & Maidment, 2005, Sakshaug, 2003), and a corresponding decline in the suitability of sea ice habitat for polar bears (Durner et al., 2009). The southern Beaufort Sea coastal plain contains two large oil fields, Prudhoe Bay and Kuparuk, as well as the National Petroleum Reserve-Alaska. Additionally, the communities of Barrow, Nuiqsut, and Kaktovik are spaced along the coast. Some residents of these communities harvest bowhead whales (*Balaena mysticetus*) in the fall for subsistence purposes. Unused remains are aggregated at centralized locations attracting polar bears and providing opportunities for commercial viewing (Herreman & Peacock, 2013, Miller, Wilder & Wilson, 2015).

2.2 | Animal capture

Polar bears in the southern Beaufort Sea have been monitored since the mid-1980s to assess population dynamics and overall health of the population (Atwood et al., 2016, Bromaghin et al., 2015). For this study, bears were encountered from a helicopter opportunistically on the sea ice from late March to early May, 2013–2015, and immobilized with the drug tiletamine hydrochloride plus zolazepam hydrochloride (Telazol®; Fort Dodge, Overland Park, KS, USA; Warner-Lambert Company, New York, NY, USA). Captured bears were uniquely identified with ear tags, a corresponding lip tattoo, and weighed and body characteristics measured. Age was determined by multiple methods following Ramsay and Stirling (1988) and Calvert and Ramsay (1998). Hair samples were collected from multiple locations on the rump to create a composite sample. Hair growth (and so likely hormone deposition) occurs over a time scale of weeks and months in the spring and summer (Meyer & Novak, 2012), reflecting the time period when animals are growing a new coat. Hair was stored in sealed plastic bags at room temperature until processed (see below). Blood samples from sub-adult and adult bears were drawn into additive-free evacuated tubes by venipuncture of either the femoral or jugular veins. All samples were centrifuged at 1,500g for 5 min on the day of collection to divide sera and plasma (Kirk, Amstrup, Swor, Holcomb, & O'Hara, 2010), aliquoted into 2 ml cryovials, stored at –20°C and then stored at –80°C upon return from the field. Samples remained frozen at –80°C until assays were performed. Plasma samples from 2014 and 2015 were analyzed within five months of storage. Plasma from 2013 was analyzed with samples from 2014, which was approximately 17 months after collection due to logistical constraints. However, though we did not see a decrease in immune capability in 2013, which would have indicated degradation of sample, we cannot rule it out. The number of polar bears sampled for this study are as follows: 2013 $N = 53$, 2014 $N = 39$, 2015 $N = 40$ (Table 2).

2.3 | Ethics statement

This research was approved under the Marine Mammal Protection Act and Endangered Species Act with U.S. Fish and Wildlife Service

TABLE 2 Physiological and body condition data from polar bears (*Ursus maritimus*) sampled in the southern beaufort sea, 2013–2015

	Sex		Age Class			Reproductive State		Year		
	Female	Male	Adult	Sub-Adult	With Cubs	Without Cubs	2013	2014	2015	
Bactericidal ability (%)	62.8 ± 5.3 N = 69	46.4 ± 6.1 N = 48	57.4 ± 4.6 N = 88	53.8 ± 8.5 N = 28	70.6 ± 7.8 N = 27	59.3 ± 9.4 N = 24	60.9 ± 6.3 N = 43	23.7 ± 5.3 N = 36	81.4 ± 6.1 N = 38	
ROS (mM of H ₂ O ₂)	11.0 ± 0.4 N = 69	14.0 ± 0.9 N = 48	12.3 ± 0.6 N = 88	12.1 ± 0.7 N = 28	9.8 ± 0.7 N = 27	12.2 ± 0.7 N = 24	11.9 ± 0.6 N = 43	13.0 ± 1.0 N = 36	11.9 ± 0.7 N = 38	
Oxidative barrier (mmol/l)	349.2 ± 1.7 N = 70	351.8 ± 1.94 N = 48	349.9 ± 1.4 N = 88	351.7 ± 3.0 N = 29	346.1 ± 3.4 N = 27	351.9 ± 1.9 N = 24	354.7 ± 1.0 N = 43	336.2 ± 2.3 N = 36	358.3 ± 1.7 N = 39	
Lysis (CH ₅₀ units/ml)	1.7 ± 0.2 N = 55	1.6 ± 0.2 N = 41	1.8 ± 0.1 N = 74	1.5 ± 0.3 N = 21	1.9 ± 0.2 N = 22	1.73 ± 0.2 N = 18	1.6 ± 0.2 N = 37	2.2 ± 0.2 N = 34	1.1 ± 0.2 N = 25	
Urea to creatinine ratio (UC ratio)	36.1 ± 5.51 N = 20	18.3 ± 5.3 N = 18	23.4 ± 4.3 N = 36	14.7 ± 3.4 N = 2	39.7 ± 9.1 N = 9	34.7 ± 7.5 N = 10	20.1 ± 4.6 N = 17	39.6 ± 9.6 N = 9	29.4 ± 8.0 N = 12	
Storage energy ((x ₁ (body mass (kg)) - x ₂ (standard body length (m)) ³))	1086.1 ± 67.4 N = 68	1991.6 ± 199.4 N = 43	1704.2 ± 121.0 N = 78	942.7 ± 86.5 N = 26	1309.2 ± 113.3 N = 25	1161.9 ± 101.6 N = 22	1427.7 ± 170.7 N = 39	1528.8 ± 175.5 N = 35	1360.0 ± 159.2 N = 37	
BMI (body mass (kg)/(standard body length (m)) ²)	41.0 ± 0.7 N = 68	50.3 ± 1.9 N = 46	47.8 ± 1.2 N = 79	39.8 ± 1.3 N = 28	43.0 ± 1.0 N = 25	41.7 ± 0.9 N = 22	44.2 ± 1.8 N = 41	46.4 ± 1.7 N = 35	43.8 ± 1.6 N = 38	
Cortisol (µg/g hair)	31.0 ± 1.1 N = 77	27.5 ± 1.1 N = 55	27.68 ± 1.0 N = 87	33.9 ± 1.7 N = 34	27.3 ± 2.0 N = 25	31.6 ± 1.6 N = 25	28.9 ± 1.1 N = 53	29.7 ± 1.5 N = 39	30.3 ± 1.7 N = 40	

Sample sizes for each metric are given. Data are presented as the mean ± 1 standard error.

(USFWS) permit number MA690038. Capture and handling protocols were reviewed and approved by the U.S. Geological Survey (USGS) Institutional Animal Care and Use Committee.

2.4 | Physiological metrics

Given constraints of the individual sample taken (i.e., amount of hair or plasma), we could not run all physiological assays for each bear. Therefore, the actual sample size for each assay may be slightly different and is outlined in Table 2. Samples were separated by year, but individual samples from each year were all assayed together. With the exception of the lysis assay, which was run in singlicate, all samples were run in duplicate.

2.5 | Bactericidal ability

We performed the bactericidal assay following the protocol outlined in French and Neuman-Lee (2012). Briefly, we combined a 1:3 dilution of plasma with CO₂-independent media (Gibco, Grand Island, NY, USA) plus 4 nM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), and 10⁵ CPU (colony producing unit) *Escherichia coli* (EPower™ Microorganisms ATCC 8739; MicroBioLogics, St. Cloud, MN), and agar broth on a 96-well microplate. We calculated the background absorbance using BioRad xMark microplate reader (Hercules, CA, USA). After a 12 hr incubation, we again read the absorbance and calculated the bactericidal ability by dividing the mean absorbance for each sample (run in duplicate) by mean absorbance for the positive controls (containing only media and bacterial solution), and multiplying by 100. This provides the percent bacteria killed relative to the positive controls. Negative controls (containing media only) were also run to ensure contamination was absent. Inter-assay variation between plates was 1.7%.

2.6 | Lysis

To measure lysis in the plasma, we adapted the protocol of Matson et al., (2005), in which 20 μ l of plasma and 20 μ l of phosphate-buffered saline (PBS) were serially diluted (1:2) across a 96-well round (U) bottom microplate for resulting dilutions of 1:1 to 1:2,048. Six positive controls of dH₂O (100% lysis) and six negative controls of PBS (0% lysis) were present on each plate. Following the serial dilution, we added 20 μ l of 1% washed sheep red blood cells (Hemostat SBH 100) in PBS to every well. Plates were covered with parafilm, vortexed at 190 rpm for 60 sec, incubated at 37°C for 90 min, and finally incubated for 60 min at room temperature. We then centrifuged plates for 5 min at 57g, aspirated the supernatant, and placed the supernatant in a clean 96-well microplate to measure absorbance at 405 nm. To standardize results, hemolytic-complement activity (lysis) is expressed as CH₅₀ units/ml serum, or the dilution of plasma that causes 50% lysis of sheep red blood cells.

2.7 | Reactive oxygen species

To measure ROS in the plasma, we followed the protocol included with the d-ROMs Test kit (Diacron, Grosseto, Italy). Briefly, we mixed the provided R₁ and R₂ reagents in a 1:100 dilution to create an acidic

buffered solution with a chromogen. Five microliters of sample plasma was added into separate wells of a 96-well microplate and 100 μ l of the R₁/R₂ solution was added to each well. Positive and negative controls were run using provided serum and Nanopure water, respectively. We measured absorbance at 505 nm after a 90-min incubation at 37°C. The resulting units are in mM of H₂O₂. The intra-assay variation was below 3.3% for all plates. The inter-assay variation between plates was 1.3%.

2.8 | Oxidative barrier

We followed the protocols included with the OXY-Adsorbant Test kit (Diacron) to measure the antioxidant capacity of the plasma. Samples were diluted with Nanopure water in a 1:50 ratio and added to separate wells of a 96-well plate. The samples were then subjected to an oxidative challenge of hypochlorous acid. Absorbance was measured at 505 nm for each row immediately after a chromogen was added to determine the ability of the plasma to defend against the oxidative challenge. Intra-assay variation was below 3.4% for all assays and the inter-assay variation between plates was 7.2%.

2.9 | Cortisol

Cortisol was measured using a protocol modified from Davenport, Tiefenbacher, Lutz, Novak, and Meyer, (2006) and Macbeth et al., (2012). All hair samples were analyzed at the same time. Briefly, hair samples were cleaned by washing with HPLC-grade methanol three times, allowed to dry, then weighed. Sample weight averaged 133.3 \pm 58.4 mg SD. In cases where the sample weight was below 150 mg, the entire sample was used. The hair was then ground using a Restch ball mill (Restch, Verder Scientific, Germany) with 7 mm steel grinding balls. Samples were ground for 10 min at 30 Hz. Balls and vials were cleaned thoroughly with ethanol, and dried before grinding the next sample. The ground hair was weighed and transferred to an Eppendorf tube. We added 3 ml of methanol to the sample, vortexed it for 1 min, then placed samples on a slow vortexer for 24 hr. After vortexing, samples were then spun for 10 min at 914g, and 1.5 ml of the supernatant was removed and placed in a clean glass tube. Samples were dried under a stream of nitrogen and then resuspended in an assay dilutant (supplied by Salimetrics, State College, PA; see below).

We determined the concentration of cortisol in the samples using an enzyme immunoassay (Salimetrics), following the manufacturer's instructions. Briefly, we pipetted the samples, controls, and zeros (blanks to control for nonspecific binding) onto the supplied plate and added the enzyme conjugate (diluted to 1:1,600). After mixing for 5 min and incubating at room temperature for 1 hr, the plate was washed four times with the supplied wash buffer using a plate washer. TMB Substrate solution was added, mixed for 5 min, and then incubated for another 25 min at room temperature. Finally, the stop solution was added, the plate was mixed, and the plate was read at 450 nm, with a secondary filter correction at 490 nm, using a BioRad xMark Microplate reader. Standard curves, controls, and controls for non-specific binding were run on each plate. Cross-reactivity to other steroid hormones is less than 0.25%, with the highest cross-reactivity

to corticosterone at 0.21%. Intra-assay variation averaged 2.4% and inter-assay variation was 4.2%.

Prior to running samples, we validated this method. Average linearity for samples yielded an $R^2 = 0.996$ (Supp. Figure S1) and interference tests with known spikes yielded an average recovery of 92.8%.

2.10 | Body condition and nutritional status

A small subset of individuals was selected for body condition analyses (Table 2). Body condition and nutritional status (e.g., fasting or feeding regularly), as determined via morphometric based indices, are important and widely used indicators of individual fitness and health (Nelson, Thomas, & Steiger, 1984; Robbins, Ben-David, Fortin, & Nelson, 2012). Individuals that are in poor body condition and/or nutritionally stressed may experience lower immunocompetence than individuals that are feeding regularly. Accordingly, we estimated body condition and nutritional status of bears to determine if they influenced immune capabilities. We calculated two condition indices: body mass index (BMI) ($BMI = \text{body mass (kg)}/\text{standard body length (m)}^2$) and storage energy (SE) ($SE = x_1(\text{body mass (kg)}) - x_2(\text{standard body length (m)})^3$), following procedures described in Cattet, Caulkett, Obbard, and Stenhouse, (2002) and Molnár, Klanjscek, Derocher, Obbard, and Lewis, (2009). Both express body condition as a measure of individual energy reserves and are routinely applied to ursids (e.g., Cattet et al., 2002).

We used the ratio of urea to creatinine (UC ratio) in blood serum to index nutritional status. Like the condition indices, the UC ratio has been frequently used to determine the nutritional status of ursids (Cherry, Derocher, Stirling, & Richardson, 2009; Derocher, Nelson, Stirling, & Ramsay, 1990; Nelson et al., 1984). To derive the UC ratio, we first quantified levels of blood urea nitrogen (BUN) and creatinine in serum samples using an Abaxis Vetscan VS2 analyzer (Abaxis, Inc., Union City, CA). We then calculated serum urea by multiplying BUN values by the constant 0.466, and dividing the resulting value by serum creatinine (Nelson et al., 1984). Studies on polar bears have used UC ratios of ≤ 10.0 as indicative of fasting for ≥ 7 days (Derocher et al., 1990).

2.11 | Statistics

To determine what explanatory variables may have an impact on the physiological metrics, we compared general linear models with fixed effects for sex, age, and year. For reproductive females, we analyzed year and reproductive status. We used the Akaike information criterion (AIC) to determine the best fitting model (Akaike, 1974). For every physiological metric, the AIC for all models was lowest for the models without interactions (sex, age, and/or year), or not different from these models ($\Delta AIC < 2.0$). The interaction between year and reproductive status for reproductive females was not significant for any variable ($P > 0.08$), and the best model was that with the main effect of reproductive status. Therefore, we performed further analyses with each explanatory variable as described below.

Because assumptions of normality were not met for measures of bactericidal ability, ROS, oxidative barrier, lysis, UC ratio, SE, and BMI, we used Wilcoxon signed-rank test for analyzing for differences among

sex (females, males), age (adult, sub-adult), and reproductive status (females with cubs, females without cubs), and we used a Kruskal–Wallis test to examine differences among years. For measures of cortisol, we conducted a Student's *t*-test for differences between sex, age, and reproductive status and an analysis of variance (ANOVA) to examine differences among years. With the exception of SE and BMI, there was no difference among the females with different numbers of cubs ($P > 0.3$), thus we combined the females with one or two cubs into one category of females with cubs. While there may have been a difference between females with dependent versus yearling cubs, the samples were too small to make statistical comparisons. To examine the relationships between the continuous variables, we performed Spearman's correlations. We removed three observations for SE, as they were negative (McKinney et al., 2014); analyses setting these three values to "0" did not change the statistical outcome.

All of the statistical analyses were performed using R (version 3.3.0) in RStudio (version 1.0.136 (R Development Core Team, 2016)). For the Kruskal–Wallis tests, we used the *pgirmess* package (Giraudoux, 2016) and for the Spearman's correlations, we used the *pspearman* package (Savicky, 2014). Analyses were considered significant if they were below an $\alpha = 0.06$ (Burnham & Anderson, 2014).

3 | RESULTS

When we analyzed immune metrics by sex, we found females had higher bactericidal ability (Figure 1A and Tables 2 and 3), but lower levels of ROS compared with males (Figure 1B and Tables 2 and 3). Lysis and the oxidative barrier were not different between females and males (Tables 2 and 3). There were no differences between any of the immune metrics between adult and sub-adult bears (Figures 1A–D and Tables 2 and 3). When we examined the immune metrics for females with or without cubs, we found that females without cubs had higher levels of ROS (Figure 1B and Tables 2 and 3), but there were no other differences between the two groups for the other physiological measures (Figures 1A–D and Tables 2 and 3).

Bactericidal ability significantly varied among the 3 years (Figure 1A and Tables 2 and 3). The highest bactericidal ability was measured in 2015, with the lowest measured in 2014. Lysis and oxidative barrier were also significantly different among years (Tables 2 and 3). For oxidative barrier, post-hoc tests revealed that 2013 and 2015 were significantly different from 2014, but 2013 and 2015 were different from one another. For lysis, only 2014 and 2015 were different from each other. ROS production was not different among the 3 years (Figure 1B and Tables 2 and 3). Accumulated cortisol in hair was not related to any immune metric ($r < 0.2$; $P > 0.2$), and did not vary annually (Tables 2 and 3). Cortisol levels were significantly higher in females compared with males (Figure 1C and Tables 2 and 3) and sub-adults had higher levels than adults (Figure 1C and Tables 2 and 3). Cortisol levels were similar between females with or without cubs (Figure 1C and Tables 2 and 3). Cortisol was correlated with the UC ratio ($r_s = 0.32$, $P = 0.03$; Figure 2A).

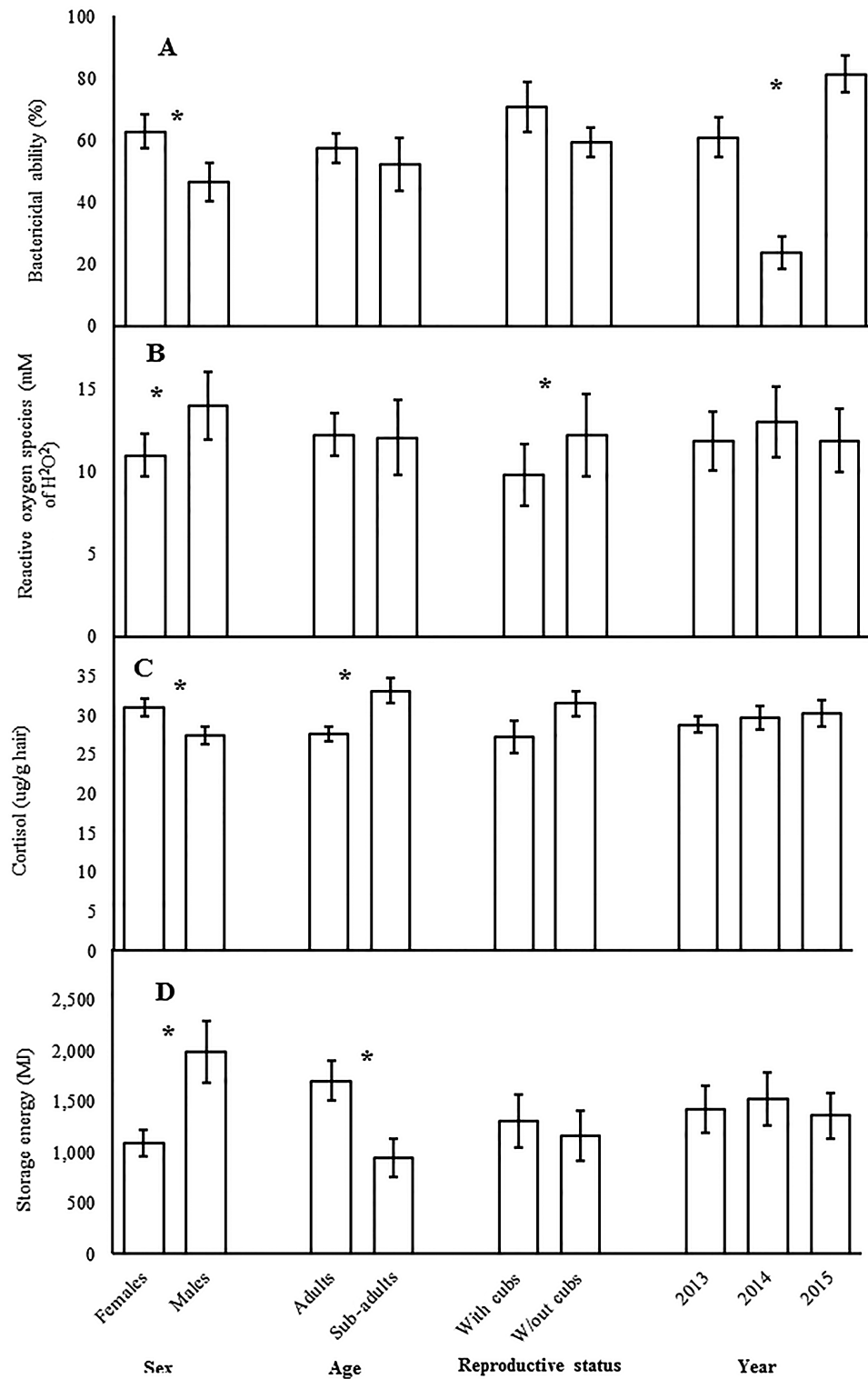


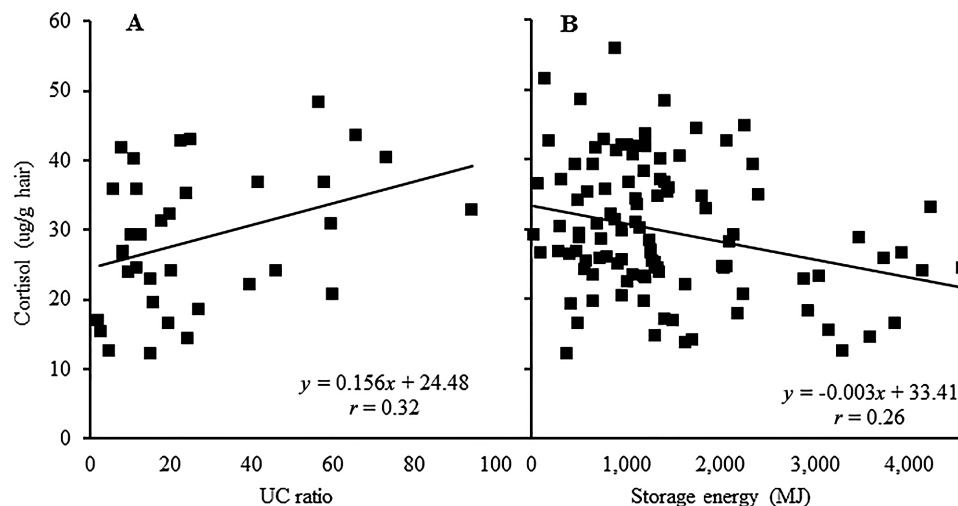
FIGURE 1 Comparison between sexes, age, reproductive status, and among years in polar bears (*Ursus maritimus*) from the southern Beaufort Sea for bactericidal ability (A), reactive oxygen species production (B), hair cortisol (C), and energy storage (D). Error bars indicate standard error. Asterisks indicate significant differences.

The UC ratio was significantly correlated with ROS production ($r_s = 0.35$, $P = 0.03$) and cortisol ($r_s = 0.32$, $P = 0.03$) but exhibited no correlation with lysis, bactericidal ability, or oxidative barrier (all P values > 0.1). Female bears had a higher UC ratio than males (Tables 2 and 3), but it was not influenced by age, reproductive status, or year (Tables 2 and 3). SE and BMI were highly

correlated ($r_s = 0.96$, $P < 0.0001$), but neither were correlated with UC ratio ($r_s < 0.05$, $P > 0.5$). SE was negatively correlated with cortisol ($r_s = -0.26$, $P < 0.01$; Figure 2B) but not correlated with any other metric ($P > 0.1$). BMI had a positive correlation with ROS ($r_s = 0.22$, $P = 0.03$) and a negative correlation with cortisol ($r_s = -0.24$, $P = 0.01$). No other immune metric was correlated with BMI ($P > 0.1$). SE and BMI

TABLE 3 Statistical relationships between sex, age group, reproductive status, and among years in polar bears (*Ursus maritimus*) sampled in the southern beaufort sea, 2013–2015

	Sex	Age Class	Reproductive State (Females Only)	Year
Bactericidal ability (%)	W = 1994.5 P = 0.05	W = 1338.5 P = 0.68	W = 366 P = 0.4	$\chi^2 = 31.58$ df = 2 P < 0.01
ROS (mM of H ₂ O ₂)	W = 1088 P = 0.002	W = 1258 P = 0.91	W = 185 P = 0.008	$\chi^2 = 0.58$ df = 2 P = 0.75
Oxidative barrier (mmol/l)	W = 1717.5 P = 0.84	W = 1258 P = 0.70	W = 292 P = 0.55	$\chi^2 = 54.22$ df = 2 P < 0.01
Lysis (CH ₅₀ units/ml)	W = 1175 P = 0.73	W = 971.5 P = 0.17	W = 208.5 P = 0.79	$\chi^2 = 13.13$ df = 2 P = 0.00
Urea to creatinine ratio	W = 283 P = 0.00	W = 44 P = 0.64	W = 48 P = 0.84	$\chi^2 = 4.72$ df = 2 P = 0.09
Storage energy ((x ₁ (body mass (kg)) - x ₂ (standard body length (m)) ³)	W = 1096 P = 0.007	W = 2188 P < 0.001	W = 312.5 P = 0.43	$\chi^2 = 0.36$ df = 2 P = 0.83
BMI (body mass (kg)/(standard body length (m)) ²)	W = 828 P < 0.001	W = 2212 P < 0.001	W = 322.5 P = 0.32	$\chi^2 = 1.61$ df = 2 P = 0.45
Cortisol (μg/g hair)	t = 2.18 df = 130 P = 0.03	t = -3.39 df = 130 P < 0.01	t = -1.67 df = 48 P = 0.10	F _(2,129) = 0.29 P = 0.75

**FIGURE 2** Correlation between cortisol concentrations in hair and UC ratio (A) and energy storage (B) in polar bears (*Ursus maritimus*) from the southern Beaufort Sea

were significantly higher for male bears (vs. females) and adult bears (vs. sub adults; Tables 2 and 3). However, SE and BMI were lower in females with two cubs versus one cub ($\chi^2 = 3.56$, $P = 0.06$; $\chi^2 = 3.77$, $P = 0.06$, respectively), but there was no difference among all three groups of females (no cubs, one cub, two cubs; Tables 2 and 3).

When we examined the relationships among the immune metrics, we found that oxidative barrier was positively correlated with bactericidal ability ($r_s = 0.31$; $P < 0.001$) and negatively correlated with lysis ($r_s = -0.24$; $P = 0.02$). Bactericidal ability was weakly correlated with lysis ($r_s = 0.2$, $P = 0.06$). There were no other correlations among the immune metrics ($r_s < 0.15$; $P > 0.2$).

The models examining the interactions of sex-year-age, sex-year, sex-age, and year-age were not significant for any physiological variable ($P > 0.08$), except the interaction between age and sex in SE ($t = -2.860$, $P < 0.01$).

4 | DISCUSSION

The present study revealed significant differences in immune signatures between sex and reproductive status, as well as yearly differences in polar bears in the southern Beaufort Sea.

Ecoimmunological tools, such as bactericidal ability, lysis capabilities, indicators of oxidative stress (ROS), and antioxidant capacity, were able to elucidate the innate immune relationships between these free-living bears and their sex, presence/absence of cubs, and yearly changes. Our predictions were generally supported by our findings: females had increased immune capabilities, which were positively correlated with bear nutritional status. However, novel findings, such as a decrease in ROS production in females with cubs and no immune differences between adults and sub-adults, reinforce the need for biologists to continue to examine diverse biological systems with as many ecoimmunological and physiological tools as possible. Not surprisingly hair cortisol, a retrospective hormone measure, was not related to relatively shorter-term physiological measures in the blood, likely because the metrics evaluate physiological processes on different time scales.

We found that bactericidal ability was elevated in females compared with males, a phenomenon that seems to be widespread in vertebrates as demonstrated by other studies (Grossman, 1985, Nalbandian & Kovats, 2005, Neuman-Lee, & French, 2017, Zuk & McKean, 1996). The differences seen between the sexes may be related to many factors, including genetic differences or differences in steroid hormone concentrations (reviewed in Oertelt-Prigione, 2012). For example, estradiol is considered immunoenhancing (Di Matteo, Baccari, Chieffi, & Minucci, 1995, Sthoeger, Chiorazzi, & Lahita, 1988), although this is not always the case (reviewed in Grossman, 1985, Mondal & Rai, 2002, Watanuki, Yamaguchi, & Sakai, 2002). While progesterone can play a role in suppressing immunity function (Graham, Earley, Guyer, & Mendonça, 2011, Watanuki et al., 2002), this typically occurs only during pregnancy to prevent rejection of the fetus (Butts & Sternberg, 2008, Grossman, 1985). Alternatively, relatively higher levels of circulating testosterone in males may be immunosuppressive (Greives et al., 2006). However, results supporting the association between testosterone and immunity are mixed (Muehlenbein & Bribiescas, 2005, Peters, Delhey, Denk Angelika, & Kempnaers, 2004).

Ecoimmunologists generally agree that immune activation is a costly endeavor (Bonneaud et al., 2003, Hasselquist, & Nilsson, 2012, Martin, Scheuerlein & Wikelski, 2003), which makes interpretation of ROS and oxidative barrier somewhat difficult. Increases in these values can indicate that there has been an elevation in metabolism and/or an increase in immune activity (Finkel & Holbrook, 2000, McCord, 2000). Our sampling period does overlap with the hyperphagic period when the main prey of polar bears (seal pups) are abundant, resulting in increased predation, and a possible increase in metabolism (Stirling & McEwan, 1975). Therefore, in the absence of explicit manipulation, all of our observations about ROS must be viewed with the understanding that these processes are interconnected.

Lower levels of ROS are expected in females, given that oxidative stress can be ameliorated by estrogens (Behl et al., 1997, Sawada et al., 1998) and progesterone (Roof, Hoffman, & Stein, 1997). Additionally, females seem to have the ability to modulate ROS production by increasing detoxifying enzymes and using different substrates than males for phosphorylation (Lagranha, Deschamps, Aponte, Steenbergen, & Murphy, 2010). However, the role of sex in predicting oxidative stress is often inconsistent (Lopez-Ruiz, Sartori-Valinotti, Yanes,

Iliescu, & Reckelhoff, 2008). This is possibly linked to the fact that oxidative stress is known to vary with growth, metabolic rates, and body size (Finkel & Holbrook, 2000, Kawaoka et al., 2003), which differ in sexes of sexual dimorphic species, including polar bears (Atkinson, Stirling & Ramsay, 1996, Stirling & Øritsland, 1995). Given the high degree of sexual dimorphism in this species (Derocher, Andersen, & Wiig, 2005), we also expected to see differences in oxidative stress. The hypothesis that body size is related to ROS production is reinforced by our findings that two of the metrics of resource availability (UC ratio and BMI) were positively correlated with ROS.

While we hypothesized that females with cubs would have an increase in ROS compared to females without cubs, we found the opposite to be true. In many species, there is an increase in ROS during reproduction (Al-Gubory, Fowler, & Garrel, 2010, Alonso-Alvarez et al., 2004, Wang, Salmon, & Harshman, 2001), but there is also often an increase in the antioxidant barrier (Al-Gubory et al., 2010). We did not detect an increase in antioxidant barrier. Most of these studies have examined reproductive females during gestation or vitellogenesis, depending on species, and thus are not necessarily representing the reproductive state of the females we measured. Given that the timing of physiological sampling in the field was during the mating season, the females without cubs could be actively preparing for mating and reproduction. Furthermore, as progesterone increases during pregnancy (Palmer, Nelson, Ramsay, Stirling, & Bahr, 1988), we would expect to observe oxidative stress to vary with pregnancy stage. For example, estrogens and progestins seem to mediate a reduction in H_2O_2 production in the mitochondria, as described in isolated rat vasculature by Stirone, Duckles, Krause, and Procaccio (2005). Another potential effector of ROS in wild animals, particularly apex predators such as polar bears, is contamination. (Muir et al., 1999). The lower concentration of ROS in females overall, and in females with cubs in particular, may be the result of the off-load of ROS-inducing contaminants (Debieer et al., 2003, Ercal, Gurer-Orhan & Aykin-Burns, 2001, Srinivasan, Lehmler, Robertson & Ludewig, 2001).

Unexpectedly, we did not find a difference in immune function between sub-adults and adults. However, there are several possible explanations. First, the sample size is low, which may mask potential differences. Secondly, sub-adults do not include cubs (which did not have their blood drawn for analyses). Therefore, the sub-adults may have already fully developed their immune system proficiency. There may be immunological differences present between sub-adult and adult polar bears in immune components not measured in the present study. For example, much of the work examining immune differences during ontogenetic stages in birds and mammals, including humans in medical research, has focused on transfer of maternal antibodies and development of the humoral immune system (Brandenburg et al., 1997, Burgio, Lanzavecchia, Plebani, Jayakar, & Ugazio, 1980, Samukawa, Yamanaka, Hollingshead, Klingman, and Faden, 2000, Siegrist, 2001), which we did not examine in this study. Because of the innate immune system's role in primary defense, many components develop relatively early (Arriero et al., 2013). There are also possible ecological reasons for the lack of a difference in immunity between sub-adults and adults. Sub-adult polar bears remain with mothers for up to 2 years and thus their condition is largely dependent on maternal foraging and

condition. In addition, they are not yet subjected to the stress of breeding and reproduction.

The annual variation in lysis, bactericidal ability, and oxidative barrier demonstrate the value in multi-year sampling of populations. Immune activation can be highly dependent upon a variety of factors, such as environmental conditions, population density, disturbance (stress), current infection status, antigens present in the individual, and resource availability (Budischak et al., 2012, Dhabhar, 2009, French, DeNardo, Greives, Strand & Demas, 2010, Mugabo, Perret, Decenci re, Meylan & Le Galliard, 2014, Nelson & Demas, 1996, Neuman-Lee et al., 2015). Unexpectedly, we did not find a relationship between the measures of resource availability (UC ratio, BMI, and SE) and these yearly differences. Therefore, the total resource availability and animal condition differences are likely not explaining the yearly differences.

Cortisol can be another energy indicator in animals and is known to influence the immune system (Dhabhar, 2009, Sapolsky et al., 2000). Cortisol did not vary across years. This finding is contrary to results in Greenland polar bears, where hair cortisol concentrations were related to the North Atlantic Climate Oscillation index over time (Bechsh ft et al., 2013). The discrepancy between the two studies is likely due to both regional environmental differences and different time scales (3 years in the present study versus 20 years in the study by Bechsh ft et al., 2013).

In the present study, cortisol did differ between males and females and between sub-adults and adults, but was not directly related to immunity. This lack of a relationship between immune metrics and cortisol was unexpected, but, while there is a theoretical relationship between cortisol and metrics of fitness, such as immune function, empirical evidence of this relationship is not always seen in animal systems (Bonier, Martin, Moore, & Wingfield, 2009). This indicates the strong need for studying endocrinology in wild organisms to determine patterns and detect deviations (McCormick & Romero, 2017).

There was also a strong negative correlation between cortisol and the long-term energy metrics of BMI and SE, and a positive correlation between the short-term energy metric of UC ratio. These differing relationships between hair cortisol and the two energy measures may be in part due to the different time scales of the metrics and the different life history or environmental conditions over the course of those different time scales. For example, hair regrowth, and so likely cortisol and long-term energy storage, are both longer duration measures that look retrospectively at the previous summer-fall (Derocher, 2012) when resources are more plentiful versus the short-term energy storage is more recent spring months when resources are more scarce. The higher levels of cortisol in female bears is an expected pattern, and has been demonstrated in another population of polar bears (Bechshoft et al., 2015). Higher levels of cortisol in sub-adult bears may indicate that they are more sensitive to disturbance than adults. There have been no clear ontogenetic patterns in wildlife with regard to GCs, such as cortisol, likely because different species undergo a variety of changes that affect hormone production at different points in their life (Baker, Gobush, & Vynne, 2013). We found a negative relationship between hair cortisol levels and long-term

energy reserves indicating that bears with better body condition were likely less physiologically stressed and/or mobilizing fewer energy reserves. However, interpretations of hair cortisol are still in infancy due to the paucity of published data on how complex interactions affect concentration. In studies examining fecal and feather concentrations of GCs, factors such as storage methods, animal diet, and size of the sample can dramatically alter concentrations (Lattin, Reed, DesRochers, & Romero, 2011, Millsbaugh & Washburn, 2004).

The more short-term indicator of UC ratio was positively correlated with hair cortisol. Given cortisol's role as an energy mobilizer, this relationship may be expected as an individual with lower levels of resource availability may be catabolizing the given resources (Cockrem Potter, & Candy, 2006, Romero & Wikelski, 2001). However, these two metrics are potentially not related as they are occurring on a different time scale. When examining the relationships between the physiological metrics, we must recognize that these metrics are representing bear status on different time scales. Therefore, direct comparison must be viewed in this light. The factors of bactericidal ability, lysis, and UC ratio are physiological markers representing physiological conditions on the order of hours to days (Cherry et al., 2009, Demas et al., 2011, Neuman-Lee & French, 2014). In contrast, the other metrics (ROS, oxidative barrier, cortisol, SE, and BMI) represent conditions on the order of weeks to months (Cattet et al., 2014, Finkel & Holbrook, 2000, McCord, 2000, Meyer & Novak, 2012, Moln r et al., 2009). One of the benefits of examining multiple immune and physiological metrics is to measure animal's condition over a broad time scale.

This study demonstrated the importance of examining multiple immune characteristics from a diverse array of individuals in a population. By gathering data on individuals of different sexes, ages, and reproductive status, we are able to provide a much clearer picture of the physiological state of the population as a whole. Further, this research demonstrated the need for sampling over multiple years to ensure a more holistic view of the population is achieved. Finally, by utilizing multiple physiological metrics, we can start to parse the relationships and immune effects that different groups of individuals may undergo during their life cycle.

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ORCID

Susannah S. French  <http://orcid.org/0000-0001-8923-9728>

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