Reference intervals for blood-based biochemical analytes of southern Beaufort Sea polar bears

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Accurate reference intervals (RIs) for commonly measured blood-based analytes are essential for health monitoring programmes. Baseline values for a panel of analytes can be used to monitor physiologic and pathophysiologic processes such as organ function, electrolyte balance and protein catabolism. Our reference population includes 651 serum samples from polar bears (Ursus maritimus) from the southern Beaufort Sea (SB) subpopulation sampled in Alaska, USA, between 1983 and 2016. To establish RI for 13 biochemical analytes, we defined specific criteria for characterizing the reference population and relevant subgroups. To account for differences in seasonal life history characteristics, we determined separate RI for the spring and fall seasons, when prey availability and energetic requirements of bears differ. We established RI for five subgroups in spring based on sex, age class and denning status, and three subgroups in fall based on sex and age class in females only. Alkaline phosphatase activities were twice as high in subadult as in adult polar bears in spring ($z_{\text{males}} = 4.08, P_{\text{males}} < 0.001, z_{\text{females}} = 3.90, P_{\text{females}} < 0.001$) and did not differ between seasons. Denning females had significantly higher glucose concentrations than non-denning females ($z = 4.94, P < 0.001$), possibly reflecting differences in energy expenditure during lactation. A total of 10 of the 13 analytes differed significantly between seasons in either males or females; however, the physiologic importance of these differences may be minimal. Establishing these RIs allows for temporal monitoring of polar bear health in the SB and may prove useful for assessing and monitoring additional polar bear subpopulations in a changing Arctic environment.

Key words: Arctic, blood biochemistry, polar bear, reference interval, serum analytes, wildlife health

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Introduction

Climate change is rapidly affecting the Arctic region. Arctic ocean temperatures have risen at over twice the average rate of global warming with models suggesting that the Beaufort Sea could increase 4°C above the 1981–2010 average by 2040 (Overland et al., 2018), accelerating abiotic and biotic changes (IPCC, 2018). With warming
temperatures and changes in sea ice phenology, polar bears (Ursus maritimus) are being exposed to novel stressors related to changes in habitat, nutrition, competition, and pollutants (Burek et al., 2008). Observed effects associated with environmental changes in polar bears include increased rates of fasting (Cherry et al., 2009; Rode et al., 2018), declines in body condition and cub recruitment, (Rode et al., 2010, 2012, 2014; Obbard et al., 2016) and declines in survival and abundance (Regehr et al., 2007; Bromaghin et al., 2015; Obbard et al., 2018). However, the effects of chronic environmental stressors on metabolic processes, physiologic function and health are poorly understood (Atwood et al., 2015; Bowen et al., 2015; Fagre et al., 2015; Patyk et al., 2015). Thus, there is a critical need to describe biomarkers that can be used as a component in monitoring polar bear health (Friedrichs, 2009; Patyk et al., 2015).

A common method for assessing physiologic function and pathology in animals is to measure blood-based analytes, which include measures of organ system function, electrolytic balance, enzyme activity, protein abundance and nutrition. Deviations from expected values of blood-based analytes are commonly used to ascertain pathologic states (Friedrichs et al., 2012). A precursor to effectively using such indices is establishment of reference intervals (RIs), which are baseline values for each analyte derived from a normal, healthy reference population. Grasbeck and Saras (1969) first introduced the concept of theoretical RI as values obtained under controlled conditions with ‘healthy, normal’ individuals as the reference population (Grasbeck, 1990). A RI is mostly commonly delimited by the central 95% of the reference population with the low and high limits bounding the interval (Geffré et al., 2009; Friedrichs et al., 2012). Hanks (1981) outlined the usefulness of blood-based variables to assess physical condition and health status of wildlife as well as to assess disease status and changes in the environment. Friedrichs (2009) further suggested that RI could be used to assess the physiologic health of individuals, populations or ecosystems.

When calculating RI, it is important to consider life history variables that may influence blood-based analytes of individuals. In wild, free-living animals, this may include seasonal impacts. Seasonal fluctuations in blood biochemistry can result from a variety of factors, including diet and nutrition, reproduction, behaviour and metabolic requirements (Lathi, 2004; Friedrichs et al., 2012). Ursids demonstrate substantial variation in biochemical values depending on habitat, behaviour and diet (Lee et al., 1977; Matula et al., 1980; Nelson et al., 1983; Brannon, 1985; Schroeder, 1987; Franzmann and Schwartz, 1988; Ramsay et al., 1991; Tryland et al., 2002). Identifying seasonal changes in RI is especially important for polar bears, given the extreme seasonality of their life history and physiologic adaptations, such as hyperphagia in the spring and extended fasts in other seasons (Atkinson and Ramsay 1995; Cherry et al., 2009; Rode et al., 2018).

Our objective was to use the southern Beaufort Sea (SB) subpopulation of polar bears to define RI that can be used to monitor the health of the SB subpopulation and for comparisons to other subpopulations. Specifically, we used polar bear blood chemistry values collected over 34 years (1983–2016) to define RI for 13 common serum analytes that measure liver and kidney function and status, immune system activity, dietary intake and electrolyte and mineral balance. We also examined variation in analytes across subgroups, such as denning status, age and sex, in both spring and fall.

**Materials and methods**

Polar bears were captured, sampled and released on the sea ice of the SB, Alaska, as part of a long-term research programme. Spring captures most commonly occurred on sea ice from 1983 to 2016 typically between March 20th and May 5th. Fall captures took place on sea ice and on land between August and November, intermittently from 1983 to 2009. The study area included the Alaska portion of the SB subpopulation, bounded by Icy Cape, Alaska, to the west and the United States–Canada border on the east and extended from the coast to ~90 km over sea ice in most years (Fig. 1). Polar bears were located from a helicopter and immobilized with a rapid-injection dart (Palmer Cap-Chur Equipment, Douglasville, Georgia, USA) containing Sernylan or M-99 prior to 1987 and thereafter, zolazepam-tiletamine (Telazol® or Zoletil®; Stirling et al., 1989). Immobilized bears were aged, weighed to the nearest kg and marked with an ear tag number and a unique tattoo on the upper lip. Polar bears ≥5 years old were classed as adults, and 3- and 4-year-old polar bears were classed as subadults. Denning status was ascertained when a female polar bear was captured with young of the year. Capture and handling of polar bears were conducted under appropriate research permits, including Marine Mammal Research Permit MA690038-17.

We collected blood into evacuated plain tubes (Vacutainer; BD Biosciences, Franklin Lanes, NJ) by venipuncture of the femoral vein. Whole blood was stored in a cooler with chemical heat packs to prevent freezing until returning from the field, at which point serum was separated from blood by centrifugation at 1500 g for 5 min (TRIAC; Clay Adams, Parsippany, NJ) and frozen at −20°C. At the conclusion of the field season, sera were stored at −70°C until analysed. Sera were analysed using a VetScan VS2 Biochemistry Analyser (Abaxis, Union City, CA) to measure the following analytes: alanine aminotransferase (ALT), alkaline phosphatase (ALP), albumin (ALB), blood urea nitrogen (BUN), calcium (CA), creatinine (CREA), glucose (GLU), phosphorus (PHOS), potassium (POT), sodium (NA), total bilirubin (TBIL) and total protein (TP). Globulin (GLOB) was calculated by subtracting ALB from TP. These analytes comprise the comprehensive diagnostic profile defined by Abaxis. The functional and interpretive characteristics of each analyte are summarized in Table 1 (Stockham and Scott, 2013). We established RI based on
the guidelines of the American Society of Veterinary Clinical Pathology (Friedrichs et al., 2012). We calculated RI for each of the 13 serum analytes using the Excel macros Reference Value Advisor (Geffré et al., 2011). Outliers were removed based on Dixon’s range statistic (see Geffré et al., 2011). In addition, individuals with two or more outliers in their analytic panel were excluded from the reference population under the assumption that this may indicate a deviation from health.

We defined subgroups based on age class, sex and denning status, each of which may influence physiologic processes (Friedrichs et al., 2012) as well as samples size. To reflect the life history traits of polar bears, spring RIs were calculated for five subgroups (females: non-denning adults, denning adults and subadults; males: adults and subadults (Table 2), and fall RIs were calculated for three subgroups (female adults, female subadults and males). Males were not further subdivided by age class in fall in order to maintain a sample size ≥20 (Friedrichs et al., 2012). The decision to create only one subgroup for males in fall was strengthened because none of the analytes had a difference in means >25% (Sinton et al., 1986), and confidence intervals between the two age groups overlap for all analytes with the exception of BUN. All samples were independent; an individual polar bear was only in a subgroup once.

RIs were calculated using non-parametric methods when samples sizes were adequate (n ≥ 40). We used parametric analyses when 20 < n < 40 and the distribution was Gaussian. We used a BoxCox transformation with parametric analysis when transformation to a Gaussian distribution was necessary (Daly et al., 2017). Upper and lower confidence intervals were calculated using non-parametric bootstrap methods when 20 ≤ n ≤ 120 and according to tables when 120 ≤ n ≤ 370 (CLSI 2008; Geffré et al., 2011). When data could not be transformed to a Gaussian distribution, RIs were defined as the minimum and maximum values with lower and upper 90% confidence intervals excluded. To assess statistical differences between subgroups and season, we compared the means of each analyte using a generalized linear model with Tukey’s multiple comparison of the means. We assessed physiologic importance of differences in RI using the upper and lower confidence intervals between subgroups and seasons; if the upper or lower reference limit was bounded by the comparative subgroup confidence interval, the RIs were considered to have limited physiologic difference.

Figure 1: Between 1983 and 2016, polar bears were captured within the International Union for Conservation of Nature defined boundary (thin line) for the SB subpopulation between Icy Cape, Alaska, and the United States–Canada Border.
Table 1: Summary of blood-based analytes

| Analyte | Tissue source or function | Brief interpretive use |
|---------|---------------------------|------------------------|
| ALT     | Liver and muscle          | Increases in some hepatic and severe muscle disorders |
| ALB     | Synthesized by liver, source of amino acids, acts as carrier protein | Increase with dehydration; decreases in some liver, renal, and inflammatory disorders |
| ALP     | Primarily liver and bone  | Increases in some liver and bone disorders, increases during active bone growth (juveniles) |
| TBIL    | Product of erythrocyte catabolism, processed by the liver and eliminated in bile | Increases with hemolysis or in disease of the liver and biliary system |
| BUN     | Product of protein catabolism, source of nitrogen for protein synthesis, eliminated primarily by kidney | Decreases with low protein intake and liver failure, increases with high protein meals and with decreased renal elimination (↓ GFR) |
| CA      | Structural component of bone; important cation for enzymatic, neurologic and muscular function | Approximately 50% bound to ALB, may be altered by vitamin D disorders |
| PHOS    | Structural component of bone, important anion for energy generation (ATP) | Increases with decreased renal elimination (↓ GFR) |
| CREA    | Catabolic product of muscle, eliminated through kidney | Low muscle mass results in lower basal concentrations, increases with decreased renal elimination (↓ GFR) |
| GLU     | Energy metabolite derived from food intake and hepatic synthesis, stored as glycogen in the liver | Strictly regulated by insulin, glucagon and other hormones, increased by glucocorticoid secretion (termed a stress response) |
| NA      | Important cation for osmoregulation | Strictly regulated by several hormonal systems and renal function |
| POT     | Important cation for neurologic and muscular activity | Strictly regulated by several hormonal systems and renal function |
| TP      | Comprised of ALB and many different GLOB molecules | Changes in TP are reflected by changes in ALB, GLOBs or both |
| GLOB    | Comprised of many different protein molecules that function in immunity and coagulation and as carrier molecules | Increased GLOBs indicate an immune response of significant duration (several days or more), individual GLOBs can be measured for specific information |

GFR means glomerular filtration rate, a measure of kidney function; GFR decreases with dehydration, renal failure and urinary bladder obstruction.

*Functional and interpretive characteristics are described by Stockham and Scott (2013).

Table 2: Number of polar bears sampled by season

|         | Spring | Fall |
|---------|--------|------|
|         | Females | Males | Females | Males |
| Adults (non-denning) | 184     | 161   | 114     | 18    |
| Subadult | 43      | 30    | 38      | 15    |
| Denning adults | 48      | —     | —       | —     |

Results

Our reference population included 651 polar bear samples (Table 2). Bears in the reference population had a body condition score ≥3 (ranking 1–5, with 5 = obese; Stirling et al., 2008) and had unremarkable physical exams. A summary of RIs, including sample size, summary statistics and 90% upper and lower confidence intervals for each of the 13 analytes, is reported in Table 3 for female polar bears and Table 4 for male polar bears as well as statistically significant differences between subgroups. We report both statistical and physiologic differences in our results. Outliers were identified in the analysis of 32 out of 104 RIs. In 20 RI calculations, the outliers represented <9% of the reference population, and in cases where outliers represented a greater percentage of the reference population, the sample size was small (n < 8). Outliers were distributed throughout the duration of the study.
Table 3: RIs for female polar bears from the SB

| Analytes | Units | Season | Subgroup | n  | Mean | Standard deviation | Median | Minimum | Maximum | RI     | 90% Confidence interval for lower limit | 90% Confidence interval for upper limit |
|----------|-------|--------|----------|----|------|-------------------|--------|---------|---------|--------|-------------------------------------|--------------------------------------|
| ALT U/l  |       | Spring | Adult    | 162| 50   | 25                | 43     | 11      | 160     | 16–126 | 11–25                              | 96–160                               |
|          |       |        | Denning<sup>a</sup> | 32 | 33   | 9                 | 35     | 13      | 55      | 14–53  | 9–18                               | 48–58                                |
| Fall     |       | Subadult<sup>c</sup> | 33 | 27   | 9                 | 25     | 14      | 48      | 13–49  | 11–15                              | 42–58                                |
|          |       | Adult  | 110 | 28   | 12                | 27     | 11      | 70      | 12–65  | 11–13                             | 50–70                                |
|          |       | Subadult | 37 | 27   | 8                 | 26     | 15      | 43      | 14–45  | 12–16                             | 40–51                                |
|          |       | Adult<sup>c</sup> | 179| 5.5  | 0.4              | 5.6    | 4.4     | 6.4     | 4.7–6.3 | 4.4–4.9 | 6.2–6.4                    |                                       |
| Spring   |       | Denning<sup>c</sup> | 48 | 5.3  | 0.4              | 5.2    | 4.3     | 6.0     | 4.4–6  | 4.4–4.7 | 5.9–6                                 |
| ALB g/dl |       | Subadult<sup>a</sup> | 42 | 5.5  | 0.4              | 5.4    | 4.7     | 6.2     | 4.7–6.2 | 4.7–5.1 | 6–6.2                              |                                       |
|          |       | Fall   | Adult  | 111 | 5.5  | 0.5              | 5.6    | 4.2     | 6.5     | 4.3–6.5 | 4.2–4.7                             | 6.3–6.5                             |
|          |       | Subadult | 38 | 5.6  | 0.3              | 5.6    | 5.0     | 6.2     | 4.9–6.3 | 4.8–5.1 | 6.1–6.4                             |                                       |
|          |       | Adult  | 165 | 40   | 27               | 32     | 6       | 139     | 8–116  | 6–10                          | 91–139                               |
|          |       | Denning | 45 | 32   | 29               | 20     | 5       | 126     | 5–122  | 5–8                          | 94–126                               |
| ALP U/l  |       | Subadult<sup>c</sup> | 38 | 66   | 26               | 68     | 26     | 132     | 12–120 | 1–24                         | 108–132                              |
|          |       | Fall   | Adult<sup>c</sup> | 106 | 32   | 25               | 24     | 3       | 127     | 6–115  | 3–9                         | 88–127                               |
|          |       | Subadult | 36 | 64   | 41               | 50     | 10      | 173     | 11–181 | 7–18                      | 141–229                              |
|          |       | Adult  | 187 | 16.7 | 13.1             | 12.0   | 2.0     | 57.0    | 2.4–48.8 | 2–2.7                      | 45–57                               |
|          |       | Denning | 49 | 15.0 | 12.3             | 11.0   | 1.0     | 47.3    | 1.4–46  | 1–3                      | 39.5–47.3                             |
| BUN mg/dl |       | Subadult | 42 | 16.6 | 14.2             | 11.8   | 1.0     | 53.5    | 1–53.5 | 1–3                        | 44–53.5                              |
|          |       | Fall   | Adult  | 113 | 16.6 | 18.0             | 9.5    | 2.7     | 114.5   | 3–70.3 | 2.7–3.9                 | 53.2–114.5                           |
|          |       | Subadult | 32 | 10.2 | 5.9              | 8.0    | 4.0     | 24.3    | 4.1–28.9 | 3.9–4.6                 | 20.2–38.3                           |
|          |       | Adult  | 176 | 9.9  | 0.7              | 9.9    | 7.5     | 12.5    | 8–11.5 | 7.5–8.8 | 11.1–12.5                       |                                       |
|          |       | Denning | 48 | 9.8  | 0.7              | 9.8    | 7.8     | 12.4    | 7.8–12 | 7.8–8.8 | 10.7–12.4                       |                                       |
| CA mg/dl |       | Subadult | 42 | 10.2 | 0.4              | 10.1   | 9.4     | 11.1    | 9.5–11 | 9.5–9.6 | 10.7–11.1                       |                                       |
|          |       | Fall   | Adult  | 109 | 10.1 | 0.9              | 10.2   | 7.3     | 12.7    | 7.8–11.9 | 7.3–8.2              | 11.5–12.7                           |
|          |       | Subadult | 37 | 10.4 | 0.6              | 10.5   | 9.3     | 11.7    | 9.3–11.6 | 9.1–9.6              | 11.3–11.9                           |

(Continued)
| Analytes | Units | Season | Subgroup | n  | Mean | Standard deviation | Median | Minimum | Maximum | RI   | 90% Confidence interval for lower limit | 90% Confidence interval for upper limit |
|----------|-------|--------|----------|----|------|-------------------|--------|---------|---------|------|--------------------------------------|--------------------------------------|
| GLU      | mg/dl | Fall   | Adult    | 111| 1.0  | 0.3                | 1.0    | 0.6     | 1.8     | 0.4  | 1.1–1.1                              | 1.2–1.1                              |
|          |       | Subadult | 38  | 1.0  | 0.3                | 1.0    | 0.6     | 1.9     | 0.6  | 1.1–1.2                              | 1.3–1.2                              |
|          |       | Fall   | Adult    | 111| 1.0  | 0.3                | 1.0    | 0.6     | 1.9     | 0.6  | 1.1–1.2                              | 1.3–1.2                              |
|          |       | Subadult | 38  | 1.0  | 0.3                | 1.0    | 0.6     | 1.9     | 0.6  | 1.1–1.2                              | 1.3–1.2                              |
|          |       | Spring | Denning  | 48 | 1.3  | 0.5                | 1.3    | 0.2     | 2.3     | 0.2  | 0.9–1.3                              | 1.6–2.3                              |
| CREA     | mg/dl | Fall   | Adult    | 111| 0.9  | 0.2                | 0.9    | 0.4     | 1.7     | 0.4  | 0.6–1.5                              | 1.4–1.7                              |
|          |       | Subadult | 42  | 0.9  | 0.2                | 0.9    | 0.5     | 1.4     | 0.5  | 0.7–1.4                              | 1.2–1.4                              |
|          |       | Fall   | Adult    | 111| 0.9  | 0.2                | 0.9    | 0.5     | 1.4     | 0.5  | 0.7–1.4                              | 1.2–1.4                              |
|          |       | Subadult | 42  | 0.9  | 0.2                | 0.9    | 0.5     | 1.4     | 0.5  | 0.7–1.4                              | 1.2–1.4                              |
|          |       | Fall   | Adult    | 111| 0.9  | 0.2                | 0.9    | 0.5     | 1.4     | 0.5  | 0.7–1.4                              | 1.2–1.4                              |
|          |       | Subadult | 42  | 0.9  | 0.2                | 0.9    | 0.5     | 1.4     | 0.5  | 0.7–1.4                              | 1.2–1.4                              |
|          |       | Fall   | Adult    | 111| 0.9  | 0.2                | 0.9    | 0.5     | 1.4     | 0.5  | 0.7–1.4                              | 1.2–1.4                              |
|          |       | Subadult | 42  | 0.9  | 0.2                | 0.9    | 0.5     | 1.4     | 0.5  | 0.7–1.4                              | 1.2–1.4                              |
Table 3: Continued

| Analytes | Units | Season | Subgroup | n  | Mean | Standard deviation | Median | Minimum | Maximum | RI  | 90% Confidence interval for lower limit | 90% Confidence interval for upper limit |
|----------|-------|--------|----------|----|------|--------------------|--------|---------|---------|-----|--------------------------------------|---------------------------------------|
| **NA**   | mmol/l| Spring | Adult    | 178| 137  | 4                  | 137    | 124     | 154     | 128–148 | 124–130                             | 144–154                               |
|          |       |        | Denning  | 44 | 136  | 4                  | 137    | 129     | 145     | 129–145 | 129–130                             | 141–145                               |
|          |       |        | Subadult | 40 | 140  | 2                  | 139    | 136     | 146     | 136–146 | 136–137                             | 143–146                               |
|          |       | Fall   | Adult    | 112| 143  | 10                 | 142    | 112     | 180     | 123–168 | 112–130                             | 162–180                               |
|          |       |        | Subadult | 38 | 143  | 6                  | 141    | 133     | 159     | 133–159 | na                                  | na                                    |
|          |       |        | Adult    | 181| 0.3  | 0.1                | 0.3    | 0.1     | 0.7     | 0.2–0.5 | 0.1–0.2                             | 0.5–0.7                               |
|          |       |        | Subadult | 49 | 0.3  | 0.1                | 0.3    | 0.2     | 0.5     | 0.2–0.5 | na                                  | 0.4–0.5                               |
| **TBIL** | g/dl  | Spring | Denning  | 41 | 0.3  | 0.05               | 0.3    | 0.2     | 0.3     | 0.2–0.3 | na                                  | na                                    |
|          |       |        | Subadult | 41 | 0.3  | 0.05               | 0.3    | 0.2     | 0.3     | 0.2–0.3 | na                                  | na                                    |
|          |       | Fall   | Adult    | 108| 0.3  | 0.1                | 0.3    | 0.2     | 0.7     | 0.2–0.6 | na                                  | 0.5–0.7                               |
|          |       |        | Subadult | 38 | 0.3  | 0.1                | 0.3    | 0.2     | 0.5     | 0.2–0.5 | na                                  | na                                    |
|          |       |        | Adult†   | 180| 7.0  | 0.4                | 6.9    | 5.4     | 8.4     | 6–7.9  | 5.4–6.4                             | 7.6–8.4                               |
|          |       |        | Denning† | 46 | 6.6  | 0.4                | 6.6    | 5.6     | 7.4     | 5.7–7.4 | 5.6–6                               | 7–7.5                                 |
|          |       |        | Subadult | 41 | 6.8  | 0.2                | 6.8    | 6.4     | 7.3     | 6.5–7.3 | 6.5–6.6                             | 7.2–7.3                               |
|          |       | Fall   | Adult    | 111| 7.6  | 0.9                | 7.5    | 5.6     | 10.4    | 5.8–9.8 | 5.6–6.3                             | 9.2–10.4                              |
|          |       |        | Subadult | 37 | 7.3  | 0.5                | 7.2    | 6.4     | 8.2     | 6.3–8.2 | 6.1–6.5                             | 8–8.4                                 |

*Non-denning females are defined as ‘adult’.
*Subgroups with different letters are within the same season are significantly different (P < 0.01).
Table 4: RIs for male polar bears from the SB

| Analytes | Units | Season | Subgroup | n  | Mean | Standard deviation | Median | Minimum | Maximum | RI    | 90% Confidence interval for lower limit | 90% Confidence interval for upper limit |
|----------|-------|--------|----------|----|------|-------------------|--------|---------|---------|-------|----------------------------------------|---------------------------------------|
| ALT      | U/l   | Fall   | Adult    | 33 | 27   | 9                 | 25     | 14      | 48      | 11    | 9-18                                   | 48-58                                 |
|          |       |        | Subadult | 32 | 33   | 9                 | 35     | 13      | 55      | 14-53 | 9. -18                                 | 48-58                                 |
| ALB      | g/dl  | Spring | Adult    | 162| 5.5  | 0.4               | 5.5    | 3.8     | 6.4     | 11    | 3.8-4.8                                | 6.1-6.4                               |
|          |       |        | Subadult | 31 | 5.5  | 0.3               | 5.4    | 4.9     | 6.2     | 4.9   | 4.7-5                                  | 6-6.3                                 |
| ALP      | U/l   | Fall   | Adult    | 32 | 59.2 | 29.6              | 53.3   | 14.0    | 123.7   | 123   | 14-123.7                               | na                                    |
|          |       |        | Subadult | 32 | 53.1 | 6.4               | 6.2    | 4.8     | 6.4     | 4.9   | 4.7-5                                  | 6-6.4                                 |
| BUN      | mg/dl | Spring | Adult    | 162| 11.3 | 1.1               | 7.8    | 1.0     | 65.0    | 1.3  | 1-2                                    | 30-65                                 |
|          |       |        | Subadult | 31 | 14.5 | 1.0               | 10.2   | 1.0     | 38.7    | 1-3  | 33-62                                  | 0.4-3                                 |
| CA       | mg/dl | Fall   | Adult    | 32 | 17.6 | 1.2               | 13.0   | 3.0     | 49.0    | 2.6  | 2.3-3.9                                | 39-66.6                               |
|          |       |        | Subadult | 31 | 10.1 | 0.5               | 10.1   | 0.5     | 9.4     | 1-1  | 10.9-11.4                              | 10.9-11.4                             |
| CREA     | mg/dl | Spring | Adult    | 162| 1.3  | 0.3               | 1.3    | 0.5     | 2.3     | 0.7  | 0.5-0.8                                | 1.8-2.3                               |
|          |       |        | Subadult | 32 | 1.0  | 0.2               | 0.9    | 0.4     | 1.5     | 0.5  | 0.4-0.6                                | 1.3-1.5                               |
| GLOB     | g/dl  | Fall   | Adult    | 33 | 1.8  | 0.5               | 1.8    | 1.1     | 3.0     | 0.9  | 0.7-1.1                                | 2.6-3                                 |
|          |       |        | Subadult | 32 | 1.5  | 0.4               | 1.5    | 0.5     | 2.3     | 0.6  | 0.4-0.8                                | 2.1-2.5                               |
| GLU      | mg/dl | Spring | Adult    | 161| 125  | 2.5               | 124    | 59      | 199     | 80   | 59-86                                  | 167-199                               |
|          |       |        | Subadult | 31 | 123  | 26                | 124    | 72      | 188     | 69   | 56-82                                  | 164-192                               |
|          |       |        | Adult    | 30 | 119  | 24                | 124    | 47      | 148     | 51   | 47-82                                  | 148-162                               |

(Continued)
### Table 4: Continued

| Analytes | Units | Season | Subgroup | n  | Mean | Standard deviation | Median | Minimum | Maximum | RI   | 90% Confidence interval for lower limit | 90% Confidence interval for upper limit |
|----------|-------|--------|----------|-----|------|-------------------|--------|---------|---------|------|---------------------------------------|----------------------------------------|
| PHOS     | mg/dL | Spring | Adult    | 162 | 6.1  | 1.2               | 6.2    | 3.1     | 9.4     | 3.7  | 8.6                                   | 3.1–4.1                                |
|          |       |        | Subadult | 28  | 6.3  | 0.8               | 6.3    | 5.1     | 7.9     | 4.8  | 7.3                                   | 4.4–5.2                                |
|          |       | Fall   | Adult    | 33  | 5.8  | 1.6               | 5.5    | 3.4     | 9.1     | 3.2  | 9.6                                   | 2.8–3.7                                |
|          |       |        |          |     |      |                   |        |         |         |      |                                       |                                       |
| POT      | mmol/L| Spring | Adult    | 158 | 4.6  | 0.3               | 4.6    | 3.8     | 5.4     | 4   | 5.2                                   | 3.9–4.1                                |
|          |       |        | Subadult | 31  | 4.5  | 0.4               | 4.6    | 3.6     | 5.3     | 3.7  | 5.3                                   | 3.5–3.9                                |
|          |       | Fall   | Adult    | 33  | 4.6  | 0.5               | 4.6    | 3.4     | 5.5     | 3.6  | 5.5                                   | 3.4–3.9                                |
|          |       |        |          |     |      |                   |        |         |         |      |                                       |                                       |
| NA       | mmol/L| Spring | Adult    | 162 | 139 | 3                 | 139    | 119     | 155     | 132  | 150                                  | 119–133                                |
|          |       |        | Subadult | 32  | 140 | 3                 | 140    | 133     | 146     | 133  | 145                                  | 132–145                                |
|          |       | Fall   | Adult    | 32  | 140 | 3                 | 140    | 133     | 146     | 133  | 144                                  | 132–144                                |
|          |       |        |          |     |      |                   |        |         |         |      |                                       |                                       |
| TBIL     | g/dL  | Spring | Adult    | 161 | 0.3  | 0.1               | 0.3    | 0.1     | 0.4     | 0.2  | 0.4                                  | 0.1–0.2                                |
|          |       |        | Subadult | 32  | 0.3  | 0.1               | 0.3    | 0.2     | 0.6     | 0.2  | na                                    | na                                     |
|          |       | Fall   | Adult    | 33  | 0.3  | 0.1               | 0.3    | 0.2     | 0.6     | 0.2  | na                                    | na                                     |
|          |       |        |          |     |      |                   |        |         |         |      |                                       |                                       |
| TP       | g/dL  | Spring | Adult    | 162 | 7.2  | 0.4               | 7.2    | 5.5     | 8.3     | 6.5  | 7.9                                  | 5.5–6.7                                |
|          |       |        | Subadult | 30  | 7.0  | 0.3               | 7.1    | 6.3     | 7.8     | 6.3  | 7.8                                  | 6.2–6.5                                |
|          |       | Fall   | Adult    | 33  | 7.3  | 0.6               | 7.3    | 6.3     | 8.9     | 6.1  | 8.7                                  | 5.9–6.4                                |

*Subgroups within the same season are significantly different (*P* < 0.01).

∗Reference intervals for male polar bears in Fall include both adults and subadults.
Spring RIs
We partitioned females captured in spring into three subgroups: non-denning adults, denning adults and subadults, consistent with expectations based on behaviour and physiology. Mean ALP activities of subadult females were nearly twice that of adult female polar bears and significantly different from both denning and non-denning female bears (Table 3; $Z_{\text{subadult/adult}} = 5.64$, $P < 0.001$, $Z_{\text{subadult/denning}} = 6.24$, $P < 0.001$). Denning females had significantly lower mean concentrations of ALB, POT and TP and mean ALT activity than non-denning adult females and significantly higher mean concentrations of GLU and CREA (Table 3).

Physiologic differences between females in spring based on the lower and upper confidence intervals of RI suggest limited differences in ALB levels, with denning females having a lower clinical decision interval (Friedrichs et al., 2012). Similarly, denning females had higher minimum GLU values than non-denning adults and subadult females in spring. GLOB levels showed physiologic difference within female bears based on age and denning status, with denning females having lower GLOB levels than both non-denning adults and subadults.

Males sampled in spring were partitioned into adult and subadult age classes. The means of ALT, ALP, CA, CREA and GLOB were significantly different between the two age classes (Table 4). Similar to subadult females in spring, subadult males had significantly higher ALP activities, with an upper reference limit for adults and subadults. BUN in subadult females was significantly different from all other analytes where the mean differed significantly between both denning and non-denning adult females (Table 3; $Z_{\text{subadult/adult}} = 5.44$, $P < 0.001$, $Z_{\text{subadult/denning}} = 5.96$, $P < 0.001$). Such differences in ALB levels, with denning females having lower activities than non-denning adults and subadult females in spring. GLOB levels were significantly different between the two age classes. The means of ALT, ALP and TP were significantly different from both adult and subadult females. BUN in subadult females was significantly different from all other analytes where the mean differed significantly between both denning and non-denning adults and subadults.

Fall RIs
Fall sample sizes were smaller than spring sample sizes but still provided adequate numbers to calculate RI using an iterative (robust) statistical approach (Friedrichs et al., 2012). Females were grouped into adults and subadults (Table 3). Differences in ALP between age classes were consistent across seasons, with subadult females having significantly higher mean ALP activities ($\bar{X} = 64.02$ U/l) than adult females ($\bar{X} = 31.65$ U/l, $t = 5.69$, $P < 0.001$). The higher upper confidence limit of ALP activity suggests a physiologic difference between the two age groups in fall. GLOB concentrations were the only other analyte where the mean differed significantly between subadult and adult females in fall ($z = −2.68$, $P = 0.018$). The upper limit of GLOB concentration in adult females suggests a physiologic difference between the two age classes. A single RI for each analyte for males in fall is reported in Table 4.

Seasonal differences in RIs
Adult females were separated into non-denning and denning females in the spring and combined in the fall (Fig. 2). We found significant differences in seasonal means for CREA ($\bar{x}_{\text{fall}} = 1.04$ mg/dl, $\bar{x}_{\text{spring}} = 0.95$ mg/dl; $t = −2.96$, $P < 0.01$), POT ($\bar{x}_{\text{fall}} = 4.75$ mmol/l, $\bar{x}_{\text{spring}} = 4.42$ mmol/l; $t = −5.01$, $P < 0.001$), TP ($\bar{x}_{\text{fall}} = 7.57$ g/dl, $\bar{x}_{\text{spring}} = 6.96$ g/dl; $t = −7.45$, $P < 0.001$), NA ($\bar{x}_{\text{fall}} = 142.79$ mmol/l, $\bar{x}_{\text{spring}} = 137.53$ mmol/l; $t = −6.23$, $P < 0.001$) and GLOB ($\bar{x}_{\text{fall}} = 2.01$ g/dl, $\bar{x}_{\text{spring}} = 1.43$ g/dl; $t = −9.15$, $P < 0.001$) in adult females. Mean seasonal differences of analytes in subadult females were often statistically significant but minimal in magnitude, with the exception of BUN concentrations, suggesting limited seasonal differences on physiologic function. Subadult females showed greater seasonal variation with significant differences between seasonal means for BUN ($\bar{x}_{\text{fall}} = 9.78$ mg/dl, $\bar{x}_{\text{spring}} = 16.58$ mg/dl; $t = 2.60$, $P < 0.01$), CREA ($\bar{x}_{\text{fall}} = 1.02$ mg/dl, $\bar{x}_{\text{spring}} = 0.88$ mg/dl; $t = −2.70$, $P < 0.01$), POT ($\bar{x}_{\text{fall}} = 4.81$ mmol/l, $\bar{x}_{\text{spring}} = 4.51$ mmol/l; $t = −3.44$, $P < 0.001$), NA ($\bar{x}_{\text{fall}} = 143.14$ mmol/l, $\bar{x}_{\text{spring}} = 139.73$ mmol/l; $t = −3.38$, $P < 0.01$), TP ($\bar{x}_{\text{fall}} = 7.26$ g/dl, $\bar{x}_{\text{spring}} = 6.81$ g/dl; $t = −5.72$, $P < 0.001$) and GLOB ($\bar{x}_{\text{fall}} = 1.69$ g/dl, $\bar{x}_{\text{spring}} = 1.29$ g/dl; $t = −6.01$, $P < 0.001$). Of these statistically different analytes, only GLOB concentration suggests a physiologic difference between spring and fall in both adults and subadults. BUN in subadult females was the only analyte to significantly increase in fall, all other significantly different analytes showed decreased activity and concentration in spring regardless of age.

Males were separated into subadults and adults in the spring and combined in the fall (Fig. 3). For adult males, ALP was greater in fall ($\bar{x} = 59.17$ U/l) than spring ($\bar{x} = 35.61$ U/l, $t = −3.69$, $P < 0.01$), as was CA ($\bar{x}_{\text{fall}} = 10.26$ mg/dl, $\bar{x}_{\text{spring}} = 9.70$ mg/dl; $t = −4.16$, $P < 0.001$) and BUN ($\bar{x}_{\text{fall}} = 17.58$ mg/dl, $\bar{x}_{\text{spring}} = 11.70$ mg/dl; $t = −2.91$, $P < 0.01$). In each of these cases, the RI shifted to the right in fall, suggesting a physiologic difference. ALT also showed a significant difference between spring and fall ($\bar{x}_{\text{fall}} = 26.51$ U/l, $\bar{x}_{\text{spring}} = 47.87$ U/l; $t = 5.10$, $P < 0.001$) with the upper limit of ALT activity in spring being more than twice as high as fall activity (Fig. 3). Mean CREA concentration was significantly lower in fall ($\bar{x}_{\text{fall}} = 1.00$ mg/dl, $\bar{x}_{\text{spring}} = 1.24$ mg/dl; $t = 4.09$, $P < 0.001$); however, the physiologic importance of this difference is likely minimal.

Discussion
Although previous research has reported blood analyte values for polar bears, these reports have examined fewer analytes and smaller numbers of bears (e.g. Lee et al., 1977; Nelson et al., 1983; Derocher et al., 1990; Ramsay et al., 1991; Tryland et al., 2002; Rode et al., 2014; Whiteman et al., 2017, 2018). Our goal was to utilize a large data set to create robust RI based on a well-studied subpopulation that can serve as a
Figure 2: Seasonal differences of biochemical analytes for female polar bears with significance between like subgroups reported as * $P < 0.05$, ** $P < 0.01$. 
Figure 3: Seasonal differences of biochemical analytes for male polar bears with significance between like subgroups reported **P < 0.001
foundation for relating biochemical analytes and polar bear health in this and other subpopulations.

Assessment of health in reference subjects is of paramount importance is establishing RI and yet is challenging in free-living wildlife owing to a single point-in-time examination. Inclusion of unhealthy subjects has the potential to widen the RI, rendering it less sensitive for detecting deviation from healthy analyte distributions (Johansen and Christensen, 2018). In order to minimize inclusion of potentially unhealthy subjects, specific criteria were defined in order to exclude potentially unhealthy subjects (see Materials and methods). Our examination and exclusion of outliers from the reference population warranted our inclusion of samples from the last four decades despite accelerating rates of environmental change and habitat perturbation in the Arctic (Harr et al., 2018).

Our results were consistent with related work on large carnivores that found higher ALP activity in subadult/juveniles than adults: wolves (Canis lupus, Thoresen et al., 2009), grizzly bears (Ursus arctos horribilis, Brannon, 1985) and polar bears (Lee et al., 1977; Tryland et al., 2002). ALP is an enzyme in both liver and bone and is involved in bone growth and remodelling. ALP is thus expected to be higher in subadults regardless of season. BUN concentrations were lowest in denning females, which likely reflects extended fasting and the energetic demands of raising young. Females with cubs of the year are often captured shortly after leaving the den, leaving little time for hunting prior to capture (Derocher et al., 1990). As access to food in spring increases, we would expect BUN concentrations to increase. Denning females also had significantly higher GLU concentrations than both adults and subadult females in spring. This difference may be related to increased GLU requirements during lactation (Bell and Bauman, 1997). These results are inconsistent with the findings of Halloran and Pearson (1972) and Matula et al. (1980) in brown and black (Ursus americanus) bears, respectively, but both authors note inconsistencies among published reports relating blood GLU concentration to denning and lactation (e.g. Lee et al., 1977; Franzmann and Schwartz, 1988; Stenvinkel et al., 2013).

Seasonal differences in analytes are likely a response to changes in nutrition and behaviour. In the western Hudson Bay subpopulation, polar bears are forced on shore when the sea ice melts in summer and have little access to food until the ice re-forms in the fall (Atkinson and Ramsay, 1995). Ramsay et al. (1991) reported a pronounced seasonal variation in BUN concentrations for western Hudson Bay bears, which averaged 48.4 ± 1.8 mg/ml for individuals captured on sea ice in spring and 19.1 ± 5.4 mg/ml for those captured on land in summer. In the SB, season-specific BUN and CREA RI were lower than those reported elsewhere (Nelson et al., 1983; Ramsay et al., 1991; Tryland et al., 2002). Our BUN RI for adult females in spring was 2.4–48.80 mg/dl with a mean of 16.7 mg/dl. Thus, our maximum spring value equaled the mean spring value reported for western Hudson Bay, while our mean spring value matched that reported for western Hudson Bay bears in the summer that had been fasting on land. Similarly, spring and fall CREA RIs from our study were substantially lower than spring and summer CREA ranges and RI previously reported for the western Hudson Bay and Barents Sea subpopulations (Nelson et al., 1983; Ramsay et al. 1991; Tryland et al., 2002). Explanations for these differences between the SB and other subpopulations could be due to disparate ice conditions during the respective study periods (Stroeve et al., 2012) or to differences in biological productivity between subpopulations (Rode et al., 2018).

Many researchers have used BUN and CREA to assess fasting in polar bears. Recently, Rode et al. (2018) documented declines in the ratio of BUN to CREA, which is an index of feeding over the previous 7 days and found increased rates of fasting in SB polar bears between 1983 and 1999 and 2000 and 2016. Pagano et al. (2018) and Whiteman (2018) supported this finding noting increases in metabolic rates due to increased energy expenditure and declines in hunting opportunities related to deteriorating sea ice habitat. While not the goal of this research, our work provides a basis from which to continue investigations into physiologic adjustments resulting from a changing climate. Using deviations from RI, we can better understand how abiotic and biotic conditions such as changes in sea ice are impacting polar bears and determine the best metrics for surveillance and monitoring.

Our work adds to the understanding of the blood biochemistry of polar bears. Our large sample size permitted biologically appropriate subgrouping, allowing us to examine differences in age class and reproductive status, the classifications used for managing polar bear populations. Nevertheless, our study has certain inherent limitations. For example, the declining availability of sea ice in the SB during summer and fall precluded the continuation of safe captures limiting our ability to calculate summer RI that included data beyond 2009. We caution that although we report a number of statistical differences for analytes across subgroups and between seasons, it is important to consider the functional importance of these differences. For example, mean TP levels showed significant differences between all subgroups for females in spring. However, the calculated values suggest minimal influence on physiologic function and critical decision limits. To clarify the functional significance of the differences we have documented, it would be useful to determine how the analytes we measured vary with known disease states. To inform relationships between disease and blood biochemistry, we suggest examining zoo-managed polar bears as well as wild polar bears with known pathological conditions (Atwood et al., 2015) to establish critical values for these physiologic markers.
We also acknowledge that RI created for one subpopulation using one analytical system may not reflect the variability of values observed in other subpopulations or by other methods. However, we provide a well-documented and robust resource for comparisons within and across the circumpolar population of polar bears. Our work is therefore most relevant to monitoring the SB subpopulation of polar bears, including detecting changes in physiologic function that may reflect subclinical and clinical disease in individuals and populations. In general, RIs provide a baseline for assessing health, and deviation from these RIs may signal an adaptive physiologic response. The SB subpopulation of polar bears is one of the most well studied; therefore, associations between stressors and physiologic responses documented for the SB subpopulation can be used to inform monitoring and management decisions both for this population and potentially for other subpopulations even with different baseline values. Furthermore, combining baseline physiologic data such as ours with complementary data on hematology (Kirk et al., 2010) and transcriptomics (Bowen et al., 2015, 2015b), as well as data on diet and nutrition (McKinney et al., 2017), reproduction (Rode et al., 2010), behaviour (Whiteman et al., 2015; Atwood et al., 2016; Lillie et al., 2018; Pagano et al., 2018) and pathogen exposure (Atwood et al., 2015, 2017) could help identify how polar bears might react and adapt to external stressors such as infectious diseases, environmental catastrophes and climate change (Stroeve et al., 2012; Laidre et al., 2015). This set of RI for SB polar bears provides a robust foundation necessary to make temporal and spatial observations on the overall health of polar bears as well as comparisons both within and among subpopulations facing myriad ecological challenges.

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