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Normal pre- and post-prandial bile acids and protein C values vary by age in harbor seal pups (*Phoca vitulina richardsi*) undergoing rehabilitation

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OBJECTIVE
To establish normal values for pre- and post-prandial bile acids and protein C in Pacific harbor seal (*Phoca vitulina richardsi*) pups.

ANIMALS
45 harbor seals undergoing rehabilitation at the Vancouver Aquarium Marine Mammal Rescue Centre, 0 to 16 weeks, and deemed healthy aside from malnutrition or maternal separation.

PROCEDURES
Venous blood was collected from the intervertebral extradural sinus in fasted seals and again 2 hours after a fish meal.

RESULTS
The reference interval (90% CL, confidence limit) for pre-prandial (fasting) bile acids was 17.2 µmol/L to 25.4 µmol/L, post-prandial bile acids were 36.9 µmol/L to 46.4 µmol/L, and protein C was 72.3% to 85.4%, across ages. For comparison between developmental ages, pups were grouped into 3 age classes: < 14 days, 5 to 8 weeks, and 10 to 16 weeks. Age affected pre- and post-prandial bile acids; pups < 14 days had significantly higher pre-prandial bile acids (36.0 µmol/L ± 16.5 µmol/L; $P < .0001$) than other age groups and pups 5 to 8 weeks had significantly higher post-prandial bile acids (50.4 µmol/L ± 21.9 µmol/L; $P < .001$). Protein C was also affected by age, with seals < 14 days having significantly lower values (mean, 51.8% ± 16.7%; $P < .0001$).

CLINICAL RELEVANCE
This study established normal reference intervals for bile acids in harbor seal pups and offered a preliminary investigation into protein C in pinnipeds. The bile acid values from 0- to 16-week-old seal pups were well above established normal ranges for domestic species, highlighting the utility of age- and species-specific reference ranges. The values presented here and the differences across age classes will aid clinicians in accurately diagnosing hepatobiliary disease in harbor seal pups.

Harbor seals (*Phoca vitulina richardsi*) are commonly rescued and undergo rehabilitation along the Pacific coast of North America. Most frequently, these patients are rescued as stranded neonates and pups. The Vancouver Aquarium Marine Mammal Rescue Center (MMR) is located in Vancouver, British Columbia, Canada. Each year, the facility rescues and rehabilitates over 150 marine mammals, the majority of which are harbor seals. In 2016, the MMR rescued 171 animals; of these, 169 were harbor seal neonates and pups.

Upon admittance to rehabilitation, harbor seal neonates and pups often have elevations in total bilirubin on serum chemistry panels. While previous studies have described normal serum chemistry values in wild and stranded harbor seals,[1–4] there are no previous reports describing other liver function tests in this species. Occasionally, diseases affecting hepatic function, including portal vascular anomalies like portosystemic shunts, have been suspected by marine mammal clinicians, but a definitive diagnosis can be difficult. In cases of elevated total bilirubin, with or without additional liver enzyme elevations, clinicians may need to perform additional diagnostics to determine if the hyperbilirubinemia is physiologic and transient due to young age or if it is pathologic.

The phenomenon of physiologic hyperbilirubinemia of neonates has previously been described in harbor seals.[5] Similar to human neonates, harbor seal neonates can experience physiologic jaundice, which is the development of unconjugated serum bilirubin levels during the first week of life. Several factors can contribute to this elevation, including increased bilirubin production, impaired uptake by hepatocytes with a premature liver, defective conjugation of bilirubin in the liver, and increased enterohepatic circulation due to immature gastrointestinal flora and subsequent reabsorption of deconjugated bilirubin. In the previous study by Dierauf et al, 67% of harbor seal pups under 28 days of age showed hyperbilirubinemia and 14% were clinically jaundiced.[5] Interestingly, by 28 days of age, bilirubin levels decreased to normal levels and clinical jaundice disappeared, as the liver presumably began functioning to capacity and intestinal bacterial flora became established to break down bilirubin. If elevations in serum bilirubin or liver values persist past this neonatal period, or the clinician suspects the changes to be pathologic rather than transient or normal, then further diagnostics may be pursued to differentiate these causes. On routine bloodwork, serum chemistry is useful as it contains several values indicative of liver function: glucose, urea, albumin, and cholesterol. In addition, liver enzyme values including ALT (alanine transaminase), AST (aspartate aminotransferase), ALP (alkaline phosphatase), GGT (gamma-glutamyl transferase), and total bilirubin can be evaluated on serum chemistry. Beyond basic serum chemistry, there are additional blood tests that can be considered to better diagnose and characterize liver disease. Serum ammonia concentration is highly sensitive and specific for evaluating liver disease and detecting portosystemic shunts; however, this is not always a practical test in the rehabilitation setting as there are few reference laboratories to run this test, it must be run soon after collection, and samples require specific handling, such as shipping on dry ice. Serum bile acids and protein C activity are 2 practical diagnostic tests, that can be run on banked serum and plasma samples, respectively, and can aid in further evaluating patients for liver disease or suspected portal vascular anomalies.

Bile acids are physiologic detergents produced by the liver, which aid in the intestinal phase of lipid digestion and absorption by solubilizing lipids in bile and helping digest fats. After working in the gastrointestinal tract, they are absorbed through the intestines and enter portal circulation. With normal hepatic function, they are removed with very high efficiency by the liver, with over 95% removed on first-pass circulation in domestic species and humans.[6,7] Typically, there are very small amounts of bile acids in fasting blood samples in dogs and cats due to this efficiency of ileal resorption and hepatic extraction. Any decrease in hepatic flow or damage to hepatocytes will cause an increase in systemic bile acid concentrations, most commonly related to intrahepatic disease or portosystemic shunts. Therefore, the measurement of serum bile acid concentration is a good indicator of hepatobiliary function and portal blood flow.[8]

When bile acids are measured, it is preferred to evaluate both a pre-prandial and a post-prandial sample, 2 hours after a meal. This effectively challenges the liver, as the meal will stimulate the contraction of the gall bladder and the release of bile acids into circulation. If the liver is functioning normally, these will be soon reabsorbed; however, if there is damage to hepatocytes or abnormal hepatic blood flow, larger quantities of bile acids may remain in the serum. In domestic animals, pre-prandial and post-prandial serum bile acid concentrations are highly specific for diagnosing hepatobiliary disease; Center et al showed that using the cutoff of > 20 µmol/L for pre-prandial samples and > 25 µmol/L for post-prandial samples was 100% specific for diagnosing hepatobiliary disease in dogs.[7] It should be noted that measurement of serum bile acids is non-specific, as it is reflective of overall hepatic transport function, and this can be affected by a variety of diseases. Further testing is often needed to help diagnose liver abnormalities if bile acid testing is abnormal.

Protein C is a Vitamin-K dependent protein that is synthesized by the liver (and not to be confused with C Reactive Protein). Protein C's major role in the body is as an anticoagulant by forming a complex with protein S and activated protein C, which exerts anticoagulant effects on the coagulation cascade. It also has several minor roles in the body including promotion of fibrinolysis, modulating inflammation, and inhibiting apoptosis.[9,10] Protein C levels can aid in diagnosing liver disease and assessing portal blood flow non-invasively. In humans and domestic animals, low protein C has been associated with thrombotic disorders and is a negative prognostic indicator in septic patients.[9–13] Low Protein C can be inherited, which rarely occurs in humans and dogs, or can be acquired.[14] Acquired low protein C is seen with conditions such as liver disease, acute phase inflammation, septicemia, DIC (disseminated intravascular coagulation), and Vitamin K deficiency.[12] Studies have shown that protein C activity in dogs is significantly lower in those with portosystemic shunts, as it reflects the inadequacy of hepatic portal perfusion.[12] In addition, it can be used as a monitoring tool after the ligation of shunts, as protein C increases after shunt ligation and is a better indicator than bile acids.[12] Despite its potential utility as an indicator of hepatic function and coagulation, there are no published reports of protein C activity in pinnipeds to the authors' knowledge.

The objective of this prospective pilot study was to establish normal values for bile acids and protein C activity in harbor seal pups undergoing rehabilitation. We hypothesized that normal values would differ across pup age classes. This study aims to improve our understanding of liver function tests in normal harbor seals across age classes, so that when suspected cases of hepatobiliary disease and portosystemic shunts are encountered they may be accurately diagnosed.

Materials and Methods

The subjects of this study were 45 harbor seals (*Phoca vitulina richardsi*) undergoing rehabilitation at the Vancouver Aquarium Marine Mammal Rescue Center during the 2016 and 2017 rescue seasons (14 females, 31 males; 16 seals with lanugo coats at the time of rescue and 29 without). The ages of pups were determined based on pelage, umbilicus, dentition, and mass, as described previously.[15–17] The inclusion criteria for this study were: patients that were deemed healthy aside from malnutrition and maternal separation, with no hematological or physical exam abnormalities; patients not on any systemic medications for 6 weeks before blood sampling; and patients who had not received any antibiotics for more than 7 days at any point. Blood sampling is part of the normal medical care of seals undergoing rehabilitation, and samples for this study were opportunistically collected in conjunction with routine sampling; the study design was reviewed by Vancouver Aquarium and MMR veterinary staff and did not require Institutional Animal Care and Use Committee (IACUC) approval.

Venous blood was collected from the intervertebral extradural sinus using a 1.5-inch, 19-gauge needle attached to a BD Vacutainer (Becton, Dickinson and Company) **(Figure 1)**. For measurement of serum bile acids, samples were collected in a serum separator tube (BD Vacutainer SST) after a 12-hour fasted period (pre-prandial sample) and 2 hours after a meal (post-prandial sample) of frozen-thawed Pacific herring (*Clupea pallasii*). These samples were centrifuged and sent to a reference laboratory (Idexx Laboratories Ltd), where the standard enzymatic assay was performed using the Bile Acids-L3K method, run on a Hitachi 717 instrument.

For measurement of serum protein C activity, blood samples were collected in sodium citrate tubes (BD Vacutainer®), centrifuged, and citrated plasma samples were shipped on ice to Cornell University Animal Health Diagnostic Center. In the protein C activity assay, plasma is treated with a venom-derived activator, and the amount of functional protein C produced is measured in a chromogenic assay system. Results are expressed as a percent protein C activity against a control. In this study, seal protein C activity was measured against a human 100% standard.

Figure 1—Venipuncture from the intervertebral extradural sinus of a harbor seal pup undergoing rehabilitation. One person is gently restraining the neck and hindlimbs while the second person is collecting blood. Photo courtesy of Vancouver Aquarium Marine Mammal Rescue Centre.

Statistical analysis was performed using Prism 7 software (GraphPad Software Inc), except when otherwise specified. A *P*-value of ≤ .05 was used as the threshold for significance. A ROUT analysis was used to identify outliers within the data. To assess normality, a D'Agostino & Pearson test for Gaussian distribution was utilized. One-way ANOVAs were used to test for significant effects of age within each treatment group (pre- and post-prandial).

For comparison between developmental ages, pups were grouped into 3 age classes: < 14 days of age, 5 to 8 weeks, and 10 to 16 weeks (n = 15 for each group). Following ASCVP Guidelines[18] when the data were broken down by age and the sample sizes were < 40, only descriptive statistics were reported rather than a reference interval. When data across age classes were combined, reference intervals were derived by calculating the 90% confidence limits (CL) around the mean using 1,000 iterations of the "Boot" package in R 3.1.2 statistical software.

Results

Bile acids

ROUT analysis was used to identify outliers within the data for each age group (pre-and post-prandial values), resulting in the removal of 2 fasted data points and 3 post-prandial data points. This served to normalize the data, as determined by a D'Agostino & Pearson test for Gaussian distribution. The resulting bile acid values were analyzed for mean, median, and standard deviation. The reference interval (90% CL) across all seals for pre-prandial (fasting) bile acids was 17.2–25.4 µmol/L (n = 43 original values), and post-prandial bile acids was 36.9 µmol/L to 46.4 µmol/L (n = 42). After removing outliers, there were 41 paired samples of pre- and post-prandial bile acids (all ages combined). A paired *t* test showed that post-prandial bile acids were significantly higher than the pre-prandial bile acids (t = 8.406, df = 40, *P* < .0001), with a mean (±SD) pre-prandial bile acid value of 21.3 µmol/L ± 15.8 µmol/L and a mean post-prandial bile acid value of 41.7 µmol/L ± 18.3 µmol/L. The descriptive statistics for pre- and post-prandial bile acid levels, as well as those for each age group, are shown **(Table 1)**.

There was a significant effect of age on pre-prandial bile acids (ANOVA $F(2, 40) = 20.07$, $P < .0001$). A Tukey post hoc analysis revealed that average pre-prandial bile acids for the youngest age group (36.0 µmol/L ± 16.5 µmol/L) was significantly higher than for either the middle (19.2 µmol/L ± 9.7 µmol/L) or oldest age groups (8.8 µmol/L ± 5.5 µmol/L).

Similarly, there was a significant effect of age on post-prandial bile acids (ANOVA $F(2, 36) = 5.87$, $P < .006$). A Tukey post hoc analysis revealed that average post-prandial bile acids for the middle age group (50.4 µmol/L ± 21.9 µmol/L) was significantly higher than the oldest age group (29.9 µmol/L ± 7.4 µmol/L), but the youngest group (44.3 µmol/L ± 16.0 µmol/L) was not different from either other groups.

Table 1—Descriptive statistics of bile acid values from pre-prandial (fasting) and post-prandial harbor seal pups by age category and overall reference intervals.

	Preprandial bile acids (µmol/L)				Postprandial bile acids (µmol/L)			
Age group	**< 14 d**	**5–8 wk**	**10–16 wk**	**Overall**	**< 14 d**	**5–8 wk**	**10–16 wk**	**Overall**
n =	14	15	14	43	13	15	14	42
Minimum	14.8	5.5	2.6	2.6	26.6	15.2	18.6	15.2
Maximum	63.6	41.9	18.8	63.6	79.9	86.6	45.7	86.6
Median	34.7	19.8	8.3	18.8	39.4	53.3	28.9	37.2
Mean	36.0	19.2	8.8	21.3	44.3	50.4	29.9	41.7
Standard deviation	16.5	9.7	5.5	15.8	16.0	21.9	7.4	18.3
Standard error of mean	4.4	2.5	1.5	2.4	4.4	5.7	2.0	2.8
Reference interval (90% CL)				[17.2–25.4]				[36.9–46.4]

CL = confidence limit.

Pup developmental age could also be defined by the presence or absence of a lanugo coat (n = 16 with lanugo at the time of rescue, n = 29 without). Across age classes, pups with lanugo had significantly higher pre-prandial bile acid levels (30.8 µmol/L ±19.3 µmol/L) than those without lanugo (16.2 µmol/L ±10.9 µmol/L; t_{41} = 3.19, P < .003); there was no significant difference in post-prandial bile acids between pups with lanugo (44.4 µmol/L ± 16.4 µmol/L) and those without (40.4 µmol/L ± 19.3 µmol/L; t_{40} = 0.66, P = .51).

Protein C activity

ROUT analysis did not identify any outliers within any of the age groups for protein C. The data followed a Gaussian distribution, as determined by a D'Agostino & Pearson test. The mean protein C activity across all age groups (n = 43) was 78.8% ± 26.5%; these were also affected by age. Average protein C activity for the youngest age group (51.8% ± 16.7%) was significantly lower than for either the middle (90.6% ± 23.3%) or oldest age groups (96.2% ± 8.9%; ANOVA F(2, 40) = 27.18, P < .0001). The reference interval (90% CL) for protein C activity was 72.3% to 85.4% as determined by the bootstrapping operation. The descriptive statistics are shown **(Table 2)**.

Data regarding signalment, presence of lanugo coat at the time of rescue, bile acids, and protein C values for each seal are presented elsewhere **(Supplementary Table S1)**. Total bilirubin values are also included when available; bilirubin values were not analyzed in this study as data was not available for all animals.

Table 2—Descriptive statistics of protein C activity values from harbor seal pups, by age class and overall reference intervals.

	Protein C (%)			
Age	**< 14 d**	**5–8 wk**	**10–16 wk**	**Overall**
n =	15	15	13	43
Minimum	19	28	78	19
Maximum	85	125	109	125
Median	54	90	99	88
Mean	51.8	90.6	96.2	78.8
Standard deviation	16.8	23.3	8.9	26.5
Standard error of mean	4.3	6.0	2.5	4.0
Reference interval (90% CL)				[72.3–85.4]

CL = confidence limit.

Discussion

This prospective study established reference intervals for pre- and post-prandial bile acids in the neonate and pup harbor seals undergoing rehabilitation and presents a preliminary investigation into the use of protein C activity in pinnipeds. As we hypothesized, normal values differed significantly across age classes. This data will be beneficial to clinicians in interpreting patients with the suspected hepatobiliary disease or portosystemic shunts, particularly in neonates when elevations in bilirubin and bile acids may be physiologically increased and transient.

Reference intervals were calculated based on the 90% CLs when all age categories were included and the number of seals was above 40, following the ASCVP Guidelines.[18] When seals were further broken down into age categories for analysis, the smaller sample sizes precluded generation of further age-specific reference intervals; thus, descriptive statistics were reported for age categories.

The bile acid values reported in this study varied with age, with younger pups having significantly higher pre-prandial levels, well above the normal ranges defined for domestic species.[8] With increasing age class, pre-prandial bile acid values were significantly lower. This data supports an earlier study that documented physiologic hyperbilirubinemia of neonatal harbor seals and suggested that neonatal harbor seal livers may take several weeks to mature and be fully functional.[5] Harbor seals had elevated bilirubin initially and normal bilirubin by day 28 as the liver and intestines presumably began to function to capacity.[5] In the current study, total bilirubin followed similar trends, with elevations in many neonates, but was not statistically analyzed as bilirubin values were not available for all animals in the data set (Supplementary Table S1). Following a similar pattern as that reported for bilirubin, mean pre- and post-prandial bile acids in this study were well above domestic animal reference ranges in seals 5- to 8-weeks-old, and were closer to normal but still elevated at 10 to 16 weeks. The patients included in

this study had no other indications of liver disease on bloodwork or physical exam. Liver biopsies were beyond the scope of this noninvasive study, but future studies could include liver biopsies to definitely establish normal livers despite high bile acid values in these young pups.

Many of the neonatal harbor seals rescued by MMR each year are considered premature, based on the presence of a lanugo coat at the time of stranding.[15] Many of the subjects in this study (n = 16) were rescued with lanugo coats and presumed to be premature (Supplementary Table S1). When pups with lanugo coats were compared with pups without lanugo coats, pre-prandial bile acid concentrations were significantly higher in lanugo pups, but not post-prandial. The data supports that neonatal harbor seal livers may take several weeks to mature before functioning to full capacity, and it is important for clinicians to keep this in mind when analyzing blood values. The bile acid values for the neonates in this study would be interpreted as pathologic based on domestic animal standards, highlighting the merit of age- and species-specific values to aid interpretation. Future studies could include older age classes of harbor seal pups to further examine the timing of when bile acid values appear to normalize as the liver becomes fully functional.

The protein C activity values reported in this study across age classes were similar to established feline and canine reference ranges, according to the Comparative Coagulation Laboratory at Cornell University (dogs: 75% to 135%, and cats: 65% to 120%).[19] When examining by age class, the youngest seals (< 14 days) had significantly lower protein C activity (51.8% ± 16.7%) than older seal pups 5 to 8 weeks (90.6% ± 23.3%) and 10 to 16 weeks (96.2% ± 8.9%). This follows the same pattern as bile acids to suggest increasing liver function during the first several weeks of life in seals. It should be noted that the protein C activity assay run at Cornell University expresses the results as a percentage of a 100% control, and the seal protein C activity values reported in this study were measured against a human control. Further work is warranted to validate these methods and explore potential species-specific differences to optimize the assay for use in seals. Therefore, the protein C activity results presented in this study are considered a preliminary investigation and limited interpretation and clinical recommendations can be made. Protein C activity holds promise not only as a useful diagnostic test to evaluate the liver but also as an early indicator of sepsis and coagulopathies in marine mammals. Traditional coagulation testing can be difficult to interpret in marine mammals due to differences in clotting factors,[20] and protein C appears to be conserved across mammalian species.[21]

There were several limitations to this study. The inclusion criteria for this study aimed to utilize individuals who were healthy aside from maternal separation and malnutrition. However, it should be noted that the patients included in this study may not be truly representative of the wild, healthy population of harbor seal pups given that they were stranded. Another limitation of this study was the small sample size; further studies with larger sample sizes of wild-caught seals could be of benefit in future investigations.

In conclusion, this study presented reference intervals for serum bile acids (pre- and post-prandial) in harbor seal pups undergoing rehabilitation. There were significant differences seen between age categories, with seals < 14 days of age having the highest bile acid values. The bile acid values from seal pups in this study were well above established normal ranges for domestic species, highlighting the utility of age- and species-specific reference ranges. The elevated bile acids in these pups are thought to be transient and physiologic due to an immature liver. Additionally, this study presented a preliminary investigation into the use of protein C activity in seals and reported reference ranges and mean values by age class in this population of harbor seal pups. Further investigation is required to validate this assay for use in marine mammals. Protein C activity holds promise as a beneficial tool in assessing hepatic function and coagulation in marine mammals.

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Supplementary Materials

Supplementary materials are posted online at the journal website: avmajournals.avma.org.