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HEMATOLOGY AND SERUM BIOCHEMISTRY OF HARBOR SEAL (*PHOCA VITULINA*) PUPS AFTER REHABILITATION IN THE NETHERLANDS

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Abstract: Hematology and serum biochemistry profiles are used to evaluate the health status of animals ongoing rehabilitation. The aim of this project was to develop blood and biochemistry ranges for harbor seal pups (*Phoca vitulina*) after rehabilitation; thus, 22 different blood parameters in 60 animals were tested before release. The second goal was to test for differences due to sex, stranding location, body condition at admission, and presence or absence of umbilical cord. The alanine aminotransferase, ALT (or glutamate pyruvate transaminase, GPT), (ALT-GPT) differed significantly (P bq = 0.00851) between sexes. Lower leukocyte counts and higher liver enzyme values were the most remarkable findings when comparing the results of this study to other published data. This is the first study to report blood reference ranges for harbor seal pups in the Dutch Wadden Sea after rehabilitation.

Key words: Harbor seal, hematology, *Phoca vitulina*, serum chemistry.

BRIEF COMMUNICATION

Hematology and serum chemistry values are used in human and veterinary medicine to aid in patients' health assessment. In the last few decades, research[7,9,11] has been conducted to apply these diagnostic techniques to marine mammals in order to render a more accurate diagnosis in the individuals brought to rehabilitation.

Hematologic reference values comprising all age classes of harbor seals (*Phoca vitulina*) have been published[7,9,11] before, and they provide one of the main tools for health assessment and diagnosis of free-ranging and captive pinnipeds. However, many factors (i.e., diet, age, sex, stress, and type of facility or analytical instrument) may affect blood values of animals held in captivity, and these factors need to be taken into consideration during establishment of reference ranges.[7,9,11]

This study was developed to determine the hematology and biochemistry reference ranges for harbor seal pups after rehabilitation and before release to the wild at the Sealcentre Pieterburen in the Dutch Wadden Sea. The aim was also to test for significant differences between sexes in stranding location, body condition at admission, and presence or absence of umbilical cord. This is the first study that assesses the blood reference ranges of harbor seal pups from the Dutch Wadden Sea after rehabilitation.

Sixty harbor seal pups (32 males and 28 females) were sampled before release, after a period of rehabilitation that lasted between 72 and 76 days. All of the individuals stranded along the coast of the Dutch Wadden Sea (Fig. 1), mainly in the province of Groningen and the Wadden Islands, between May and June 2016 and were admitted to the Sealcentre Pieterburen for rehabilitation. When admitted, all animals were weighed (6.4–12.5 kg), presence of an umbilical cord was noted, and age class was estimated (animals younger than 1 mo of age were considered pups; factors such as presence or absence of lanugo, eruption of teeth, and status of umbilical cord or umbilicus were used to determine the age of the animal).[8] Twenty-five percent of the animals arrived with an umbilical cord present, and only four individuals had complete or partial lanugo fur upon arrival.

Animals were considered suitable for release when they had increased in weight over the course of several weeks, showed normal behavior, and lacked any evident signs of disease. The weight range prior to release was 24.5 to 36.5 kg.

Animals in the last phase of rehabilitation were housed in pools ranging from 35 m^3 to 125 m^3, with depths of 100 to 170 cm and a salinity of the water of 1.3 ppt. Animals were fed twice a day, and feeding was based on 5 kg of fish (herring [*Clupea harengus*]) per animal per day. Seals were weighed once a week to monitor weight gain.

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Figure 1. Stranding location of the animals included in this study. Each dot corresponds to an individual; the star marks the location of the Sealcentre Pieterburen. The three geographic areas used in this study are marked.

Adjustment to the amount of food in a given feeding was related to the number of seals in the pool and the amount of fish left from one feeding to the next. Because availability of food in the pool was *ad libitum* and individual food intake was not monitored, possible fasting of the animals and last feeding time prior to the blood draw were not considered for this study.

All blood samples were obtained from the epidural vertebral vein or from the tarsal sinus on the hind flippers using a 21-ga needle (0.8 × 50 mm) or a 20-ga needle (0.9 × 38 mm) and a vacutainer assemblage. Blood was collected into a tube containing ethylenediamine tetraacetic acid (3 ml) for the hematology analysis and a tube with a serum separator gel (4 ml) for the biochemistry analysis. Blood was kept at room temperature until it was processed within an hour of collection. Two animals were removed from the data set; one was considered an inappropriate reference individual based on having an elevated leucocyte count at release (i.e., possible evidence of disease, No. 16-186) and the other due to pre-analytical and analytical errors (No. 16-239).

Whole blood samples were processed with an hematology analyzer (Medonic CA620/530 vet; Boule Medical AB, SE-126 13 Stockholm, Sweden) to obtain complete blood counts of total leukocytes (WBC), lymphocytes, granulocytes, and midsize population cells (i.e., monocytes, basophils, eosinophils, blasts, and other immature cells), hematocrit (HCT), mean corpuscular volume (MCV), total erythrocyte count (RBC), hemoglobin, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW), mean platelet volume (MPV), and platelets. Manual counts were not performed because of lack of available resources.

Serum samples were processed with the Spotchem EZ PR-4430 (Arkray Global Business Inc, Nakagyo-ku, Kyoto 604-8153, Japan) to obtain biochemistry profiles, which included blood urea nitrogen (BUN), glucose, alkaline phosphatase (ALP), total protein, glutamate pyruvate transaminase (GPT, also known as alanine aminotransferase, ALT), and creatinine. This short biochemistry panel was the only resource available at the Sealcentre Pieterburen, and external lab analyses were not performed as a result of budget limitations.

Statistical analysis was performed with the statistical analysis software IBM® SPSS Statistics®. Normality of each individual parameter was tested (Kolmogorov-Sminov test). Parameters that were not normally distributed were log-

converted, and those values that did not follow a normal distribution after the log-conversion were tested with nonparametric tests. Outliers were determined following the ASVCP guidelines for Gaussian parameters and following the Tukey's interquartile fences,[5] and 18 outlier values were discarded (Table 1).

Several variables were tested for their effect on the blood parameters: the body condition at arrival was tested using the Kruskal-Wallis test, while the presence or absence of an umbilical cord at arrival and sex were tested with Mann-Whitney *U*-test. The sample size was too small to test for the different stranding locations. All of the *P*-values were two-tailed, and the significance level chosen was 5%. In order to counteract the multiple comparisons problem and avoid type 1 errors, the Sidak correction was used, and a corrected *P*-value was established for each category: hematology values and biochemistry values were considered independently. The new significance levels were established at P cbc $= 0.0032$ and P bq $= 0.00851$. None of the 22 blood parameters at release were associated with body condition or presence or absence of an umbilical cord at the time of arrival. Only the ALT differed by sex and was greater in females (ALT Md: 85M/106F; $U = 226.5$, $P = 0.0023$).

Descriptive statistics were established and reference ranges were calculated using the 95% confidence interval, following the ASVCP guidelines' suggestion of using the 2.5 and 97.5 percentiles.[5]

White blood cells, lymphocytes, and granulocytes in the pups in this study were lower than those previously reported for adult harbor seals[7,9] and likely result from the pups having a less mature immune system.[10]

Because this study was based on rehabilitated animals, normal exposure to sea or ocean water, deep diving, and swimming for long periods of time did not occur as it would happen in nature. All animals had access to water and pools in the facilities, but this access was limited according to medical condition and improvement of each individual. Following treatment, all the animals were provided access to the pools. In the case of this study, the total erythrocyte count, MCH, MCHC, hemoglobin, and HCT had ranges that were similar to the values reported[7,9,11] previously for this age class in the rehabilitation setting.

Marine mammals are known to dive deeply for long periods of time, which requires specific adaptations to be able to store oxygen.[13] Some of these adaptations can be seen in the hematological parameters: for example, larger amounts of hemoglobin are stored in the erythrocytes, which leads to an increase of the MCHC and the total hemoglobin when compared to those of terrestrial mammals.[2,14] This is confirmed by the results of this study, as the reference ranges established are similar to other published data[7,9,11] and greater than those associated with terrestrial mammals.

The range in the platelet count was similar to that reported by Greig et al,[7] although no manual count was performed in this study. Automated platelet counts are reportedly unreliable in cats because of platelet aggregation and different cell size.[12] In addition to providing a more accurate platelet count, the examination of the blood smear and a manual count should be performed in order to assess clumping and morphological abnormalities of platelets.[14]

The biochemistry values in this study had, on the one hand, similar ranges of glucose, BUN, and total proteins, and, on the other hand, higher ALT (*Md*: 85M/106F) values when compared to the ones published by Lander et al ($\bar{x}$x 45.4),[11] and Greig et al (*Md* 56).[7] Although single alterations in ALT levels may represent liver dysfunction in pinnipeds,[1,4] this enzyme can also be found in other tissues, such as kidney and skeletal and cardiac muscles.[4] As values were consistently elevated across samples in this project, this possibly indicates reasons other than liver dysfunction for the observed differences. The measurement of another enzyme (creatine kinase, CK) can be useful to help differentiate between hepatocellular damage and muscular disease.[4]

ALP is an enzyme present in multiple tissues in harbor seals, such as liver, bone, and skeletal muscle.[4] Growing individuals, such as the study subjects, have higher levels of ALP compared to adults as a result of elevated isoenzyme activity in the bones.[1,3] Thus, single elevations of ALP in young and juvenile individuals should not be considered pathological. Because this study did not differentiate between the different isoenzymes of ALP, increased values of this enzyme were considered to be related with growth, which is consistent with and similar to the results of Greig et al.[7]

Creatinine and BUN usually reveal the normal functionality of the kidneys.[6] Normal BUN levels in carnivore mammals, consistent with those in this study, are greater than in herbivore mammals, mainly because of high dietary protein and fat.[1] Creatinine levels were similar to other published data.[7,11]

Table 1. Reference ranges of the hematology and biochemistry values established by this study. Outlier values are listed as lost values. The value that differed significantly between males and females is separated in each category.[a]

Hematology Reference Intervals for harbor seal pups before the release

Analytes	SI Units	n*	Mean	SD	Median	Min	Max	Reference interval	LRL 90% CI	URL 90% CI	Distribution[b]	Method[c]
PCV (HCT)	L/L	57	0.42	0.04	0.42	0.30	0.51	0.31–0.51	0.30	0.51	G	P
RBC conc.	10^{12}/L	56	4.65	1.06	4.62	3.95	5.27	4.01–5.27	3.95	5.27	G	T, P
Hemoglobin	g/L	57	168.03	17.76	167.58	122.46	209.47	125.36–205.85	122.46	209.47	G	P
MCV	fL	58	89.53	5.57	90.20	74.50	100.00	75.02–99.48	74.50	100.00	G	P
MCHC	g/L	58	403.82	7.17	402.85	388.35	418.96	388.97–418.96	388.35	418.96	G	P
MCH	pg	58	36.15	2.27	36.42	30.29	40.61	30.98–40.38	30.29	40.61	G	P
WBC conc.	10^9/L	57	6.98	1.66	6.80	3.90	11.20	4.04–11.02	3.90	11.20	G	P
Granulocytes	10^9/L	58	4.43	1.37	4.30	2.30	9.40	2.48–8.81	2.30	9.40	G	T, P
Lymphocytes	10^9/L	57	1.44	0.51	1.40	0.40	2.70	0.45–2.57	0.40	2.70	G	P
MID cells	10^9/L	57	0.94	1.31	1.00	0.50	1.70	0.54–1.65	0.50	1.70	G	T, P
Platelet conc. (thrombocytes)	10^9/L	58			679.50	46.00	972.00	86.85–963.45	46.00	963.45	NG	NP
MPV	fL	58			9.40	8.20	12.10	8.34–11.72	8.50	10.61	NG	NP
RDW	%	57	23.33	1.42	23.40	20.50	26.60	20.55–26.55	20.87	25.18	G	P

Chemistry Reference Intervals for harbor seal pups before the release

Analytes	SI Units	n*	Mean	SD	Median	Min	Max	Reference interval	LRL 90% CI	URL 90% CI	Distribution[b]	Method[c]
Urea nitrogen	mmol/L	56	15.87	3.17	16.10	7.70	24.20	8.25–23.1	7.70	24.20	G	P
Creatinine	μmol/L	56	59.53	1.22	58.00	36.00	93.00	38.44–92.15	36.00	93.00	G	T, P
ALP	U/L	58			192.00	124.00	573.00	126.85–514.58	124.00	573.00	NG	NP
ALT (GPT) male	U/L	31			85.00	33.00	172.00	63.13–167.72	33.00	162.23	NG	NP
ALT (GPT) female	U/L	26			106.00	65.00	160.00	94.53–195.94	65.00	169.03	NG	NP
Glucose	mmol/L	57	8.83	0.92	8.80	6.90	11.20	7.13–11.2	6.90	11.20	G	P
TP	g/L	58			66.00	57.00	89.00	58.9–81.88	57.00	89.00	NG	NP

[a] LRL, lower reference limit; URL, upper reference limit; PCV, packed cell volume; RBC, red blood cell; conc., concentration; MCHC, mean cell hemoglobin concentration; MCH, mean corpuscular hemoglobin; WBC, white blood cell; MID cells, midsize population cells; MPV, mean platelet volume; RDW, red blood cell distribution width; ALP, akaline phosphatase; ALT, alanine aminotransferase; TP, total protein.

[b] G, Gaussian; NG, non-Gaussian.

[c] P, parametric; NP, nonparametric; R, T, transformed.

* Sample sizes less than 58 result from the removal of outliers.

The significant differences found between sexes in the ALT are challenging to explain. As the population studied is made of young, nonreproductive individuals of a non–sexually dimorphic species, it could be expected that sexually differing parameters would not exist. The biological significance of this single differing parameter is also questionable, as it might have no effect on the health status of the individuals. Furthermore, since no other endocrinological tests were performed, the effect of certain hormones on this value is also unknown.

The data given in this study provide the first published hematology reference ranges for harbor seal pups after rehabilitation in the Dutch Wadden Sea. These data can be used in the treatment and release criteria of future stranded harbor seals pups in this region. Future research regarding blood parameters should focus on the effects of several variables, such as age. Regarding the finding of significant differences between the sexes, more research should be done relative to this matter to assess the possible effect of hormones, age and growth, and immune status on the blood parameters of young male and female harbor seals. Possible differences between wild-caught versus rehabilitated individuals should also be studied.

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