Haematology and clinical chemistry values for harbour seals (*Phoca vitulina*) fed environmentally contaminated herring remain within normal ranges

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Abstract: Twenty-two young harbour seals (*Phoca vitulina*) were fed herring from either the relatively unpolluted Atlantic Ocean or the heavily polluted Baltic Sea as part of a 2½-year immunotoxicological study. Blood samples taken at regular intervals were analyzed for routine haematology and clinical chemistry. Minimal differences between the two groups were observed in these parameters over the course of the experiment. Of the 20 clinical chemistry parameters analyzed, slight differences were found in serum levels of urea, creatinine, magnesium, and cholesterol. In haematology profiles, red blood cell counts and haematocrit values were higher in seals fed Baltic herring, but these differences diminished over time. Neutrophil counts were also higher in this group of seals, especially during the second half of the feeding study. Factors affecting haematological and clinical chemistry parameters within feeding groups included gender, age, and season. The data collected demonstrate a relative insensitivity of clinical chemistry parameters to the effects of chronic exposure to environmental contaminants accumulated through the food chain, but suggest the induction of clear alterations in differential white blood cell counts. In addition, a comprehensive set of normal ranges for healthy harbour seals is presented.

Résumé : Au cours d'une étude immunotoxicologique d'une durée de 2½ ans, vingt-deux jeunes Phoques communs (*Phoca vitulina*) ont été nourris de harengs provenant des eaux relativement peu polluées de l'Atlantique ou des eaux très polluées de la Baltique. Des échantillons de sang prélevés à intervalles réguliers chez les deux groupes d'animaux ont été utilisés pour des analyses classiques de l'hématologie et de la chimie clinique. Les analyses n'ont révélé que des différences minimales entre les deux groupes. Vingt variables chimiques cliniques ont été analysées; les concentrations sériques d'urée, de créatinine, de magnésium et de cholestérol ont subi de légères variations. L'hématocrite et le nombre d'érythrocytes étaient plus élevés chez les phoques nourris de hareng de la Baltique, mais ces différences ont diminué graduellement. Les neutrophiles étaient également plus nombreux chez ce groupe de phoques, particulièrement durant la deuxième moitié de l'étude. Parmi les facteurs qui affectaient les variables hématologiques et chimiques chez les phoques d'un même groupe, il faut mentionner le sexe, l'âge et la saison. Les données recueillies indiquent que les variables chimiques analysées sont relativement insensibles aux effets d'une exposition chronique aux contaminants du milieu cumulés par la chaîne alimentaire, mais elles indiquent plutôt que les contaminants semblent affecter le nombre de globules blancs. On trouvera ici un tableau complet des concentrations (valeurs limites) qui assurent normalement une bonne santé aux Phoques communs.
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Introduction

In 1988, an outbreak of a highly infectious disease among European harbour seals (*Phoca vitulina*) caused the death of approximately 20 000 animals (Osterhaus and Vedder 1988). On the basis of serological studies, virus isolation and characterization, and experimental infection of dogs and seals, it was concluded that the primary cause of this epizootic was a previously unrecognized morbillivirus, phocid distemper virus (PDV) (Osterhaus et al. 1988; Mahy et al. 1988; Cosby et al. 1988; Visser et al. 1989; Barrett et al. 1992). However, the contribution of other factors to the severity of this outbreak, such as environmental contaminant related immunosuppression, could not be excluded (Osterhaus and Vedder 1989). This hypothesis became even more plausible when in subsequent years other morbilliviruses caused heavy losses among marine mammal populations inhabiting contaminated coastal waters (Visser et al. 1993).

To test the hypothesis that environmental pollutants accumulated through the food chain adversely affect immune function in marine mammals, we conducted a semifield prospective study using two groups of captive harbour seals. One group received herring (*Clupea harengus*) from the heavily polluted Baltic Sea, while the other group was fed herring from the relatively uncontaminated Atlantic Ocean. Estimated daily intakes of dioxin-like organochlorines, expressed in 2,3,7,8-TCDD toxic equivalents (TEQ) (Safe 1990), were 288 ng TEQ/day for the seals fed Baltic herring and 29 ng TEQ/day for the seals feeding on Atlantic herring (de Swart et al. 1994). Analyses of total TEQ levels in blubber biopsies of the seals taken after about 2 years on the respective diets showed 3.4 times higher levels in seals feeding on Baltic herring (62 $\pm$ 4 vs. 209 $\pm$ 12 ng/kg (mean $\pm$ SD), on lipid base; independent t test: $p < 0.01$) (Ross et al. 1995*a*). Previously, we reported an impairment of immune function in the seals feeding on polluted fish, as measured in vitro by impaired natural killer cell activity and T cell responses (de Swart et al. 1994, 1995; Ross et al. 1995*b*) and in vivo by impaired delayed type hypersensitivity responses and antigen-specific antibody production (Ross et al. 1995*a*).

Haematology and clinical chemistry parameters were measured during this feeding study for use as possible indicators of (immuno)toxic stress as well as indicators of general health. An important aspect of immunotoxicological studies is ascertaining that effects measured in specific immunological assays are directly caused by the chemical(s) under investigation, and are not due to indirect causes such as nutritional status, impaired protein synthesis, or stress. A full set of routine diagnostic parameters was therefore evaluated to control for such potential indirect effects. Since these parameters were measured repeatedly in a relatively large group of clinically healthy pinnipeds, the data collected not only shed light on the effects of chronic exposure to environmental contaminants accumulated through the food chain, but also present a useful set of normal ranges for captive young harbour seals.

Materials and methods

Seals and diets

Twenty-two harbour seals caught as weaned pups on the northeast coast of Scotland were fed herring from the Atlantic Ocean for an adaptation period of 1 year. The seals were matched for mass and gender and divided into two groups (seven females and four males in each group). At the beginning of the feeding study, the diet of the seals in the first group, which were then approximately 15 months old, was changed to herring from the heavily polluted Baltic Sea. The animals were housed at the Seal Rehabilitation and Research Centre in Pieterburen, the Netherlands, in fresh water supplemented with salt (1.5 g/L), in two similar basins with approximately 55 m^3 water and haulout platforms of approximately 24 m^2. The shared water volume of the two basins and a buffer totalled 165 m^3 and was continuously recirculated through a sand–charcoal filter at 80 m^3/h. Based upon constant monitoring of water quality, including bacterial contamination, this water was periodically replenished.

The animals were fed in groups, and both groups received weekly vitamin supplements to compensate for losses during storage of the fish at −25°C. Vitamins supplemented, with the dose per animal in parentheses, were A (12 500 IU), B_1 (50 mg), B_2 (2 mg), B_3 (80 mg), B_5 (2.5 mg), B_6 (25 mg), C (250 mg), and D_3 (2500 IU). An increase in body masses of the seals during the study has been reported previously (de Swart et al. 1994). Blood samplings and other events during the feeding study are indicated by "week of sampling," starting with week 0 in September 1991 and ending with week 126 in February 1994.

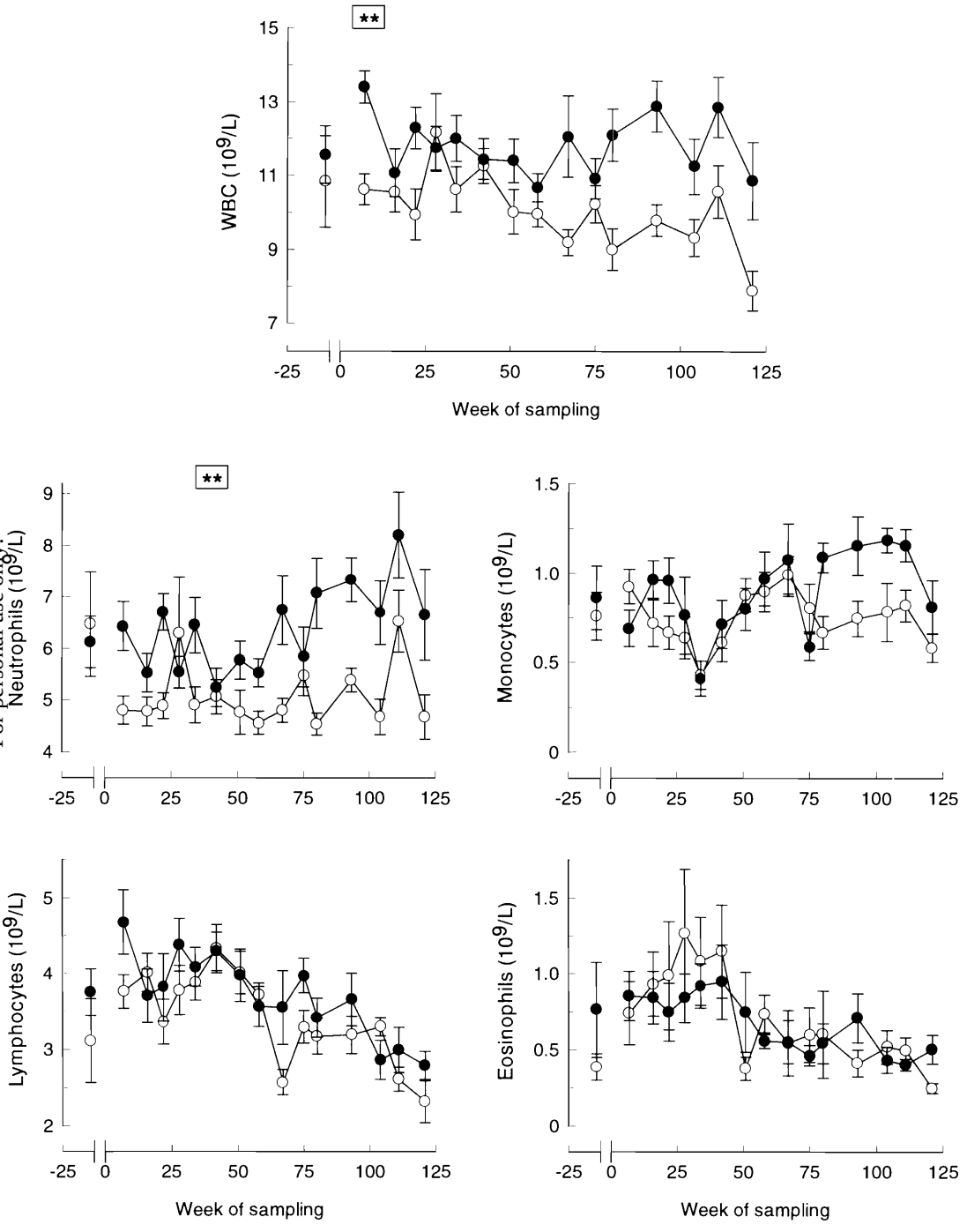
During the experiment, three different batches of herring were fed to both groups. Batch 1 of the Baltic herring was fed from weeks 0 to 50, batch 2 from weeks 51 to 80, and batch 3 from week 81 until the end of the study. Batch 1 of the Atlantic herring was fed until week 16, batch 2 from weeks 17 to 66, and batch 3 from week 67 onwards. The Baltic and Atlantic herring diets contained, on average, 75 and 69% water, 7.1 and 12.3% fat, 17.1 and 16.4% protein, and 1.7 and 2.3% minerals, respectively. The seals in the Baltic herring group were fed more fish than the seals in the Atlantic herring group to compensate for the lower fat percentage in the Baltic herring (the average daily fish intake was 5.6 and 3.7 kg/seal, respectively). This led to similar fat intakes in the two groups (0.40 and 0.45 kg/day per seal, respectively), but higher protein intakes in the Baltic herring group (0.96 and 0.61 kg/day per seal, respectively).

During captivity the seals were under veterinary supervision, and were observed by animal-care technicians at least 4 times per day. This study was undertaken in accordance with Dutch law and approved by the Animal Care Committees of the institutes involved, and in accordance with the guidelines of the Canadian Council on Animal Care.

Blood sampling

Blood samples were taken at 21 weeks before and 7, 16, 22, 28, 34, 42, 51, 58, 67, 75, 80, 93, 104, 111, and 121 weeks following the start of the feeding study. Blood was obtained from the epidural vein using a 18G3½ (1.2 × 90 mm) spinal needle and a Vacutainer tube holder with a Luer lock adapter (Becton–Dickinson). A total volume of approximately 75 mL was collected for measurement of immunological, toxicological, haematological, and clinical chemistry parameters. Samples were taken between 09:00 and 13:00, following a 15-h fasting period, with the exception of the sampling at weeks 42 and 93, when the seals were fed shortly before

Fig. 1. Longitudinal measurements of white blood cell (WBC), neutrophil, lymphocyte, monocyte, and eosinophil counts in harbour seals fed Atlantic (○) or Baltic (●) herring. Data are presented as means ± standard error measured 21 weeks before and 7–121 weeks following the start of the feeding experiment. Significant differences (ANOVA repeated measures, $p < 0.01$) between the two groups are indicated by asterisks.

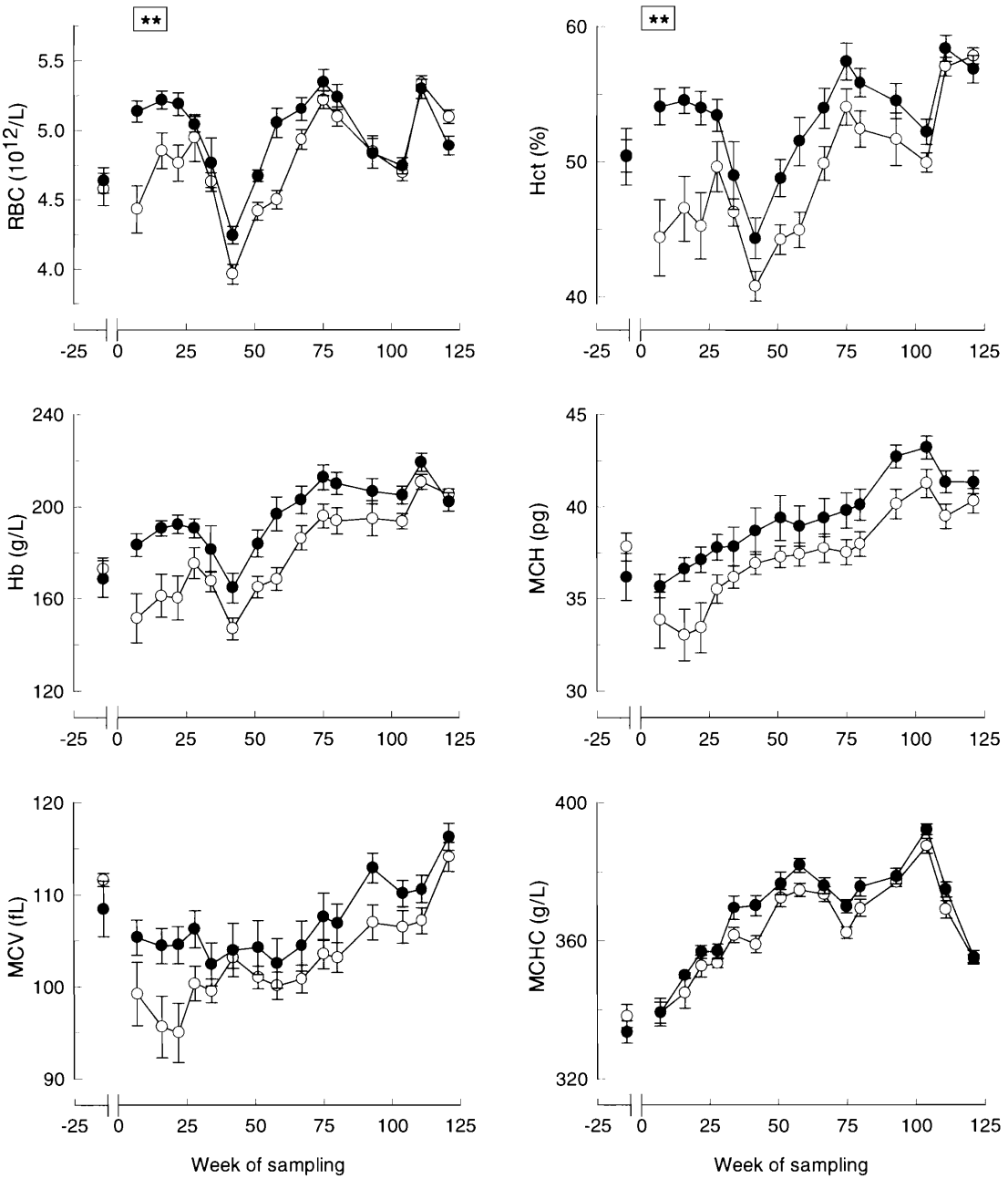


blood sampling. Samples were kept in the dark at 4°C until analysis on the same day.

Haematology and clinical chemistry analyses

Haematological analyses were carried out on blood with EDTA as an anticoagulant, using an automated haematology analyzer (Sysmex E-5000), which uses automatic windowing to differentiate between different cell subsets. Differentiation of leukocyte subsets was carried out by counting 100 leukocytes in Giemsa-stained blood smears. Clinical chemistry parameters were analyzed in serum on a selective discrete clinical chemistry analyzer (Hitachi 717). This machine measures different clinical parameters in one sample, using ion-selective electrodes (indirect potentiometry) and a spectrophotometer with a grid monochromator (12 fixed wavelengths). The machine was run at standard conditions for

Fig. 2. Longitudinal measurements of red blood cell (RBC) counts, haemoglobin levels (Hb), mean corpuscular volume (MCV), haematocrit values (Hct), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentrations (MCHC) in harbour seals fed on Atlantic (○) or Baltic (●) herring. Data are presented as means ± standard error measured 21 weeks before and 7–121 weeks following the start of the feeding experiment. Significant differences (ANOVA repeated measures, $p < 0.01$) between the two groups are indicated by asterisks.



human samples, using an incubation temperature of 30°C. Clinical chemistry values measured at week 34 were excluded from the statistical analyses. These measurements deviated strongly from normal values, and an analytical error was suspected.

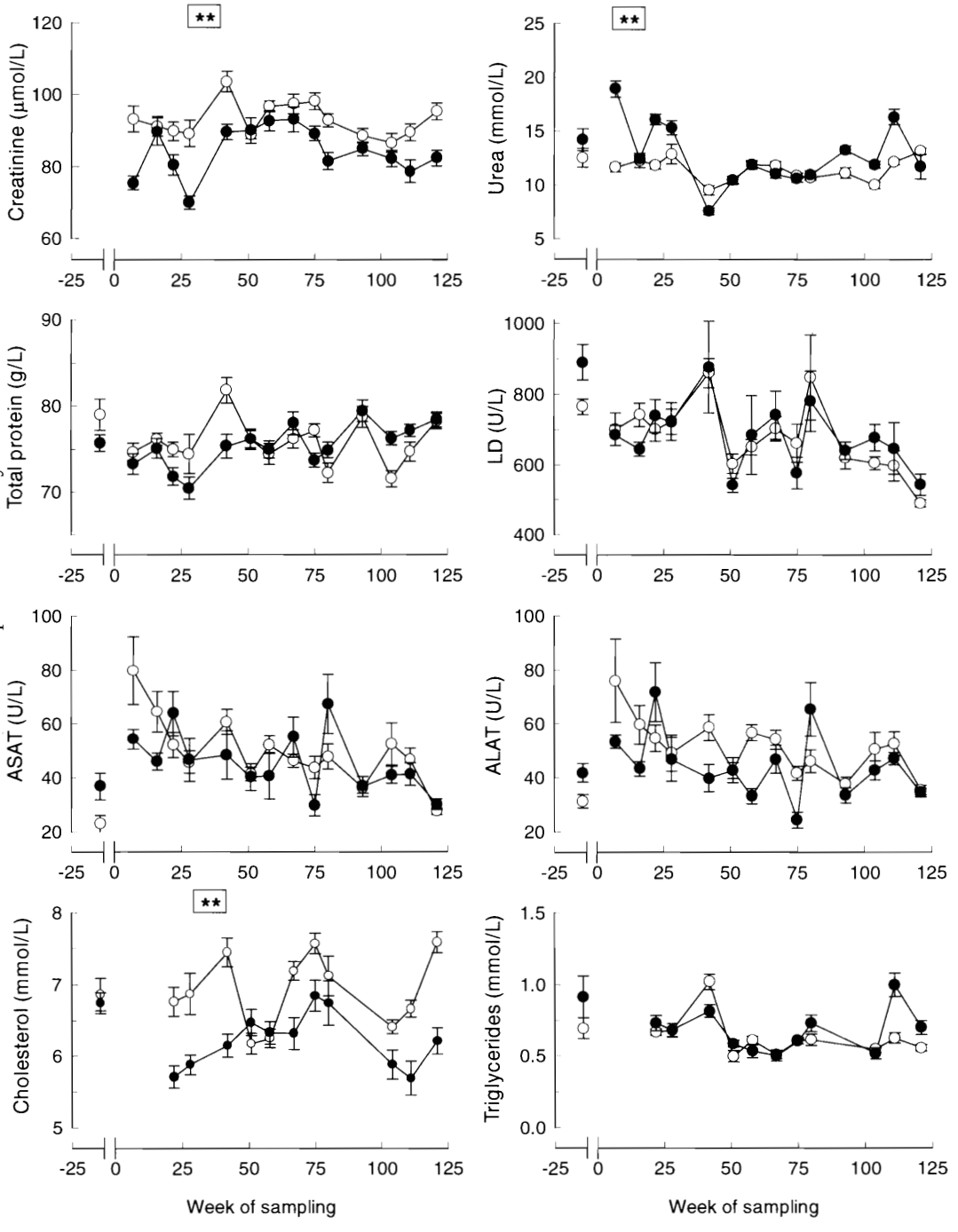
Statistical analysis

Longitudinal data were analyzed using a repeated-measures ANOVA model (BMDP module 5V) with sex and diet as between-animals grouping factors and time as a within-animal factor. The method of restricted maximum likelihood was used to estimate the parameters of the coefficients of the model. For the covariance matrix of the residuals a first-order autoregressive structure was assumed. Differences between the two groups were considered significant if p values for both the influence of diet and the interaction of diet and time were below 0.01.

Calculation of normal ranges

Normal ranges were calculated as the intervals between the 2.5 and 97.5 percentiles (mean ± 1.96 × standard deviation) of data obtained from all 22 seals at all time points, provided

Fig. 3. Longitudinal measurements of creatinine, total protein, aspartate aminotransferase (ASAT), cholesterol, urea, lactic dehydrogenase (LD), alanine aminotransferase (ALAT), and triglyceride levels in harbour seals fed Atlantic (○) or Baltic (●) herring. Data are presented as means ± standard error measured 21 weeks before and 7–121 weeks following the start of the feeding experiment. Significant differences (ANOVA repeated measures, $p < 0.01$) between the two groups are indicated by asterisks. Cholesterol and triglyceride levels measured at weeks 42 and 93 were not included in the statistical analysis, since the animals had been fed shortly before blood sampling and serum levels were elevated at this time.



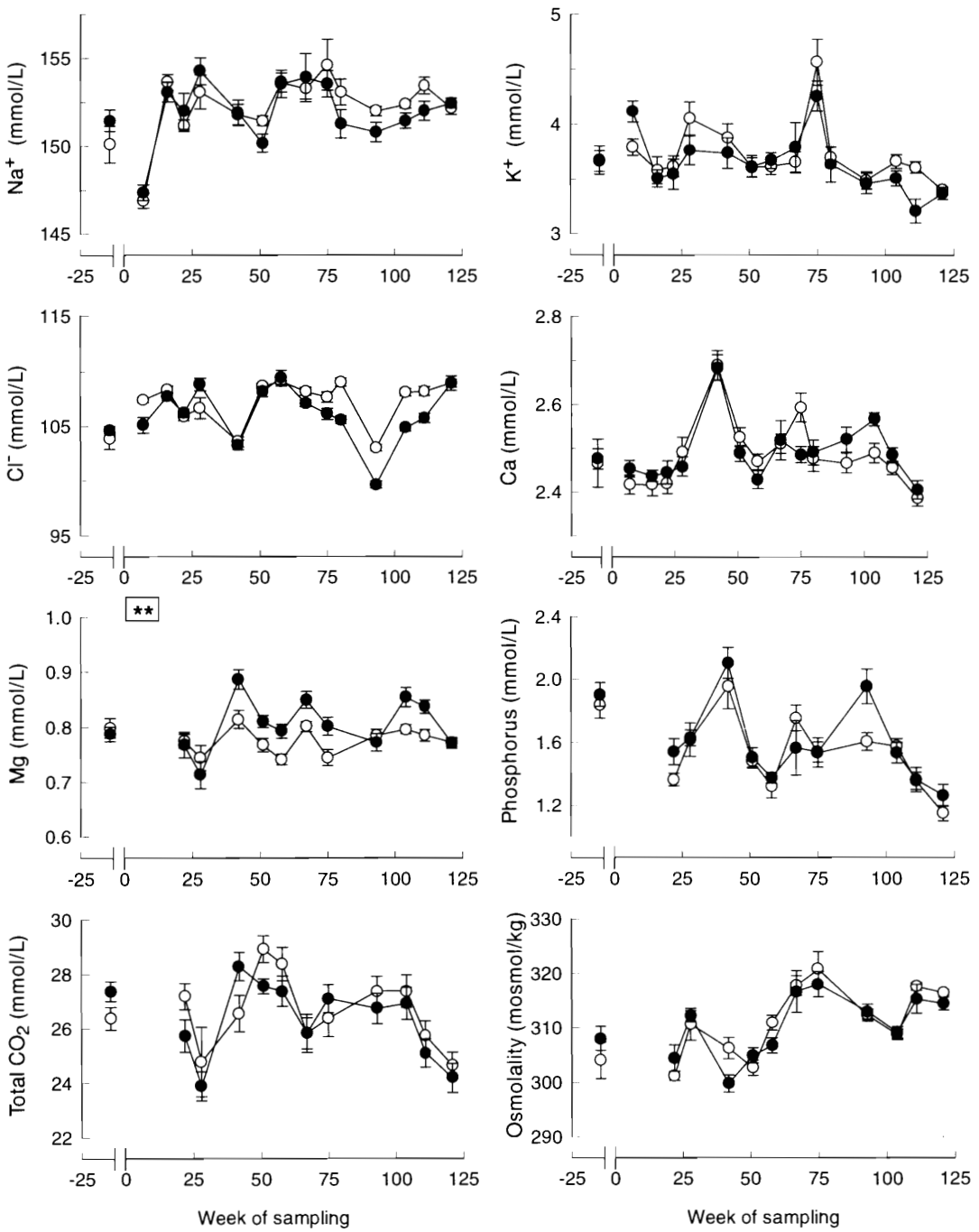
no significant differences were found between the two feeding groups. Where significant differences were found, only data from the 11 seals of the Atlantic herring group were used. The standard deviation of a single measurement was calculated as the square root of the sum of the between-animals and within-animal variance.

Results

Haematology

Longitudinal measurements of leukocyte numbers in both groups of seals are shown in Fig. 1. White blood cell (WBC) counts were significantly higher in seals fed Baltic herring.

Fig. 4. Longitudinal measurements of sodium (Na^+), potassium (K^+), chlorine (Cl^-), calcium (Ca^{2+}), magnesium (Mg^{2+}), phosphorus, and total carbon dioxide (total CO_2) levels and osmolality in the serum of harbour seals fed Atlantic (○) or Baltic (●) herring. Data are presented as means ± standard error measured 21 weeks before and 7−121 weeks following the start of the feeding experiment. Significant differences (ANOVA repeated measures, $p < 0.01$) between the two groups are indicated by asterisks.



This was particularly striking in the second half of the experiment. The higher WBC counts resulted mainly from elevated numbers of neutrophils in these animals, since no significant differences were found in the numbers of lymphocytes, monocytes, or eosinophils. Coinciding, however, with the higher neutrophil counts, monocyte counts measured during the last five samplings were also increased in Baltic herring group seals. This did not lead to a statistically significant difference between the two groups over time. Large numbers of eosinophils often coincided with the presence of heartworm larvae (*Acanthocheilanema spirocauda*) in peripheral blood mononuclear cell samples isolated from heparinized blood as previously described (de Swart et al. 1993). These microfilaria were observed amidst peripheral blood mononuclear cells isolated from seals of both groups. These parasitic infections were not treated and the animals did not show any clinical signs of illness.

Longitudinal measurements of red blood cell (RBC) parameters are shown in Fig. 2. Red blood cell counts and haematocrit (Hct) values were significantly higher in seals fed

Baltic herring. No significant differences were found in mean corpuscular volume (MCV), haemoglobin (Hb), mean corpuscular haemoglobin (MCH), or mean corpuscular haemoglobin concentrations (MCHC).

Clinical chemistry

Longitudinal measurements of serum biochemistry parameters in both groups of seals are shown in Figs. 3 and 4. Serum levels of urea and magnesium were significantly higher in seals fed Baltic herring, whereas levels of creatinine and cholesterol were significantly lower in these animals. No significant differences were found in any other levels. Cholesterol and triglyceride levels measured at weeks 42 and 93 were not included in the statistical analysis, since the animals had been fed shortly before blood sampling and serum levels of these blood constituents were elevated at this time (Fig. 3). Significant gender-related differences were found in lactic dehydrogenase (LD), aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), sodium, potassium, phosphorus levels, and osmolality (higher in males) and magnesium levels (lower in males).

Normal ranges

Normal ranges calculated from the results of this study, including clinical chemistry parameters not displayed in the figures, are listed in Table 1, together with previously reported normal ranges for captive harbour seals (Roletto 1993; Bossart and Dierauf 1990). Normal ranges of parameters for which significant gender-related differences were found are not shown separately for males and females, since the numbers of animals from which these ranges would be calculated would become relatively small. Mean haematology and clinical chemistry values for seals in both groups remained within the previously reported normal ranges, with the exception of eosinophil counts in one of the animals in the Atlantic herring group.

Discussion

Although we previously found impaired immunological responses in the seals fed Baltic herring (de Swart et al. 1994, 1995; Ross et al. 1995*a*, 1995*b*), mean values of most haematology and clinical chemistry parameters remained within previously reported normal ranges for captive harbour seals (Engelhardt 1979; Bossart and Dierauf 1990; Roletto 1993). This suggests that both the Atlantic and the Baltic herring diets were of adequate nutritional value for the captive seals, and that the impaired immunological responses observed were not an indirect effect related to nutrition.

The most prominent difference between the two groups in the parameters measured was an increase in neutrophil counts in the Baltic herring group seals (Fig. 1). Differential WBC counts can serve as indicators of problems related to infection and immunity, and are routinely included in screening programs for immunotoxicity of chemicals (van Loveren and Vos 1989). Previously, we reported significantly higher percentages of neutrophils in peripheral blood leukocytes of seals fed Baltic herring measured over a shorter period (up to week 93) (de Swart et al. 1994). We then speculated that the observed impairment of immune function in these animals, as measured in vitro by natural killer cell activity and T cell proliferation, had led to an increase in subclinical bacterial infections. However, it is equally plausible that an effect at the myeloid stem cell level is responsible. Studies using laboratory animals have demonstrated that a lack of T cell

Table 1. Normal ranges for haematology and clinical chemistry values of harbour seals.

	Unit	This study	Literature
Haematology			
WBC[a]	10^9/L	6.0–14.2	7.5–18.9[b]
Neutrophils[a]	%	33–68	45–81[b]
	10^9/L	2.2–7.9	—
Lymphocytes[c]	%	16–50	10–42[b]
	10^9/L	1.5–5.7	—
Monocytes	%	0–14	0–15[b]
	10^9/L	0–1.7	—
Eosinophils[c]	%	0–16	0–9[b]
	10^9/L	0–2.0	—
Thrombocytes	10^9/L	150–800	—
RBC[a,d]	10^{12}/L	3.9–5.7	3.9–5.5[b]
Hct[a,d]	%	35–63	39–62[b]
Hb[c]	g/L	135–240	143–229[b]
MCH[c]	pg	30.9–45.7	34.5–43.9[b]
MCHC[c]	g/L	336–396	348–388[b]
MCV[c]	fL	88–121	93–121[b]
Clinical chemistry			
Creatinine[a]	μmol/L	75–111	27–97[b]
Urea[a]	mmol/L	8.0–14.8	2.9–12.7[b]
AP	U/L	20–84	23–259[b]
LD[e]	U/L	285–1071	292–1330[b]
GGT	U/L	4.9–14.8	—
ASAT[e]	U/L	5–91	45–221[b]
ALAT[e]	U/L	6–90	36–69[f]
Total bilirubin	μmol/L	2.0–6.6	0.1–6.8[b]
Cholesterol[a]	mmol/L	5.4–8.3	5.5–7.8[f]
Triglycerides	mmol/L	0.2–1.0	0–2.9[b]
Glucose	mmol/L	6.9–9.8	4.8–10.3[b]
Total protein	g/L	67–84	57–89[b]
Na^+[e]	mmol/L	147–158	144–156[b]
K^+[e]	mmol/L	2.8–4.6	3.9–5.9[b]
Cl^-	mmol/L	101–112	99–111[b]
Ca	mmol/L	2.3–2.7	2.2–2.7[b]
Mg[a,e]	mmol/L	0.68–0.87	—
Phosphorus[e]	mmol/L	0.9–2.2	1.4–2.6[f]
Total CO_2	mmol/L	23–32	22–34[b]
Osmolality[e]	mosmol/kg	295–325	—

Note: Normal ranges calculated from the results of this study are the intervals between the 2.5 and 97.5 percentiles of longitudinal data for all 22 harbour seals (14 females and 8 males aged 15–42 months) fed Atlantic or Baltic herring, unless otherwise specified. Normal ranges reported by Roletto (1993) are the intervals between the 2.5 and 97.5 percentiles of values obtained from clinically healthy pups at a rehabilitation centre (Marine Mammal Centre, San Fransisco). Normal ranges reported by Bossart and Dierauf (1990) were obtained from adult harbour seals maintained in fresh water.

[a]Significant difference between Atlantic and Baltic group seals; the results for the Atlantic group seals were used for calculating normal ranges.

[b]From Roletto (1993).

[c]Parameter apparently influenced by age (see figures).

[d]Parameter apparently influenced by season (see figures).

[e]Significant differences found between males and females.

[f]From Bossart and Dierauf (1990).

activity (e.g., in the congenitally athymic nude rat) can be physiologically compensated for by increased activity of myeloid stem cells, leading in turn to higher production of both monocytes and granulocytes (Vos et al. 1980). The increase in monocyte counts during the last five blood samplings may support the latter hypothesis.

The significantly higher RBC counts and Hct values in seals fed Baltic herring (Fig. 2) could not be related to iron deficiency, since MCHC values were not significantly different. The chronic contaminant exposure is not likely to be the cause of these differences either, since they were most pronounced during the first half of the experiment and decreased towards the end. MCV values also appeared to be higher in Baltic herring group seals during the first half-year, perhaps indicating a shift in red cell age distribution. At the beginning of the experimental period the animals in the Baltic herring group refused to eat when their diet was changed from Atlantic to Baltic herring, leading to a sudden drop in body mass (de Swart et al. 1994). Although the masses returned to those of the control group 4–5 weeks later, it is possible that this fasting period led to a shorter life-span of their red cells, which was compensated for by increased production of erythrocytes.

The differences in cholesterol, creatinine, urea, and magnesium levels between the two groups of seals were relatively small. Estimated daily fat intake was similar in both dietary groups, and no differences were found in serum triglyceride levels. The observed differences in cholesterol levels could be related to possible differences in fatty acid composition between the two diets. Creatinine is formed in muscles during the metabolism of creatine and phosphocreatine, and enters the circulation only for transportation to the kidneys. Since daily production of creatinine is relatively constant and serum levels are unaffected by diet (Bossart and Dierauf 1990), levels are generally used as an indicator of kidney function. Since there was no indication of renal problems in the seals of our study groups, the lower creatinine values in the Baltic herring group seals may reflect slightly lower muscle mass in these animals. Serum urea levels are known to be elevated by high-protein diets (Bossart and Dierauf 1990). Although estimated daily protein intakes of the seals in the Baltic herring group were 50% higher than those of seals in the Atlantic herring group, urea levels were only slightly higher in these animals. The largest difference was found 7 weeks following the start of the experiment, and was probably related to the high fish intake of the Baltic herring group in this period relative to the fasting period that they had gone through immediately following their change of diet. The higher serum magnesium levels in the seals fed Baltic herring probably resulted directly from differences in magnesium intake.

The observed differences in haematology and serum chemistry values differ from those reported previously by Reijnders (1988). In a captive feeding study in which two groups of harbour seals were fed fish containing different levels of contaminants he found elevated lymphocyte and basophil counts and ALAT, ASAT, alkaline phosphatase (AP), and gamma glutamyl transpeptidase (GGT) levels in the seals fed on contaminated fish. Reduced levels of total bilirubin, creatinine, uric acid, calcium, magnesium, albumin, and gamma-globulin were found in these animals. These results, however, are difficult to compare with the present data, since Reijnders studied adult female harbour seals fed on two different fish species (herring and flatfish), and data were obtained from a single blood sampling.

Apart from differences between the two dietary groups, within-group differences in haematology and clinical chemistry parameters were observed. Apparently age-related changes were observed in lymphocyte and eosinophil counts, which decreased with age, and in Hb, MCH, MCHC, and MCV values, which increased with age (see Figs. 2 and 3). A decrease in lymphocyte count with increasing age has also been observed in other species like the dog, cow, and sheep (Jain 1986). Haemoglobin, MCH, MCHC, and MCV values also tend to be influenced by age in these species, although observations within species are often inconsistent (Jain 1986). Red blood cell counts and Hct values showed dips around weeks 42 and 104 (July 1992 and September 1993, respectively), indicating a seasonal trend (Fig. 2). Seasonal influences on erythrocyte parameters are also commonly found in other mammals (Jain 1986; Rietkerk et al. 1994), and may be related to temperature-dependent changes in behaviour or water balance. Since the seals spent most of their time hauled out in summer, the decreased RBC parameters might reflect a reduced oxygen requirement. Statistical analysis also revealed gender-related differences in several of the clinical chemistry parameters measured. Similar differences have been reported in other species (Audigé 1992; Rietkerk et al. 1994), although there are no discernible patterns in these data. In studies of healthy, free-ranging harbour seal mothers and their pups, factors including age, fasting, and reproductive status apparently influenced several haematological and clinical chemistry parameters (Ross 1990; Ross et al. 1994).

The data obtained during this study are important not only for their value for the immunotoxicological feeding experiment, but also for their utility as a set of normal ranges and longitudinal patterns of haematology and clinical chemistry values in healthy captive harbour seals. These values will be of particular use for the interpretation of haematology and clinical chemistry values measured in seals suspected of acute or chronic disease.

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