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PLASMA HAPTOGLOBIN LEVELS IN THREATENED ALASKAN PINNIPED POPULATIONS

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ABSTRACT: We evaluated the plasma concentration of the acute phase protein haptoglobin (Hp) from Steller sea lions (*Eumetopias jubatus*) and harbor seals (*Phoca vitulina*) in regions of Alaska (USA) where the populations of these pinnipeds were declining and compared the values with concentrations of Hp from the same species in areas where the populations were stable. Samples were collected from 1992 through 1994 at sites in Southeast Alaska, Prince William Sound, the Gulf of Alaska, and the Aleutian Islands. Significantly higher levels of Hp were found in the samples from the areas of decline compared to those from stable populations. Based on these findings, we propose that one may be able to distinguish these compromised pinniped populations using Hp as a biomedical indicator.

Key words: Steller sea lion (*Eumetopias jubatus*), harbor seal (*Phoca vitulina*), blood chemistry, health, disease, stress.

INTRODUCTION

Haptoglobin (Hp) belongs to a group of blood proteins that increases considerably in response to infection, inflammation, tumor, or trauma in what is known as the acute phase reaction. The analysis of Hp concentration is a very sensitive, although non-specific, indicator of disease used for diagnostic assessments in human and veterinary medicine (Henry et al., 1974). It is an α_2-glycoprotein which binds free hemoglobin (Hb) in a stable complex, both in vivo and in vitro (Nguyen, 1989). Haptoglobin prevents the loss of Hb from the plasma and blocks the exchange of heme between methemoglobin and albumin (Koj, 1974).

As one of the acute phase proteins, Hp increases in cases of inflammatory and infectious disease, trauma, myocardial infarction, active rheumatoid arthritis, cancer, leukemia, and tuberculosis (Gordon and Koj, 1985; Echersall et al., 1989; Cross et al., 1991). Increases in the acute-phase proteins, including Hp, have been associated to physical, psychological, and environmental stress (Aikawa et al., 1990; Kalmovarin et al., 1991; Boosalis et al., 1992). The Hp concentration is useful in the determination of acute hepatitis, anemia, and other hemolytic episodes, in which the plasma concentration of Hp is significantly reduced, due to increased removal rate of the haptoglobin-hemoglobin (Hp-Hb) complex (Laurell and Gronwall, 1962). Reduced Hp levels are also observed during paroxysmal hemoglobinuria, malaria, chronic liver disease, cirrhosis and mononucleosis, due to decreased synthesis of Hp in the liver (Giblett, 1974).

In humans, the first signs of increased synthesis of Hp after injury appear with a delay of 3 to 6 hr, maximum levels being reached about 5 to 7 days after the onset of the inflammatory process, and returning to normal levels within 4 to 6 wk (Nguyen, 1989). The Hp-Hb complex has a shorter half-life of 10 to 90 min (Loeb and Quimby, 1989). Effects of the long-term sustainment of an acute-phase reaction have not been recorded; however, based on the results of Murata and Yamamoto (1993), chronic increases in Hp may compromise the immunocompetence of an animal.

The application of acute phase protein analyses to wild populations to assess potential stressors is relatively new. Duffy et al. (1993) found elevated Hp levels in river otters (*Lutra canadensis*) 1 yr after being exposed to the *Exxon Valdez* oil spill in Prince William Sound, Alaska (USA). Sampling a year later, in 1992, however, they deserved a return to lower Hp levels (Duffy et al., 1994).

To assess the potential use of Hp anal-

yses for population health studies of marine mammals, Steller sea lions (*Eumetopias jubatus*) and harbor seals (*Phoca vitulina*) from Alaska were examined. For the past 20 yr the populations of Steller sea lions and harbor seals in Alaska have been declining (Pitcher, 1990; Loughlin et al., 1992), and a portion of the Steller sea lion population has recently been proposed as endangered (Federal Register, 1995). There is no known obvious cause for these declines. In routine hematological studies of Alaskan Steller sea lion pups, Castellini et al. (1993) and Rea (1995) found no evidence in the clinical blood chemistries of gross physiological or metabolic disturbances. Since Hp may be a much more sensitive indicator of disease or sublethal damage, Hp levels in plasma samples of Steller sea lions from the Gulf of Alaska and the Aleutian Islands, and of harbor seals from Prince William Sound (all sites where significant population declines have occurred) were compared to samples from Steller sea lions and harbor seals captured in Southeast Alaska (where the populations have been stable) (Loughlin et al., 1992; Small and DeMaster, 1995). Our working hypothesis was that plasma samples from the areas of decline may be characterized by Hp levels significantly different than control populations.

MATERIALS AND METHODS

Blood samples were taken from Steller sea lions at rookeries in the Aleutian Islands and the Gulf of Alaska during the summers of 1992, 1993 and 1994. Harbor seals were sampled in Prince William Sound, Alaska, in 1994. As comparative controls, Steller sea lions were sampled in Southeast Alaska during the summers of 1992, 1993 and 1994 and harbor seals in 1994. Additionally, blood samples were obtained from healthy Steller sea lions captured from the wild as pups in 1994 and reared at Vancouver Aquarium (Vancouver, British Columbia, Canada). We collected 128 plasma samples from Steller sea lions. These included 19 pups from the Aleutian Islands (approximately 158° to 166°W, 55°N; 13 newborns, 41 pups, and 14 adults from different rookeries in the Gulf of Alaska (approximately 147° to 154°W, 58 to 60°N); six newborns, 22 pups, four juveniles, and five adults from Southeast Alaska, including Forrester Island (133°W, 55°N); and four juveniles from Vancouver Aquarium. We also had plasma samples from 63 harbor seals, including 12 juveniles and 20 adults from Prince William Sound, Alaska (approximately 147°W, 61 N); and nine juveniles and 22 adults from Southeast Alaska (133° to 135°W, 55 to 58 N). Harbor seals were subjectively assigned into the age categories of adult (>45 kg in weight, >110 cm in length), juvenile (>25 to 45 kg, >90 to 110 cm), and pup (≤25 kg, ≤90 cm) based on their size. Steller sea lions were classified as newborn when the umbilicus was attached, and as pup if the umbilicus was no longer present but the animal was still nursing. Detailed age information was available for a subset of Steller sea lion pups which had been tagged within two days of birth. Steller sea lion adults and juveniles were darted with Telazol® (Wyeth-Ayerst Laboratories, Philadelphia, Pennsylvania, USA) (2 mg/kg; Loughlin and Spraker, 1989) and some of the adults were anesthetized further with Halothane® (Wyeth-Ayerst Laboratories) (2 to 4%; Heath et al., 1993). Steller sea lion pups were manually restrained. Harbor seals from Prince William Sound were anesthetized with ketamine (Aveco Company Inc., New York, New York, USA) and diazepam (Abbott Laboratories, North Chicago, Illinois, USA) at standard doses (2 to 3 mg/kg and 0.25 ml, respectively; Geraci et al., 1981), and seals from Southeast Alaska were manually restrained. All animals included in this study were typically sampled within 4 hr of capture. Blood samples were taken by venipuncture from either a hind flipper vein, the extradural vein, or the dorsal pelvic vein, and collected in heparinized vacuum tubes (Vacutainer, Beckton Dickinson, Rutherford, New Jersey, USA) and stored on ice. All animals were captured in collaboration with National Marine Fisheries Service (NMFS) and Alaska Department of Fish and Game (ADFG) field projects.

In all cases, plasma was separated by centrifugation within 4 hr, and samples were stored at −20 C until assayed. The Hp content of plasma samples was determined as the Hb-binding capacity of Hp, using a high resolution electrophoresis kit (Helena Laboratories, Beaumont, Texas, USA) (Moors, 1978). A known excess of a 10% solution of homologous Hb was added to each plasma sample and allowed to mix for 5 min. Samples were then applied to an agarose plate and electrophoresed (Wide Mini Sub Cell with model 3000 xi power supply, BioRad, Palo Alto, California, USA) at 110 V for 30 min. After fixing the protein complex by drying at 60 C for 15 min, the gels were stained with o-di-

anisidine (Valeri et al., 1965). The Hp-Hb complex, which migrates in a different region from Hb, was quantified in a densitometer (model 620, BioRad, Palo Alto, California). Results were expressed as mg of Hb-binding capacity per 100 ml of plasma. Data were analyzed by using non-paired *t*-tests with Bonferroni adjustment for multiple comparisons, and are presented as mean ± SE (Shott, 1990). Statistical significance was assumed when $P < 0.05$.

A separate set of tests were carried out to evaluate the possibility that handling methods and time between capture and sampling might have affected Hp levels. These hypotheses were tested in several ways. First, comparisons were made between Hp concentrations between anesthetized and manually restrained harbor seals. Second, correlations between Hp levels and known biochemical indices of short-term handling stress, including creatinine phosphokinase (CPK), alanine aminotransferase (ALT), alkaline phosphatase (AP), aspartate aminotransferase (AST) and albumin-to-immunoglobulin G (A/G) ratios in harbor seals and Steller sea lions from Southeast Alaska were analyzed. Concentrations of these metabolites were determined at Fairbanks Memorial Hospital (Fairbanks, Alaska, USA), using a Kodak Ektachem analyzer (Kodak Company, Rochester, New York), following the manufacturer's instructions. Third, changes in Hp concentrations with time elapsed between capture and sampling in harbor seals in Southeast Alaska were evaluated. Fourth, the effect of time-lag between capture and blood sampling on Hp concentration in five healthy northern elephant seal pups (*Mirounga angustirostris*) fitted with indwelling venous catheters, under controlled laboratory conditions was evaluated. Differences between time interval samples were assessed using an analysis of variance (ANOVA) for repeated measures and post-hoc analysis with use of a Tukey's multiple range test (Zar, 1984).

RESULTS

No statistically significant differences in Hp concentrations were found between 20 anesthetized (mean ± SE Hp: 136.3 ± 11.2 mg/100 ml) and 12 non-anesthetized (123.7 ± 10.5 mg/100 ml) harbor seals ($T = -0.76$, $P = 0.455$). Similarly, Hp concentrations did not change with time between capture and sampling in harbor seals in Southeast Alaska (mean ± SE Hp within the first hour after capture, 75.1 ± 5.9 mg/100 ml; 2 hr after capture, 86.6 ± 16.7 mg/100 ml; 3 hr after capture, 78.3 ± 13.1 mg/100 ml; 4 hr after capture, 81.1 ± 17.7 mg/100 ml). In parallel studies, Hp levels were not correlated with biochemical indices of short-term handling stress in harbor seals (CPK, $r = 0.077$, $P = 0.545$; AST, $r = 0.146$, $P = 0.250$; ALT, $r = 0.138$, $P = 0.276$; AP, $r = 0.019$, $P = 0.880$) or Steller sea lions (CPK, $r = -0.259$, $P = 0.122$; AST, $r = -0.205$, $P = 0.223$; ALT, $r = -0.257$, $P = 0.125$; AP, $r = -0.292$, $P = 0.079$). Finally, in northern elephant seal pups with indwelling venous catheters, there were no significant effects of time between capture, catheterization, and blood sampling on Hp concentration for up to 8 hr (mean ± SE Hp at catheterization, 80.7 ± 7.0 mg/100 ml; 1 hr later, 93.6 ± 4.4 mg/100 ml; 2 hr, 113.5 ± 7.1 mg/100 ml; 3 hr, 103.2 ± 14.9 mg/100 ml; 5 hr, 114.4 ± 14.9 mg/100 ml; 7 hr, 104.1 ± 10.2 mg/100 ml), using ANOVA for repeated measures and Tukey's post-hoc analysis.

In Steller sea lions, significantly higher Hp concentrations were found in samples from the Gulf of Alaska and the Aleutian Islands than in those from Southeast Alaska (Table 1). The Hp levels in the four Steller sea lions held at Vancouver Aquarium were comparable to those in sea lions from Southeast Alaska. Similarly, harbor seals sampled in Prince William Sound had significantly higher Hp levels than those sampled in Southeast Alaska.

The Hp levels in Steller sea lions from Southeast Alaska generally increased with age (Fig. 1), with the exception that newborns (estimated <5 days old) had relatively high Hp levels compared to animals estimated to be 10 days old.

In Steller sea lion pups, A/G ratios had a weak negative relationship with Hp levels (A/G ratio = 1.723 − 0.001 (Hp); $r^2 = 0.358$). This was due to a decrease in the albumin content. No correlation was found between Hp levels and A/G ratio in plasma samples from harbor seals ($r = 0.059$, $P = 0.643$).

TABLE 1. Haptoglobin concentration (mg Hb bound/100 ml) in plasma of Steller sea lions and harbor seals. Newborns = <5 days old; pups = 1 to 10 weeks old; juveniles = 1 to 3 years old; adults = >4 years old. Age classification was based on estimated age at sampling.

Site	Age	Mean	SE	Number sampled
	STELLER SEA LIONS			
Southeast Alaska	Adults	143.1	13.5	5
	Juveniles	97.8	14.4	4
	Pups	87.2	13.8	22
	Newborns	35.4[a]	4.6	6
Aleutian Islands	Pups	253.6[b]	16.7	19
Gulf of Alaska	Adults	250.7[b]	20.7	14
	Pups	263.0[b]	10.3	41
	Newborns	68.6[a]	21.8	13
Vancouver Aquarium	Juveniles	73.8	11.2	4
	HARBOR SEALS			
Southeast Alaska	Adults	82.9	11.3	22
	Juveniles	73.9	9.1	9
Prince William Sound	Adults	133.1[b]	11.4	20
	Juveniles	130.1[b]	10.8	12

[a] Significantly ($P < 0.05$) different compared to adult animals.
[b] Significantly ($P < 0.05$) different compared to animals from Southeast Alaska.

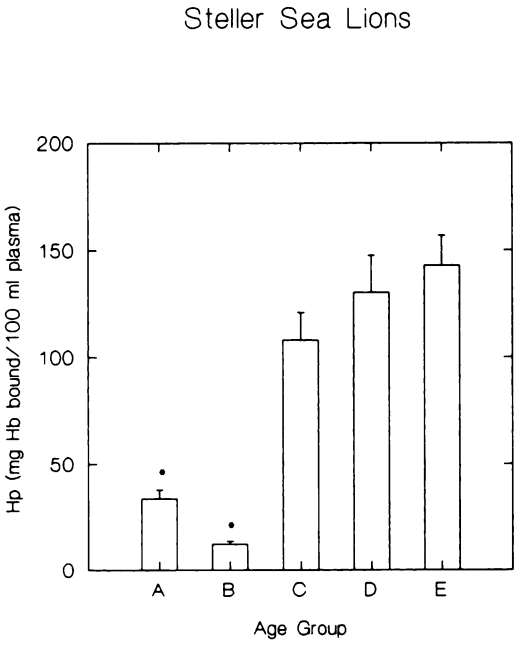

FIGURE 1. Mean circulating haptoglobin (Hp) levels in Steller sea lions from Southeast Alaska. A = newborn (<5 days old), B = pup (5 to 15 days old), C = pup (1 to 10 weeks old), D = juvenile (1 to 3 years old); E = adult (>4 years old). Vertical lines indicate ± SE. * = Significantly ($P < 0.05$) different compared to adult animals.

DISCUSSION

Based on the control studies, we believe that the differences in Hp seen in Steller sea lion and harbor seal populations were probably not due to handling differences. Furthermore, immobilization of harbor seals, white-tailed deer (*Odocoileus virginianus*), black bears (*Ursus americanus*), and raccoons (*Procyon lotor*) for over 6 hr did not result in changes in Hp levels (Seal et al., 1971).

Mean ± SE Hp concentrations in plasma samples from adult Steller sea lions and harbor seals in Southeast Alaska (Table 1) fell within the ranges reported for healthy adult humans: 93 ± 40 mg/100 ml (Kirk, 1968) and 150 mg/100 ml (Oliviero et al., 1987). Haptoglobin levels were significantly ($P < 0.05$) higher in Steller sea lions of all age classes sampled in the Gulf of Alaska and the Aleutian Islands than in sea lions sampled in Southeast Alaska, and were comparable to the mean ± SE Hp concentrations reported in serum from mice subjected to an acute phase response (297.6 ± 9.3 mg/100 ml; Waites et al., 1983). In harbor seals, Hp concentration

was higher in samples from Prince William Sound than in those obtained at Southeast Alaska. Albeit elevated relative to harbor seals from Southeast Alaska, Hp concentration in samples from harbor seals at Prince William Sound did not reach the levels observed in samples from Steller sea lions at the Aleutian Islands and the Gulf of Alaska, nor the values reported for laboratory mice (Waites et al., 1983) during the induced acute phase response.

Based on these data, we believe that the Steller sea lion and harbor seal populations in the Gulf of Alaska and the Aleutian Islands may be faced with a stressor, or a group of stressors, that are inducing an acute phase reaction, as supported by elevated Hp levels.

Mean ± SE Hp levels in adult harbor seal serum samples, collected in 1989 and 1990 following the *Exxon Valdez* oil spill, were not significantly different for oiled and non-oiled areas of Prince William Sound, or from non-oiled areas of the Gulf of Alaska (125.1 ± 11.4, n = 7; 144.0 ± 22.7, n = 5; 148.4 ± 24.7, n = 7, respectively) (Frost and Lowry, 1994). However, the mean ± SE Hp level (133.0 ± 11.3, n = 19) for these animals, sampled in 1989 and 1990, is similar to the values from this study for Prince William Sound adult harbor seals (Table 1).

In Steller sea lion pups, Hp was detectable within the first week of life. The developmental variation in Hp concentration observed in the Steller sea lion pup samples is normal in mammals. In newborn mice, serum Hp was about 50% of the adult levels; the Hp levels dropped quickly and remained at 10 to 30% of the adult range during the first 2 wk of life (Loeb and Quimby, 1989). In humans, Hp is first detected in 14-day-old infants and increases gradually with the maximum value seen by about the 20th year (Kirk, 1968; Loeb and Quimby, 1989). Hypohaptoglobinemia is characteristic of most human newborns and the normal range for Hp levels in infants is 1 to 30 mg Hb bound/100 ml (Adinolfi and Adinolfi, 1974). The Hp concentrations in all Steller sea lion pups were higher than in healthy human infants; thus, this species may be immunologically precocious compared to humans. Shaughnessy (1974) found Hp bands in electrophoretic analyses of serum samples from 1-day-old southern elephant seals (*Mirounga leonina*) and concluded that they were more immunologically advanced at birth than most mammals.

Haptoglobin acts as an immunomodulator in humans and cattle (Oh et al., 1990; Murata and Yamamoto, 1993). The negative relationship between A/G ratios and Hp levels in Steller sea lion pups may be evidence for impairment of the immune system in these animals and is consistent with the suppression of lymphocyte blastogenesis reported for bovine calves (Murata and Yamamoto, 1993). Albumin deficiency is characteristic of various liver diseases, including cirrhosis, as well as parasitic infections, chronic inflammation, and malignancy (Vido and Rezai, 1975; Keshgegian, 1984; Monzon and Villavisencio, 1990).

There are many possible disease states, as well as physical, psychological, or environmental stressors, that could induce a higher Hp level in the Steller sea lions and harbor seals. Future workers will need to address the relationship between Hp concentrations and known disease states in these species. However, based on these data, some event has occurred which differentiates the declining and stable pinniped populations on the basis of Hp levels.

Beyond disease states, it is possible that these differences in Hp concentrations may reflect differences in genetic composition or biology and natural history of harbor seals and Steller sea lions. Sufficient behavioral, phenotypic, and genotypic evidence has been recently found to distinguish the Steller sea lions in Alaska as two distinct stocks separated east and west of Cape Suckling, Alaska (144°W longitude) (Bickham et al., 1996; Loughlin, 1996). All the Southeast Alaska, as well as the Vancouver Aquarium Steller sea lions analyzed

in this study were sampled at various sites east of Cape Suckling while those from the Gulf of Alaska and Aleutian Islands were sampled at several rookeries and haul-out sites west of Cape Suckling. Harbor seals at Grand Island, Southeast Alaska, were sampled during August 1994, at which time they were molting. Further research is needed to address the possibility that differences in Hp levels found between pinnipeds in the Gulf of Alaska, Aleutian Islands and Prince William Sound and those in Southeast Alaska reflect genetic isolation between stocks, and to study changes in Hp levels related to different stages in the life cycle (such as molting and fasting) of these species. Current studies are focused on these problems in harbor seals where samples from different seasons can be obtained.

In summary, plasma concentrations of Hp from Steller sea lions and harbor seals in stable populations (Southeast Alaska) and healthy Steller sea lions (Vancouver Aquarium) were within the range reported for humans and other mammals. Compared to reported values for newborn and infant humans, Steller sea lion pups had higher Hp levels; thus, these animals may be born with an immune system developed to a higher degree than humans. Steller sea lions and harbor seals sampled at sites where populations are declining (Aleutian Islands and Gulf of Alaska, and Prince William Sound, respectively) had elevated levels of plasma Hp relative to individuals from stable populations. This is the first evidence that these stocks can be separated on the basis of an acute phase reaction that is consistent with declining Alaskan pinniped populations.

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