Clinical evaluation and biochemical analyses of thiamine deficiency in Pacific harbor seals (Phoca vitulina) maintained at a zoological facility

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Objective—To determine thiamine-dependent enzyme activities in various tissue samples of Pacific harbor seals (Phoca vitulina) and thiaminase activities in dietary fish.

Design—Cross-sectional study.

Animals—11 Pacific harbor seals with thiamine deficiency and 5 control seals.

Procedures—Seals underwent evaluation to rule out various diseases and exposure to toxins. For seals that died, measurement of thiamine-dependent enzymes in liver and brain samples and determination of mitochondrial DNA (mtDNA) copy number in liver, brain, and muscle samples were performed. Thiaminase activity in dietary fish was determined.

Results—8 seals with thiamine deficiency died. Affected seals typically had acute neurologic signs with few nonspecific findings detected by means of clinico-pathologic tests and histologic examination of tissue samples. Thiamine-dependent enzyme activities in liver samples of affected seals were significantly lower than those in control liver samples. The primary activation ratios and latencies for enzymes indicated that brain tissue was more affected by thiamine deficiency than liver tissue. Activities of pyruvate dehydrogenase were more affected by thiamine deficiency than those of transketolase and ketoglutarate dehydrogenase. For control seals, the mtDNA copy number in muscle samples was significantly lower than that for affected seals; conversely, the copy number in control liver samples was significantly greater than that of affected seals. Thiaminase activity was substantially higher in smelt than it was in other types of dietary fish.

Conclusions and Clinical Relevance—Results of analyses in this study confirmed a diagnosis of thiamine deficiency for affected seals resulting from high thiaminase activity in dietary fish, inadequate vitamin administration, and increased thiamine demand caused by pregnancy and lactation. (J Am Vet Med Assoc 2013;243:1179–1189)

Thiamine (vitamin B₁) is an essential micronutrient for humans and animals of most other species. Animals obtain thiamine primarily from their diets, and most dietary thiamine is absorbed in the proximal aspect of the small intestine. Thiamine is essential for carbohydrate, amino acid, and fatty acid synthesis and metabolism; this vitamin is a cofactor for various metabolic enzymes, including TK, PDHC, and AKGDH. Transketolase is part of the pentose phosphate pathway, which produces metabolites for fatty acid, hormone, nucleic acid, and antioxidant synthesis. Pyruvate dehydrogenase complex and AKGDH have an important role in ATP production via the Krebs cycle, and PDHC deficiency in animals is associated with impaired production of neurotransmitters, such as acetylcholine.

Thiamine deficiency has detrimental effects for animals of many species. Insufficient intake of thiamine causes neurologic disorders in humans such as encephalopathy, psy-
Aquatic animals have diets that may contain thiamine; these animals are susceptible to diet-induced thiamine deficiency. Thiamine deficiency potentially attributable to eating a diet of fish was first reported for a gray mullet (*Hillichthys grypus*) that had a diet consisting of sea smelt (*Osmerus mordax*). Other authors attributed thiamine deficiency in captive California sea lions (*Zalophus californianus*) and a bottlenosed dolphin (*Tursiops truncatus*) to ingestion of fish. Diagnoses for the deficiency in the animals of these reports were presumptive because it was difficult to determine whether thiamine concentrations were low in postmortem samples from those animals. Clinical signs and various metabolic effects of thiamine deficiency were reported for seals kept in controlled zoological settings. Polioencephalomalacia presumably attributable to thiamine deficiency, determined on the basis of clinical signs and histopathologic lesions, has been reported for captive harbor seals; however, although other causes of polioencephalomalacia (eg, toxins, pesticides, and infectious diseases) were ruled out for those seals, thiamine deficiency could not be confirmed because assays for determination of that diagnosis were not performed.

Thiamine deficiency in an animal may be suspected on the basis of history (particularly regarding diet), clinical signs, response to treatment, and histopathologic lesions. Confirmation of that diagnosis is difficult; thiamine deficiency is diagnosed for humans on the basis of detection of low TK activity in RBCs or by means of other indirect methods including analysis of dietary thiamine or organic acids in urine samples.

In the study reported here, we investigated thiamine deficiency in Pacific harbor seals (*Phoca vitulina*). No whole blood samples were available from animals after death; therefore, thiamine deficiency was evaluated on the basis of the TPP-dependent enzymatic activity in samples other than blood (ie, brain and liver samples). In tissues that have a high rate of aerobic metabolism, such as the brain, TPP-dependent enzymes (PDHC and AKGDH) other than TK are important for ATP production in mitochondria. Measurement of TK activity in erythrocytes (cells that do not have mitochondria) may underestimate the severity of thiamine deficiency in such tissues. We determined activities of 3 thiamine-dependent enzymes (TK, PDHC, and AKGDH) in brain and liver samples of seals at a zoological institution that died of suspected thiamine deficiency and control animals that died of unrelated causes (free-ranging, stranded seals that died because of infection, trauma, or acute exposure to toxic substances). In addition, we evaluated oxidative stress caused by thiamine deficiency by means of determination of mtDNA copy number in liver, muscle, and brain samples of seals with thiamine deficiency and control seals because increases in mtDNA copy number are associated with a compensatory response to oxidative stress.

An objective of the study reported here was to identify enzymes and tissue sample types that could be assayed for determination of a diagnosis of thiamine deficiency in seals. Another objective was to determine baseline activities of TPP-dependent enzyme activities in tissue samples of control (unaffected) seals. To the authors' knowledge, no data have been published regarding activities of TPP-dependent enzymes in tissue samples obtained from harbor seals.

**Materials and Methods**

**Animals**—Eleven seals with thiamine deficiency were included in the study. These seals were evaluated between April and July 2010 because of severe neurologic signs (n = 9) or sudden death with no premonitory signs (2). These seals included one 2-year-old female, one 3-year-old male, three 4-year-old females, one 6-year-old female, two 7-year-old females, one 8-year-old female, one 19-year-old female, and one 28-year-old female. These seals had been healthy with a clinically normal appetite prior to illness or death. Four of these female seals had healthy pups within 2 weeks prior to the initial evaluation, and 2 female seals had stillborn pups approximately 2 months prior to that time. Six of 9 female seals in the breeding colony that had pups during 2010 developed thiamine deficiency and were included in this study. Seals included in this study were in a breeding colony that included 29 Pacific harbor seals (11 male and 18 female seals [age range, < 1 year to > 40 years]) and 41 California sea lions (various sexes and ages) in an artificial saltwater habitat at a public zoological institution. All seals had plastic flipper tags and subcutaneous microchips just proximal to the hind flippers for identification. The habitat consisted of rocks and a shallow grotto (464 m² of dry resting area) and a large saltwater pool (170,000 L; water temperature, 18°C). Water quality standards met or exceeded USDA recommendations. Light was provided exclusively by the sun; therefore, day lengths varied.

Five control seals (2 males and 3 females) with a cause of death unrelated to thiamine deficiency were included in the study. These seals were free ranging. Two of these seals died because of boat strike, 2 seals...
Died because of infection, and 1 seal died because of neoplasia. Control samples of brain and liver tissue were obtained from these seals for analyses.

**Diet of seals**—Diets of seals with thiamine deficiency included capelin, herring, and Columbia River smelt. Fish were purchased and maintained at −20°C until feeding. Twelve to 24 hours before feeding, fish were thawed and maintained at 4° to 6°C. Fish were fed to seals or discarded within 12 hours after feeding time. Fish were fed to seals ad libitum. Seals typically consumed approximately 2.2 to 3.1 kg (4.84 to 6.82 lb) of fish/day. Twice daily, keepers attempted to hand feed each seal a fish (approx 2.27 kg [5 lb]) containing two 1.5-g vitamin tablets² (total amount of thiamine, 202 mg).

**Clinicopathologic analyses**—A blood sample (15 mL) was obtained from each live seal with thiamine deficiency (9 animals) immediately after they were brought for evaluation; a CBC and serum biochemical analyses were performed for each blood sample. For each seal, blood samples were obtained from the epidural venous sinus into blood collection tubes containing EDTA or serum-separation gel. The CBCs were performed with an automated hematology analyzer; variables determined included WBC, RBC, and platelet counts; hemoglobin concentration; Hct; and mean RBC volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration. Determination of WBC differential counts and morphology was performed by means of examination of a blood smear stained with Wright-Giemsa stain. Serum separator tubes were centrifuged, serum was collected, and serum biochemical analyses were performed with an automated chemistry analyzer.³ Serum biochemical analysis variables included glucose, BUN, creatinine, alanine aminotransferase, aspartate aminotransferase, γ-glutamyltransferase, creatine kinase, and lactate dehydrogenase activities. Globulin concentration was calculated as the total protein concentration minus the albumin concentration. Various other analyses were conducted to detect viral infections or exposure of seals to toxic substances.

**Pathological findings**—Necropsies were performed within 24 hours after death. Representative tissue samples were collected and fixed via immersion in neutral-buffered 10% formalin. Tissue samples were processed by use of routine methods, embedded in paraffin, and stained with H&E for histologic examination. Additional unfixed tissue samples were collected in sample bags and frozen at −80°C.

**Determination of TPP-dependent enzyme activities**—Thiamine deficiency status of seals that died was evaluated on the basis of TPP-dependent enzyme activities in brain and liver samples collected during postmortem examination. Approximately 50 to 100 mg (wet weight) of liver or brain tissue obtained from each seal that had died was homogenized with a mixer mill in 20 mM HEPES (pH, 7.4) containing protease³ and phosphatase inhibitors (wet weight-to-volume ratio, 1:5) with 3.2-mm stainless steel beads. Tissue homogenates were centrifuged (3,000 × g at 4°C) to remove particulate matter, and supernatant was collected for determination of enzyme activities.

Transketolase activity was measured following a published method²² with slight modifications.²⁴ Briefly, a reaction mixture was prepared that contained 1.6 mM xylulose 5-phosphate, 1.7 mM ribose 5-phosphate, 3 mM magnesium chloride, 100 U of α-glycerophosphate dehydrogenase–triose phosphate isomerase/mL, and 100 mM Tris (pH, 7.6). Fifty microliters of prepared liver or brain sample protein was added to 0.15 mL of the reaction mixture, the reaction was started by adding 0.2 mM NADH, and the oxidation of NADH was evaluated for 30 minutes at 37°C. The decrease in absorbance resulting from oxidation of NADH during the assay period was measured at 340 nm. The change in slope per minute was determined in the linear portion of the absorbance graph, which was detected from 10 to 23 minutes after the start of the reaction.

A p-iodonitrotetrazolium violet–coupled assay was performed to measure PDHC activity.²³,²⁶ The reaction mixture contained 4 mM rotenone, 6.5 mM phenazine methosulfate, 0.1 mM coenzyme A, 0.6 mM p-iodonitrotetrazolium violet, 1 mM magnesium chloride, 2.5 mM NAD⁺, 3 mM α-keto-β-methylvalerate, 5 mM pyruvate, 25 mM oxamate, 0.1% Triton, 0.1% bovine serum albumin, and 50 mM potassium phosphate buffer (pH, 7.8). The reaction was started by adding 20 µg of prepared liver or brain sample protein to 0.2 mL of reaction mixture. The absorbance of the assay mixture was measured at 500 nm in the presence or absence of 3 mM 3-fluoropyruvate (an inhibitor of PDHC) at 25°C for 15 minutes. The change in slope per minute was determined for the linear portion of the absorbance graph, which was detected from 5 to 15 minutes after the start of the reaction. The specific PDHC activity was determined to be the difference between the slope obtained with and without fluoropyruvate.

The AKGDH activity was measured by use of a published method,²³ with minor modifications. The reaction mixture contained 4 mM rotenone, 50 µM coenzyme A, 1 mM EDTA, 1 mM α-ketoisocaproate, 2 mM 2-mercaptoethanol, 5 mM magnesium chloride, 0.025% Triton, and 30 mM potassium phosphate buffer (pH, 7.4). Then, 1 mg of prepared liver or brain sample protein was added to 0.2 mL of reaction mixture, and the reaction was started by adding 1 mM NAD⁺; the increase in absorbance resulting from the reduction of NAD⁺ to NADH was measured at 30°C and 340 nm for 5 minutes.

The activities of the TPP-dependent enzymes TK and PDHC were evaluated without the addition of TPP; values were expressed as U/mg of protein (nmol of product/min•mg of protein). The PAR of the enzymes was calculated as ([100 × enzyme activity in the presence of saturating concentrations of thiamine]/[activity in the absence of additional TPP]) − 100).²⁸ Latency and PAR values were expressed as percentages. The PAR indicates relative in-
crease in enzyme activity after the addition of TPP, and latency indicates the proportion of an enzyme that is in the apoenzyme form and readily reactivated by TPP. The saturating concentration of TPP for each enzyme and tissue sample type was determined at various concentrations of TPP (from 0.5 to 20mM) in enzyme reaction mixtures until the maximum reaction rate was detected. The specific activity of AKGDH was only determined with 5mM TPP in reaction mixtures because no activity was detected in the absence of TPP.

**Determination of enzyme activity cutoff values**—For a population with a normal (Z) distribution, values within the range of 1.65 × SD higher and lower than the mean value exclude 5% of normal values. Thus, mean enzymatic activity for control seal brain and liver samples minus \( t_{0.05} \times \sqrt{SD} \) was calculated, where \( t_{0.05} \) is the tabulated critical value of \( t \) for the appropriate number of degrees of freedom. Enzyme activity values < 60% of the mean enzymatic activity for control tissue samples were considered indicative of thiamine deficiency in seals.

**Determination of thiaminase activity in dietary fish**—A diet of fish for seals, purchased from a commercial frozen seafood distributor, was assayed to detect thiaminase activity. The fish assayed included capelin, menhaden, herring (2 sizes [17.6 to 22.7 cm; 7 to 9 inches; large] and 12.6 to 20.2 cm [5 to 8 inches; medium]), and smelt. The fish were received frozen and stored at −80°C until analysis. After removal of the heads and tails, each fish was pulverized in dry ice by use of a published method, with minor modifications. The pulverized fish samples were kept at −20°C for 24 hours to allow complete sublimation of dry ice. Five milliliters of freshly prepared TCEP buffer (10mM TCEP, 100mM NaCl, and 50mM sodium phosphate [pH, 6.9]), degassed with nitrogen) was added to 1 g of pulverized fish samples, vortexed 3 times for 10 seconds, and then centrifuged at 17,200 × \( g \) for 20 minutes at 4°C. An aliquot of 1.5 mL of supernatant was collected, transferred to a centrifuge column, and centrifuged at 1,500 × \( g \) at 4°C for 10 minutes. Fish extract samples that flowed through the column were assayed for protein concentrations and thiaminase activities.

Thiaminase activities in fish extract samples were determined by use of a colorimetric method. The reaction mixture contained 0.2mM 4-nitrothiophenol, 10mM TCEP, 100mM NaCl, and 50mM potassium phosphate buffer (pH, 6.9), degassed with nitrogen) was added to 1 g of pulverized fish samples, vortexed 3 times for 10 seconds, and then centrifuged at 17,200 × \( g \) for 20 minutes at 4°C. An aliquot of 1.5 mL of supernatant was collected, transferred to a centrifuge column, and centrifuged at 1,500 × \( g \) at 4°C for 10 minutes. Fish extract samples that flowed through the column were assayed for protein concentrations and thiaminase activities.

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The mtDNA copy numbers in genomic DNA of seal liver, brain, and muscle samples were determined via quantitative PCR assays performed with asymmetric cyanidine dye. Copy numbers of the mitochondrial gene ND1 were normalized to copy numbers of APP, a single-copy nuclear gene. Seal-specific PCR assay primer sequences for APP (forward, 5′-GGAAAGAAACAGCCCTTCTC-3′; reverse, 5′-GCTCAGCTCCCCAGTTCAACA-3′) and ND1 (forward, 5′-GCAATATCAAGCCTAGCTGTCTA-3′; reverse, 5′-GGTTGTGAAAGATTTATTATGATG-3′) were determined with primer design software. The real-time PCR assay efficiencies for each gene were calculated by use of the following equation: 10^(-ΔCt/K) = k. After determination of the linear relationship between mean cycle number and template DNA amount (25, 12.5, 6.25, 3.13, and 1.56 ng...
of total DNA/reaction), PCR assay efficiencies were determined to be 114% and 97% for ND1 and APP, respectively. The PCR amplifications were performed in 96-well plates with an asymmetric cyanine dye PCR mixture containing 400nM of each primer and 5 µL (3.13 ng) of total template DNA/reaction. The PCR assays were incubated for 2 minutes at 50°C; 10 minutes at 95°C; 40 cycles of 40 seconds at 95°C, 1 minute at 60°C, and 20 seconds at 56°C; and 7 minutes at 72°C. The mean cycle time was determined with a double derivatives method and expressed as Ct values. Relative amounts of mtDNA to nuclear DNA per cell were determined via the comparative Ct method with the following equation: mtDNA/nuclear DNA = 2^(-∆Ct) (where ∆Ct = Ct value for mtDNA – Ct value for nuclear DNA).

**Total protein**—Total protein concentration in liver and brain samples of seals was determined with a protein assay kit by use of the manufacturer's instructions. Absorbance of assay mixtures was measured with a microplate reader.

**Statistical analysis**—The total protein, TK, PDHC, and mtDNA PCR assays were performed in triplicate for tissue samples obtained from control seals and seals with thiamine deficiency. The mean value for each sample assayed in triplicate was used in statistical analyses. The normality of data was tested by use of the Kolmogorov, Shapiro-Wilk, and D’Agostino tests; 95% CIs and mean values for control and thiamine deficiency–affected seals were determined after outlier values were identified and removed from analyses by use of a statistical program. Cutoff values were determined on the basis of the lowest (specific enzyme activity and percentage of mean control values) or the highest (PAR and latency) values of the 95% CI for control seals. The ORs for thiamine deficiency in seals that were pregnant during 2010 were calculated by use of the following equation: (number of pregnant seals with thiamine deficiency × number of nonpregnant control seals)/(number of nonpregnant seals with thiamine deficiency × number of pregnant seals in the control group). The 95% CIs of the ORs were determined by means of the logit method, assuming a normal approximation to the distribution of ln(OR). Statistical analyses of data were performed via the Student t test, and values of P < 0.05 were considered significant.

**Results**

**Hematologic and serum biochemical analyses**—Values of CBC and serum biochemical analysis variables for seals with thiamine deficiency in this study were within reference ranges, except for hyperglycemia detected in the 9 seals for which blood samples were obtained antemortem. Hyperglycemia in these seals was presumed to be secondary to seizure activity.

**Testing to detect toxins and diseases other than thiamine deficiency**—Brain samples obtained from seals with thiamine deficiency had negative PCR assay results for arboviral infections (West Nile virus, eastern equine encephalomyelitis virus, and western equine encephalomyelitis virus). All 11 seals with thiamine deficiency had negative serologic test results for West Nile virus, eastern equine encephalomyelitis virus, western equine encephalomyelitis virus, and Venezuelan equine encephalomyelitis virus. For seals that died, exposure to toxic substances was ruled out by means of testing of liver, brain, muscle, gastric content, and urine samples to detect heavy metals, selenium, strychnine, metaldehyde, cholinesterase, cyanide, organophosphorus and carbamate insecticides, and various other compounds that could have caused neurologic signs. Possible exposure of seals to algal toxins was also investigated; food and water samples had negative results for domoic acid, brevetoxin, saxitoxin, and anatoxin A.

**Treatments and outcomes for seals with thiamine deficiency**—Seals with thiamine deficiency were treated by means of various methods selected by attending veterinarians. For all seals, catheters were placed in the caudal extradural sinus and ≥ 2,000 mL of fluids (lactated Ringer’s solution or saline [0.9% NaCl] solution) was administered. Those seals also received various other treatments, including glucocorticoids (dexamethasone [0.5 to 1.25 mg/kg [0.23 to 0.57 mg/lb]] or prednisolone sodium succinate [5 to 10 mg/kg [2.3 to 4.5 mg/lb]], IV or IM), anticonvulsants (diazepam [0.5 mg/kg in the first 2 seals treated and 0.1 to 0.2 mg/kg [0.045 to 0.09 mg/lb]] in other animals because administration of diazepam preceded respiratory arrest in the first 2 seals treated) or phenobarbital [1.5 to 3 mg/kg [0.7 to 1.4 mg/lb]], IV or IM), mannitol (0.25 to 0.5 g/kg [0.11 to 0.23 mg/lb], IV), diphendydramine (1.5 mg/kg, IV), thiamine (1.1 mg/kg [0.5 mg/lb], IM [only 1 seal received this treatment]), ceftriaxone (20 mg/kg [9.1 mg/lb], IV), atropine (0.2 to 2 mg/kg [0.09 to 0.9 mg/lb], IV), pralidoxime (20 mg/kg, IV), and West Nile virus vaccine (IM). Seal heart rates, respiratory rates, and subjective assessments of responses to treatments (neurologic status) were determined. Six female seals with thiamine deficiency that received treatments died. Five of these 6 seals died or were euthanized within 8 hours after initiation of treatment because of respiratory arrest that was refractory to doxapram (1 to 10 mg/kg [0.45 to 4.5 mg/lb], IV) administration. One seal died after 4 days of treatment. Two female seals died acutely and did not receive treatments.

Clinical signs of thiamine deficiency resolved in 3 seals after treatment. These seals received boluses of thiamine (25 mg/kg [11.4 mg/lb]) and vitamin B complex (containing 25 to 35 mg of thiamine/kg [11.4 to 15.9 mg of thiamine/lb]) IV once. Then, seals received vitamin B complex (10 to 15 mg of thiamine/kg [4.5 to 6.8 mg of thiamine/lb]) IM 4 to 6 hours later. The next day (24 hours after the initial evaluation), all seals received thiamine parenterally twice each day and vitamin B complex each morning (10 to 15 mg of thiamine/kg, IV) and afternoon (38 to 50 mg/kg [17.3 to 22.7 mg/lb], SC). Seals received doses of vitamin B complex once each day for at least 3 additional days (administered IV, IM, or SC [route of administration determined on the basis of the severity of signs of depression and ability of personnel to manually restrain animals]). Improvement in clinical signs of these 3 seals was detected 24 hours after administration of the first dose of thiamine, and neurologic signs resolved 72 to 96 hours after that time. Resumption of full appetite varied among animals; one seal was eating well by 4 days, one by 7
days, and one (the oldest seal) by 14 days after the initial evaluation.

Necropsy findings—Necropsy of the 8 affected seals that died revealed pulmonary congestion (n = 7), mild meningeal edema (6), and cerebellar edema and acute focal hippocampal necrosis (2). Histologic examination of brain samples of the seal that died after 4 days of treatment revealed marked lesions consistent with polioencephalomalacia, which could have been attributable to the effects of thiamine deficiency.7,32–35

Enzyme activities in liver samples—Liver samples from the 8 seals with thiamine deficiency that died and the 5 control seals were analyzed to determine TPP-dependent enzyme activities. However, data for the seal that was treated with thiamine before it died were not included in the statistical analyses; therefore, data for 7 seals with thiamine deficiency and 5 control seals were included in enzyme activity analyses. The mean ± SEM specific activity of TK (without the addition of TPP) in liver samples of control animals was 1.9 ± 0.1 nmol/min/mg of protein; this value was significantly (P = 0.014) higher than the value (1.0 ± 0.2 nmol/min/mg of protein) for liver samples of seals with thiamine deficiency (Table 1). The PAR of TK in liver samples of seals with thiamine deficiency was higher than the calculated cutoff value for control seals; however, no significant differences were detected between TK latency values for liver samples of seals with thiamine deficiency and those of control seals. Transketolase activities (with and without the addition of TPP) and PAR values for liver samples of seals with thiamine deficiency supported that diagnosis. Transketolase analysis of liver samples for the seal that had received thiamine before it died indicated a PAR of 75% and a latency of 43%; these values were approximately 4-fold higher than the mean values for the other affected seals.

The mean ± SEM specific activity of PDHC (without the addition of TPP) in liver samples of control seals was 1.2 ± 0.1 nmol/min/mg of protein (Table 1); this value was significantly (P = 0.004) higher than the value (0.30 ± 0.05 nmol/min/mg of protein) for seals with thiamine deficiency. The PDHC PAR and latency values for liver samples of seals with thiamine deficiency were higher than the calculated cutoff value for control seals. These results for seals with thiamine deficiency supported that diagnosis.

The mean ± SEM specific activity of AKGDH in liver samples of control seals was 1.6 ± 0.1 nmol/min/mg of protein (Table 1). This value was significantly (P = 0.009) higher than the value (1.1 ± 0.3 nmol/min/mg of protein) for AKGDH specific activity in liver samples of seals with thiamine deficiency.

Enzyme activities in brain samples—The mean ± SEM specific activities of TK (without the addition of TPP) in brain samples of control (n = 5) and thiamine deficiency–affected (n = 7) seals were 1.7 ± 0.1 nmol/min/mg of protein and 2.1 ± 0.2 nmol/min/mg of protein, respectively (Table 2); no significant differences were detected between these groups of seals regarding TK activity in brain samples with or without the addition of TPP. However, all of the brain samples obtained from the affected seals had a significant response to the addition of TPP as indicated by the PAR and latency outcomes. For brain samples of seals with thiamine deficiency, the mean PAR of TK was 60.8%, which was significantly (P = 0.02) higher than the calculated value (11.3%) for control seals. The mean TK latency value for seals with thiamine deficiency was 36.0%, which was significantly (P = 0.008) higher than the value (10.2%) for control seals. The PAR and latency values for the brain samples of the seal that died after receiving thiamine were 73% and 42%, respectively; these values were approximately 20% higher than the mean values for the other affected seals.

The mean ± SEM specific activities of PDHC (without the addition of TPP) in brain samples of control (n = 5) and thiamine deficiency–affected (7) seals were 0.36 ± 0.06 nmol/min/mg of protein and 0.30 ± 0.05 nmol/min/mg of protein, respectively (Table 2); these values were not significantly different. The PDHC activities for brain samples increased after the addition of TPP. The PAR

Table 1—Values of TPP-dependent enzyme activities in liver samples of thiamine deficiency–affected (n = 7) and control (5) Pacific harbor seals (Phoca vitulina) housed in a zoological park.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Without TPP</th>
<th>With TPP</th>
<th>PAR (%)</th>
<th>Latency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TK</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.9 ± 0.1*</td>
<td>2.1 ± 0.1*</td>
<td>11 ± 2*</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>Affected</td>
<td>1.0 ± 0.2*</td>
<td>1.1 ± 0.2*</td>
<td>18 ± 8*</td>
<td>11 ± 5</td>
</tr>
<tr>
<td>PDHC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.30 ± 0.05*</td>
<td>0.46 ± 0.04*</td>
<td>407 ± 294*</td>
<td>43 ± 13*</td>
</tr>
<tr>
<td>Affected</td>
<td>0.29 ± 0.05</td>
<td>0.69 ± 0.04</td>
<td>407 ± 294</td>
<td>43 ± 13*</td>
</tr>
<tr>
<td>AKGDH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.6 ± 0.1*</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Affected</td>
<td>1.1 ± 0.3*</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Values for control and affected seals are mean ± SEM, and cutoff values are the lowest (enzyme specific activities) or highest (PAR and latency) values of the 95% CI for control seal values.

*Within an enzyme, values for the variable in the column are significantly (P < 0.05) different between control and affected seals.

— = Not determined.

Table 2—Values of TPP-dependent enzyme activities in brain samples of thiamine deficiency–affected (n = 7) and control (5) Pacific harbor seals housed in a zoological park.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Without TPP</th>
<th>With TPP</th>
<th>PAR (%)</th>
<th>Latency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TK</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.7 ± 0.1</td>
<td>1.9 ± 0.1</td>
<td>11.3 ± 6*</td>
<td>10.2 ± 5.4*</td>
</tr>
<tr>
<td>Affected</td>
<td>2.1 ± 0.2</td>
<td>3.3 ± 0.4</td>
<td>60.0 ± 14*</td>
<td>36.0 ± 5*</td>
</tr>
<tr>
<td>PDHC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.36 ± 0.06</td>
<td>0.39 ± 0.04</td>
<td>8.3 ± 8*</td>
<td>7.7 ± 7.4*</td>
</tr>
<tr>
<td>Affected</td>
<td>0.30 ± 0.05</td>
<td>1.1 ± 0.4*</td>
<td>259 ± 86</td>
<td>63 ± 11*</td>
</tr>
</tbody>
</table>

See Table 1 for key.
and latency values for brain samples of seals with thiamine deficiency were significantly \( (P = 0.04\) and 0.012, respectively) higher than values for brain samples of control seals.

**mtDNA copy number in brain, liver, and muscle samples**—The mean ± SEM mtDNA copy number in brain samples of thiamine deficiency–affected seals (878 ± 218) was not significantly \( (P = 0.233)\) different from the value for control seals (698 ± 79). The

![Figure 1—Mean ± SEM thiaminase activity in various types of whole fish versus thiaminase activity in eviscerated fish fed to Pacific harbor seals (Phoca vitulina) in a zoological park. Data were analyzed in a scatterplot with a best fit least-squares straight line fitted to the data points. The equation indicates the regression line. Thiaminase activity is expressed as \( \mu g \) of thiamine consumed/h of fish (wet weight).](https://example.com/figure1.png)

**Table 3—Estimated thiamine requirements for seals fed theoretical diets comprised solely of smelt or capelin.**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Amount of thiamine received by seals as fed (mg/d)</th>
<th>Amount of thiamine degraded by thiaminase in fish* (mg/d)</th>
<th>Supplementation of thiamine required by seals† (mg/d)</th>
<th>Thiamine ingested‡ (mg/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smelt</td>
<td>202</td>
<td>117</td>
<td>126</td>
<td>117.2</td>
</tr>
<tr>
<td>Capelin</td>
<td>202</td>
<td>14</td>
<td>24</td>
<td>14.2</td>
</tr>
</tbody>
</table>

* Determined on the basis of results of this study that 43.3 \( \mu g \) of thiamine was degraded in 1 g of smelt. The mean amount of fish consumed by adult seals was 2,700 g/d. Therefore, the amount of thiamine required to compensate for degradation by thiaminase would have been 14 mg/d. †Value calculated as the amount of thiamine degraded by thiaminase plus 4 times the daily thiamine requirement (2.5 mg/d) because water-soluble vitamins are typically offered in an amount 2- to 6-fold higher than the recommended daily required amount. ‡On the basis of results of this study for PDHC indicated in Table 1, seals (at the time of death) were consuming 1/10 to 1/15 the amount of thiamine required for complete saturation of PDHC. Assuming the amount of thiamine required for enzyme saturation (2.5 mg) was equal to 5 × the Michaelis constant, then the amount of thiamine = PDHC activity in affected seals relative to control seals × Michaelis constant/(1 – PDHC activity of affected seals relative to control seals); this value was 0.2 mg. Then, 0.2 mg + 117 mg (amount of thiamine degraded in smelt) = 117.2 mg of thiamine/d (which was the actual amount of thiamine ingested); a similar calculation was performed for capelin.

**mtDNA copy number in muscle samples**—The mtDNA copy number in muscle samples was 262 ± 41 for control seals and 574 ± 173 for seals with thiamine deficiency; these values were significantly \( (P = 0.044)\) different. The mean ± SEM of mtDNA copy number in liver samples of seals with thiamine deficiency (117 ± 27) was significantly \( (P = 0.003)\) lower than the value for control seals (263 ± 24).

**Pregnancy and lactation as risk factors for thiamine deficiency**—Because the high requirement for thiamine by fetuses and other tissues of females during pregnancy and production of milk during lactation can increase demand for dietary thiamine consumed by the mother, pregnancy and lactation were evaluated as risk factors for thiamine deficiency. Six of 9 females in the colony that had pups during 2010 had thiamine deficiency, but only 4 of 9 nonpregnant females in the colony had that condition. The OR for development of thiamine deficiency was 2.5-fold higher (95% CI, 0.15 to 6.8; \( P < 0.05 \)) for pregnant seals versus other seals in this study.

**Assessment of thiaminase activity in dietary fish**—Thiaminase activities for whole capelin, herring 200, herring 7, menhaden, and smelt were 5.1, 8.7, 12.1, 17.7, and 43.3 \( \mu g \) of thiamine consumed/h of fish (wet weight). Thiaminase activities in whole fish samples were approximately 5-fold higher than those in eviscerated fish (ie, fish without intestinal tracts, Figure 1).

To determine whether high thiaminase activity in fish could cause thiamine deficiency in seals, the amount of thiamine ingested by seals was estimated on the basis of the TPP-dependent enzyme activities in tissue samples of seals and thiaminase activities in fish (Table 3). Assuming that the TPP-dependent enzymes follow Michaelis-Menten kinetics and the saturating concentration of thiamine was 5% the value of the Michaelis constant, seals with thiamine deficiency had a thiamine deficit of approximately 10 mg/d. Thus, the seals would have needed to consume 126 mg of thiamine/d for saturation of all TPP-dependent enzymes and receive 4 times the estimated daily thiamine requirement when consuming a thiaminase-rich smelt diet. The required amount of supplemental thiamine would have been equivalent to 47 mg of thiamine/kg (21.4 mg of thiamine/lb) of fish fed (for seals consuming a diet with high amounts of thiaminase).

**Discussion**

For humans, thiamine deficiency may be diagnosed via detection of low specific activity of TK in erythrocytes or a significant activation of TK by TPP in vitro.\(^3\) Investigators in other studies\(^37\)–\(^40\) have evaluated activities of TPP-dependent enzymes in tissues other than RBCs. In the present study, we identified thiamine deficiency in seals maintained in a zoological institution by means of determination of the specific activities of 3 TPP-dependent enzymes in liver samples (TK, PDHC, and AKGDH) and 2 such enzymes in brain samples (TK and PDHC) of seals and by means of calculation of PAR and latency values for these enzymes.

In liver samples of seals with thiamine deficiency, the enzyme with the lowest specific activity relative to values for control seals was PDHC, followed by TK and AKGDH (24%, 54%, and 69% of the values for control...
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to values reported for animals of other species; this samples determined with and without TPP were similar brain tissue. These findings suggest that the rate of brain is similar to the calculated rate of thiamine turnover in cerebral cortex. Brain tissue requires a continuous supply of thiamine because transport of thiamine across the blood-brain barrier is a saturable process. The maximum rate of uptake of thiamine in brain tissue was 2.4 times that of liver tissue. This rate is similar to the calculated rate of thiamine turnover in brain tissue. These findings suggest that the rate of brain transport may be substantial but not substantially higher than the rate needed to support a normal amount of brain function.

Results of the present study indicated the PDHC activity in brain samples of thiamine deficiency–affected seals was 73% of the value for controls seals. Brain sample TK activities for seals with thiamine deficiency and control seals were not significantly different. The PAR for seals with thiamine deficiency relative to that for control seals was substantially higher for PDHC (approx 32-fold higher than the value for control seals) than it was for TK (approx 5.5-fold higher than the value for control seals). In contrast to results for liver samples, all samples with thiamine deficiency could be identified via the PAR and latency values for PDHC or TK in brain samples; however, the low mean activities of those enzymes could not be used to identify such animals. The mean PAR of PDHC in brain tissue was 4 times that of TK, consistent with the results obtained for liver samples, suggesting a higher turnover or faster degradation of the apoenzyme form of TK than for the apoenzyme form of PDHC.

Results of this study suggested that brain tissue was more sensitive than liver tissue to the effects of thiamine deficiency in seals. Livers typically have higher reserve amounts of thiamine than brains of seals. Severe thiamine deficiency typically causes encephalopathy rather than liver disease in foxes and mink. Thiamine deficiency has substantial effects on the function of cellular pathways in neural tissues, and thiamine is an essential cofactor for processing pathways are required to maintain mtDNA integrity and copy numbers. Despite the finding that mtDNA copy numbers were low in liver samples of thiamine deficiency–affected seals in the present study, the values were not indicative of a mtDNA depletion syndrome in the affected seals.

Clinical signs in seals in this study were consistent with thiamine deficiency, and severe TPP-dependent enzyme deficiencies were detected in brain (TK and PDHC) and liver (TK, PDHC, and AKGDH) samples (values ranged from 4% to 63% of the values for control seal tissue samples). These findings suggested that seals died because of thiamine deficiency. In addition, 3 affected seals in this study improved after they received high doses of thiamine and vitamin B complex via parenteral routes of administration.

The estimated thiamine intake for seals in this study was low because of high thiaminase activity in the diet and other contributing factors such as inadequate amounts of vitamins administered and a high dietary requirement for thiamine during pregnancy and lactation. Although thiamine deficiency has been suspected in seals in other studies, to the authors’ knowledge, the present study is the first in which enzyme assays were available to confirm such a diagnosis.

The keepers of seals in the present study reported that ≤ 50% of the animals received their intended amount of vitamins (approx 37 mg of thiamine/kg [16.8 mg of thiamine/lb] of fish) each day. Changes in social dynamics among seals attributable to the introduction of several large breeding male California sea lions to the exhibit may have contributed to the difficulty of consistent feeding of fish containing supplemental vitamins. New keepers in the pinniped area and variable communication regarding which seals received and ate supplemental vitamins during each feeding period may also have contributed to that problem. The supplemental vitamin formulation for pinnipeds at the facility had recently been changed to include lutein, presumed to be beneficial for ocular health; this change resulted in seals requiring 2 vitamin tablets/d rather than 1 tablet/d to receive the intended amount of thiamine. Also, keepers were preparing fish with supplemental vitamins when buckets of feed were prepared. Seals may not have been fed for 1 to 2 hours after food preparation; water-soluble vitamins (eg, thiamine) that are easily degraded or inactivated were likely not received by the seals in the intended amounts. The finding of the present study that more female seals than male seals seemed to be affected with thiamine deficiency may have been attributable to the function of the pentose phosphate pathway (and in particular fatty acid synthesis) during pregnancy and lactation.

Requirements for thiamine are higher during pregnancy and lactation than they are at other times for humans and other animals.
Thiaminase activity in fish samples evaluated in the present study was similar to reported values. Thiaminase activities in smelt samples in this study were similar to those determined in another study by use of a radiometric assay (44 μg of thiamine/h•g of fish). Similar to results of other studies, thiaminase activities in smelt samples in the present study were substantially higher than those of other evaluated fish samples. Findings of the present study suggested that thiaminase activity was primarily attributable to bacteria in intestinal tracts of the fish. This finding is similar to those of another study indicating activities of 1,902 and 362 pmol of thiamine degraded/min•g of smelt for whole and eviscerated fish, respectively. The required amount of supplemental thiamine determined for seals in this study was equivalent to 47 mg of thiamine/kg of fish fed (for seals consuming a diet with high amounts of thiaminase). This value was 1.5-fold lower than that reported for a carp preparation in which 26 mg of thiamine was degraded/kg of fish; that value is the theoretical quantity of thiamine necessary to maintain marine mammals on thiaminase-containing diets.

For 3 seals in this study, clinical signs of thiamin deficiency resolved after parenteral administration of high doses of thiamine for at least 96 hours. Such treatment was similar to that required to resolve signs of thiamin deficiency in animals of other species; resolution of such signs is typically detected after daily administration of high doses of thiamine for up to several months. Intraperitoneal administration of multiple doses of thiamine (for up to 4 weeks) are required to improve signs of thiamin deficiency in rats, however, such rats have damage to the brainstem and cortical and subcortical tracts and also have high amounts of serotonin and norepinephrine and low amounts of γ-aminobutyric acid and glutamine in the brain weeks after resolution of clinical signs. These findings suggest early treatment may be necessary for full resolution of clinical signs. Humans recover from mild thiamin deficiency after 16 days to 3 months of treatment, and severe thiamin deficiency can result in death, despite treatment. The seal in the present study that died despite administration of thiamin may have had severe thiamin deficiency because TK activities in liver and brain samples of that animal were higher than values for other seals with clinical signs of that disease. Histologic examination of brain samples of that seal indicated lesions consistent with polioencephalomalacia, which is associated with thiamin deficiency. However, the histopathologic changes were mild and not as severe as those typically detected in cats and ruminants with the disease.

Feeding of supplemental vitamins in fish did not ensure adequate thiamine intake for seals in this study. Variation in feeding protocols may have caused variability in amounts of vitamins consumed by seals. Thiamine is not extensively stored in bodies of seals, and requirements for that vitamin increase during perinatal periods. Female seals are commonly anorectic during the perinatal period; therefore, female harbor seals may be highly susceptible to thiamin deficiency during that period. We modified preventative medicine protocols for seals in the colony of the present study so that harbor seals with anorexia for > 48 hours received thiamine or vitamin B complex parenterally. Other modifications for prevention of thiamine deficiency in seals included improvements in record keeping, ensuring that seals received an adequate amount of supplemental vitamins. In addition, protocols were changed so that vitamin tablets were added to fish just prior to feeding of seals. Additionally, protocols were changed so that all harbor seals, especially breeding age females, receive an additional 250 mg of capsulated thiamine PO once/day; that dose is increased to 500 mg daily 1 month prior to and during parturition and lactation (mid-March to June [determined on the basis of the seasonal reproductive cycle]). Although removal of intestinal tracts of fish before feeding of seals might prevent thiamine deficiency (because of a reduction in dietary thiaminase), the number of fish fed to animals each day precluded that procedure and, more importantly, whole fish contain various important nutrients (eg, vitamin A). Therefore, administration of thiamine may be the best management practice for prevention of thiamine deficiency in seals.

Thiamine deficiency should be considered as a differential diagnosis for seals with neurologic signs or those that acutely die, especially if such animals are fed fish that contain thiaminase. Clinical signs and pathologic findings may not clearly indicate a diagnosis of thiamine deficiency for seals; therefore, toxicological and biochemical analyses should be performed for such animals. The methods used in the present study provided information that would be useful for postmortem determination of a diagnosis of thiamine deficiency in seals. Determination of activity of TK in RBCs in blood samples of seals with suspected thiamine deficiency could be considered as a minimally invasive and reliable antemortem approach to early determination of a diagnosis of thiamine deficiency. Further studies are warranted to determine methods for rapid diagnosis of that disease in seals; potentially useful methods may include determination of PDHC and TK activities in peripheral mononcytic blood cells, rather than determination of TK activity in erythrocytes.
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References

Effects of dietary vitamin A content on antibody responses of feedlot calves inoculated intramuscularly with an inactivated bovine coronavirus vaccine

Junbae Jee et al

**Objective**—To investigate effects of low dietary vitamin A content on antibody responses in feedlot calves inoculated with an inactivated bovine coronavirus (BCoV) vaccine.

**Animals**—40 feedlot calves.

**Procedures**—Calves were fed diets containing high (3,300 U/kg) or low (1,100 U/kg) amounts of vitamin A beginning on the day of arrival at a feedlot (day 0) and continuing daily until the end of the study (day 140). Serum retinol concentrations were evaluated in blood samples obtained throughout the study. Calves were inoculated IM with an inactivated BCoV vaccine on days 112 and 126. Blood samples obtained on days 112 and 140 were used for assessment of BCoV-specific serum IgG1, IgG2, IgM, and IgA titers via an ELISA.

**Results**—The low vitamin A diet reduced serum retinol concentrations between days 112 and 140. After the BCoV inoculation and booster injections, predominantly serum IgG1 antibodies were induced in calves fed the high vitamin A diet; however, IgG1 titers were compromised at day 140 in calves fed the low vitamin A diet. Other isotype antibodies specific for BCoV were not affected by the low vitamin A diet.

**Conclusions and Clinical Relevance**—Dietary vitamin A restriction increases marbling in feedlot cattle; however, its effect on antibody responses to vaccines is unknown. A low vitamin A diet compromised the serum IgG1 responses against inactivated BCoV vaccine, which suggested suppressed T-helper 2–associated antibody (IgG1) responses. Thus, low vitamin A diets may compromise the effectiveness of viral vaccines and render calves more susceptible to infectious disease.