PLASMA CONCENTRATIONS OF VITAMIN A1, B1, D3, AND E IN HUMBOLDT PENGUINS (SPHENISCUS HUMBOLDTI) BEFORE AND AFTER DIETARY VITAMIN SUPPLEMENTATION OF THEIR FISH DIET

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PLASMA CONCENTRATIONS OF VITAMIN A_1, B_1, D_3, AND E IN HUMBOLDT PENGUINS (*SPHENISCUS HUMBOLDTI*) BEFORE AND AFTER DIETARY VITAMIN SUPPLEMENTATION OF THEIR FISH DIET

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Abstract: In a practical feeding trial at Ouwehand Zoo, plasma concentrations of vitamin A_1, calcidiol (D_3), α-tocopherol (E), and B_1 in 17 Humboldt penguins (*Spheniscus humboldti*) were measured before and after supplementation to gain insight into the effect of supplementing these vitamins in animals being fed thawed frozen-fish diets. None of the penguins received vitamin supplements for at least 6 mo before the supplementation trial, which was conducted prior to their normal nesting and molting period. During the trial period, eight penguins received daily vitamin A_1, D_3, tocopheryl acetate, and B_1 supplementation placed in their fish immediately prior to feeding and nine control penguins received no supplementation. Concentrations of vitamins A_1, D_3, α-tocopherol, and B_1 were also measured in the thawed ready-to-feed fish. Concentrations of vitamins B_1 and α-tocopherol were below the Association of Zoos and Aquariums (AZA) recommendations for penguin diets, while concentrations of vitamins A_1 and D_3 were far above AZA recommendations. At the start of the study and after 70 days of supplementation, plasma concentrations were determined for these vitamins. Vitamin B_1 concentrations in plasma increased significantly ($P < 0.05$) between Day 0 (mean 39.9 μg/L) and day 70 (mean 160.5 μg/L) in the supplemented group. Plasma vitamin D_3 and α-tocopherol did not show a significant change. Vitamin A_1 levels in the supplemented group decreased significantly from 1.65 mg/L on day 0 to 1.4 mg/L on day 70. In the control group no significant changes were observed. The results of the study support the necessity of supplementing vitamin B_1 in penguins fed thawed frozen fish. Depletion of vitamin A and E concentrations in frozen food fish over time support recommendations to regularly measure vitamin concentrations in different batches of frozen fish.

Key words: Feeding trial, Humboldt penguins, *Spheniscus humboldti*, supplementation, vitamins.

INTRODUCTION

Vitamin supplementation in zoo animals is a topic of much debate and research. Little is known about vitamin requirements in Humboldt penguins (*Spheniscus humboldti*). Captive penguins are fed primarily thawed-fish diets, and it is usually advised to prophylactically supplement thawed-fish diets with vitamin B_1 and vitamin E.[5,7,8,14,19] These recommendations are based on enzymatic activities in the tissues of the fish causing a decrease in these vitamins during storage as frozen fish and during the thawing process.[5]

Dietary vitamin recommendations and requirements for zoological birds are often extrapolated from poultry data.[2,18] Penguins are piscivorous and therefore may have markedly different vitamin requirements from those of granivorous birds. A wide range of multivitamins and formulations of vitamin E and B_1 in tablets or gels are marketed for penguins.[16] This study was performed to help understand the effect of simultaneous supplementation (as recommended by the Association of Zoos and Aquariums [AZA] penguin handbook) of vitamins A_1, calcidiol (D_3), α-tocopherol (E), and B_1 for 70 days on the plasma concentrations of these vitamins in Humbolt penguins.[2,18] This study focuses on the four vitamins for which the AZA penguin handbook has established minimum requirements.[2]

A group of 17 Humboldt penguins was used at Ouwehand Zoo (Utrecht, Rhenen, the Netherlands). The goal of this study was to determine any effect on plasma vitamin concentrations after adding the minimal required amount and thus determining if supplementing the minimum has any effect.

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MATERIALS AND METHODS

Animals and housing

Seventeen adult Humboldt penguins representing the entire population of Ouwehand Zoo were

included in this trial conducted between mid-April 2016 and mid-December 2016. The penguins ranged from 8 to 30 yr of age. No deaths or abnormalities were noted during yearly screenings and all animals were clinically healthy. Routine supplementation of the penguins prior to the study consisted of vitamin B_1 and tocopheryl acetate (Akwavit Basic Liquid 100/25, Kasper Faunafood, 3440AA Woerden, the Netherlands). From mid-April 2016 onwards, the supplementation of the fish was halted. All penguins were marked with colored ty-raps (Thomas & Betts Netherlands BV, 2991 LG Barendrecht, the Netherlands) at their proximal wings. Eight penguins, five males and three females (5 : 3) with a mean age of 13.7 yr, that were easy to hand-feed were assigned to the supplemented group. The remaining penguins, three males and six females, (3 : 6), with a mean age of 17.4 yr, were assigned to the control group. All penguins in both groups were housed together in an outdoor enclosure consisting of a large recirculating basin containing 75,000 L of fresh water, which is filtered using a sand filter combined with ultraviolet light to reduce microorganisms. To ensure water quality, monthly measurements of nitrite, nitrate, ammonia, pH, and colony-forming unit counts are performed. None of the measured water parameters were outside the normal range before and during the trial. The enclosure also contains a land area of 750 m^2 (including a 300-m^2 grass area with chestnut trees) with artificial rock formations that conceal 25 nesting boxes.

Diet and supplements

Each penguin in the test group received the same amount of supplemental vitamins. No other dietary changes were made. Each penguin was offered 50 g of mackerel (*Scomber scombrus*) 200 g herring (*Clupea harengus*), 150 g sprattus (*Sprattus sprattus*), and 100 g capelin (*Mallotus villosus*) divided over two feeding times during the day. Three liquid vitamin supplements were used. Based on the available literature and the AZA penguin husbandry manual and to ensure a minimum intake of the upper limit of the requirements, the following vitamin supplement doses were chosen: 6,000 IU/kg dry matter (DM) vitamin A_1 (vitamin A oil, Holisan BV, 8226NA Lelystad, the Netherlands), 1,000 IU/kg DM vitamin D_3, (Davitamon cholecalciferol, Omega Pharma Nederland BV, 3062CE Rotterdam, the Netherlands) 400 IU/kg DM tocopheryl acetate, and 120 mg/kg DM vitamin B_1 (Akwavit Basic Liquid 100/25, Kasper Faunafood, 3440AA Woerden, the Netherlands). The fish were injected with the previously mentioned concentrations. This supplemented fish was always the first fish given to the penguins in the supplemented group during the morning feeding. The time between injecting the fish and feeding was less than 5 min. No supplements were given in the fish during the feeding in the afternoon.

Fish is normally received at the zoo in frozen condition in large packets ($40 \times 50 \times 10$ cm) stored at −20 °C and defrosted under refrigeration (4 °C) for 48 hr immediately prior to feeding. The same batch of fish was used throughout the entire trial. During the trial, 2 kg of fish in this study was submitted as a frozen package to a laboratory (Nutricontrol, 5460 AC Veghel, the Netherlands) for nutrient analysis. The laboratory defrosted the fish under the same conditions as in the zoo prior to analysis. After thawing, the entire sample was homogenized and concentrations of vitamins A_1, D_3, E (α-tocopherol), and B_1 were measured. Vitamin B_1 was assayed by high performance liquid chromatography (HPLC). Vitamins A_1 and E were measured using liquid chromatography and fluorescence detection. The vitamin D_3 levels in the fish were determined using liquid chromatography-tandem mass spectrometry detection.

Plasma samples

On day 0 and day 70, blood samples were collected from the right jugular vein 3 hr after the morning feeding, and placed in sodium ethylenediaminetetraacetate (EDTA). All samples were wrapped in aluminum foil to reduce vitamin degradation caused by UV radiation. Samples were stored at 5–7 °C and sent the same day overnight on ice packs in a Styrofoam shipping container to Vet Med Labor GmbH (Ludwigsburg, Germany). Measurements were performed using a Dionex Ultimate RS LC 3000 instrument (ThermoFisher, Waltham, MA 02452, USA). The measurements of the vitamins in the EDTA plasma samples were performed for vitamin A_1, B_1, and E by ultra-high-performance liquid chromatography (U-HPLC) and for vitamin D (25-OH) by high-performance liquid chromatography (HPLC) after protein precipitation and solid phase extraction (SPE).Vitamin B_1 (thiamin) was measured from EDTA blood that was derivatitized to thiamine pyrophosphate (TPP) and separated by U-HPLC for analysis using a fluorescence detector (Wielders and Mik, 1993 Standard Operating Procedure, SOP, Idexx Laboratories, 71636 Ludwigsburg, Germany).

Table 1. The mean and standard deviation of the vitamin concentrations in the plasma of *S. humboldti* for the supplemented and control groups on day 0 and 70. [a] Paired *t*-test with $P < 0.05$, [b] student *t*-test with $P < 0.05$.

	Control	Supplement
	Means (±SD)	
Vitamin A day 0 (mg/L)	1.56 ± 0.26	1.65[a] ± 0.35
Vitamin A day 70 (mg/L)	1.28 ± 0.44	1.4[a] ± 0.28
Vitamin E day 0 (mg/L)	21.2 ± 4.3	26.9 ± 12.8
Vitamin E day 70 (mg/L)	22.9 ± 7.8	29.7 ± 13.9
Vitamin D_3 day 0 (nM/L)	20 ± 8.7	19 ± 3.81
Vitamin D_3 day 70 (nM/L)	25.8 ± 15.9	20.3 ± 7.2
Vitamin B_1 day 0 (μg/L)	49.6 ± 8.9	39.9[a] ± 8.8
Vitamin B_1 day 70 (μg/L)	79.2[b] ± 34.4	170.5[a,b] ± 45.2

Statistics

A paired *t*-test was used to determine whether there were significant differences ($P < 0.05$) between day 0 and day 70 within the control and supplemented groups. A student *t*-test was used to determine significant differences between the groups on day 0 and day 70.

RESULTS

At the start of the experiment both groups had similar plasma D_3 and E (α-tocopherol) concentrations. During the sampling on day 0, vitamin B_1 samples from two females in the control were not suitable for analysis due to too little sample material obtained. Vitamin B_1 in the remaining day 0 samples from the control and groups were not significantly different. Vitamin B_1 concentrations showed a significant increase ($P < 0.05$) in the supplemented group on day 70 (Table 1). Vitamin A concentrations in the supplemented group decreased significantly ($P < 0.05$) from day 0 (1.65 ± 0.35 mg/L) to day 70 (1.4 ± 0.28 mg/L) (Table 1). Concentrations of vitamins E (α-tocopherol) and D_3 were not statistically significantly different between time 0 and time 70 in the supplemented group nor between the supplemented and the control groups at either time point (Table 1). Table 2 shows the vitamin nutrient analysis of the unsupplemented fish diet.

Table 2. Analyzed vitamin A_1, D_3, α-tocopherol, and B_1 concentrations in the daily feed offered to *S. humboldti* per kg dry matter (DM).

	Vitamin concentration feed
Vitamin A_1	30,160 IU/kg DM
Vitamin D	7,760 IU/kg DM
α-tocopherol	29.25 IU/kg DM
Vitamin B_1	<0.25 mg/kg DM

DISCUSSION

In this study, results indicate that oral supplementation for 70 days of vitamin B_1 results in a statistically significant increase in the circulating plasma vitamin B_1 in the supplemented group (Table 1). This data corresponds with other research on the effects of vitamin B_1 supplementation on circulating plasma B_1 levels in penguins.[14,19] Based on the low vitamin B_1 found in the analyzed fish (<0.25 mg/kg DM), supplementation with vitamin B_1 is necessary (Table 2). Plasma vitamin B_1 concentrations in the control group were stable during the 70-day trial and were higher than one would expect given the measured concentrations of vitamin B_1 in the analyzed diet. One possible explanation is that naturally occurring thiaminase may have affected laboratory samples during processing and homogenizing prior to analysis.[16] Only the unsupplemented fish was sent for analysis and the interval between supplementing the fish and feeding was so small that no significant change was expected for the vitamin concentrations within the fish.

Vitamin A_1 plasma concentrations in the supplemented group decreased significantly from day 0 to day 70. Decreasing vitamin A blood concentrations in supplemented penguins has been described previously.[4] Retinol concentrations are normally very high in whole fish. [6] High concentrations of dietary vitamin A_1 may impair vitamin E absorption. A previous study in northern fur seals showed adding high concentrations of vitamin A to their diets caused a decrease in circulating vitamin E concentrations.[15] During digestion of food, fat-soluble vitamins are incorporated with other lipids to form mixed micelles that are essential for absorption by the enterocytes. In rats it has been shown that adding vitamin E decreases the absorption of carotenoids up to 50%.[12] This process could very well also exist in penguins. In most animals there seems to be a limit to the absorption capacity of fat-soluble vitamins. By simultaneously supplementing three fat-soluble vitamins, the maximum absorption

capacity of the penguins may have been overcome. This would explain the lower plasma vitamin A_1 concentrations in the supplemented group. The unsupplemented daily diet fed to the penguins in this study already contained a high concentration of vitamin A_1 (30,160 IU/kg DM); therefore, supplementation of vitamin A_1 would likely not have had a beneficial effect. When comparing this study to free-ranging Humboldt penguin's vitamin A_1 plasma concentrations, lower plasma concentrations are found in free-ranging Humboldt penguins. The average vitamin A_1 plasma concentration in free-ranging Humboldt penguins was 1.11 mg/L with no significant difference throughout the seasons.[20] In another penguin study, the plasma vitamin A_1 concentrations in 17 adult zoo jackass penguins (*Spheniscus demercus*) receiving a daily supplement of 2.04 mg retinol and 185 mg vitamin E were 0.86 ± 0.4 mg/L.[11] These plasma concentrations were also lower than those found in this study. Yet another penguin study looked at seasonal changes of vitamin A_1 plasma concentrations in gentoo (*Pygoscelis papua)* and rockhopper (*Eudyptes crestatus)* penguins, ranging from 0.58 mg/L to 1.14 mg/L.[17] These lower plasma concentrations could represent differences between species, but more likely, might also be due to higher vitamin A_1 levels in the fish fed to the penguins in this study. When feeding whole fish containing high concentrations of vitamin A_1, the supplementation of vitamin A should be discontinued. Hepatic retinol concentrations in captive penguins have been shown to be 20 times lower than those measured in free-living birds; furthermore, no strong correlation between liver and plasma vitamin concentration has been identified.[9] Vitamin A_1 storage in the liver has not been taken into account during this study. This study was designed to evaluate changes in plasma vitamin concentrations but further research into Vitamin A_1 metabolism and the actual requirement for appropriate plasma and liver concentrations is needed. The authors recommend that each batch of fish be analyzed for concentrations of vitamin A_1 and E (α-tocophenol) prior to feeding.

In this study, plasma vitamin D_3 levels did not change significantly in either the control or supplementation groups. Bernard and Allen describe vitamin D_3 concentrations ranging from 2,500 IU/kg DM to 16,800 IU/kg DM in the fish species usually fed to penguins.[3] In this study, vitamin D_3 concentrations of the fish was within this range (7,760 IU/kg DM). Supplementing vitamin D_3 can be considered unnecessary when feeding a diet that already contains the recommended 1,000 IU/kg DM vitamin D_3. There have been reports of possible vitamin D deficiency, including a group of jackass penguins (*Spheniscus demersus*). This group of penguins was housed indoors and consumed a diet exclusively of capelin that had low concentrations of vitamin D when analyzed.[13] Another report describes the presumed physiological calcidiol plasma level in Humboldt penguins of 3.7 ± 2.4 nM/L.[1] These values are considerably lower than in this experiment. The proposed explanation by the author is that there is uncertainty about the validity and usefulness of a commercially available acetonitrile extraction–equilibrium radioimmunoassay kit.[1] It is difficult to compare plasma vitamin D_3 levels due to different detection methods. When feeding multiple fish species, the risk of hypovitaminosis D_3 is lower compared with feeding one fish species. Another factor in this study possibly contributing to stable vitamin D_3 plasma concentrations is the fact that the penguins were housed in an outdoor enclosure and had exposure to sunlight before and during the course of the trial.

α-tocopherol concentrations of the fish assayed in this study (29.25 IU/kg DM) are relatively low in comparison with other reported research.[2,4,14] Concentrations of vitamins A_1 and E decrease after freezing and storage over time.[3,5,6,16] The fish in this study was stored for a maximum of 10 mo (Parlevliet, 2235 SE Valkenburg, the Netherlands). Plasma α-tocopherol concentrations in both the supplemented and control groups were similar to those reported for free-ranging Humboldt penguin with an α-tocopherol plasma concentration 18.77 mg/L in April and 22.89 mg/L in September.[20] Also, when comparing α-tocopherol concentrations to other penguins species like the jackass, macaroni (*Eudyptes chrysolophus*), gentoo, and rockhopper penguins, similar plasma concentrations are seen.[10,11,17] Despite the lack of increase in circulating α-tocopherol concentrations after supplementing tocopheryl acetate in supplemented penguins in this study, due to the plethora of literature supporting supplementation and the varying concentrations of vitamin E in frozen stored fish, the authors recommend supplementing vitamin E to penguins fed thawed frozen-fish diets. If supplementation is needed on a daily basis, it should be researched more extensively. A possible outcome could be to supplement vitamin A and E separately within a schedule to maximize the intestinal vitamin absorption.

A concern in this study regarding the plasma vitamin concentrations of the penguins is that the supplemented and control groups were not randomly selected. Ideally, the treatments would have been sorted evenly across gender and age because reproduction status could have had an effect on the plasma concentrations. To minimize the effect of reproduction, the trial was scheduled before the normal nesting and molting period. Due to practical reasons, it was not possible in this setting to randomly select the animals.

In summary, the results from this study corroborate previous results that supplementing thawed frozen fish with vitamin B_1 is prudent. While the results of this study do not demonstrate significant differences, the authors also recommend supplementing with vitamin E. Supplementing vitamin D_3 may be advisable if the diet contains an inadequate amount of this vitamin. Supplementation with vitamin A cannot be recommended based on the results of this study, but when diets being fed to penguins are found to be low in vitamin A_1, supplementation may be necessary. In those circumstances, plasma levels should be measured before and after supplementation to ensure circulating vitamin A levels do not decrease after supplementation. Due to changing vitamin A and E concentrations over time, regular measurement of vitamin concentrations in different batches of frozen fish is also advised.

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