Zoo Biology 30:32–51 (2011)

RESEARCH ARTICLE

Evaluation of an Alternative to Feeding Whole Frozen Fish in Belugas (*Delphinapterus leucas*)

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Feeding fish to captive piscivores can be challenging owing to cost, availability, variability in nutrient, and caloric composition, as well as handling and storage concerns. This trial evaluated the response of three belugas to being fed Fish Analog, an alternative to frozen fish. Body condition, gut transit time, serum chemistry and metabolic hormone analytes, immune function, and behavioral motivation were the dependent variables. Belugas ($n = 3$) were fed various levels of Fish Analog (0–50%) over a 6-month period, and follow-up studies were conducted to further examine several dependent variables. When provided in gradually increasing amounts, belugas consumed the Fish Analog, with only minor fecal consistency changes and without behavioral responses indicative of gastric discomfort. Axillary girth and blubber thickness were positively correlated, and did not differ significantly with changes in the percentage of Fish Analog fed. Individual animal variation in initial passage time, some serum chemistry analytes, and immune function differences were noted following feeding of Fish Analog. Feeding Fish Analog reduced blood n9 fatty acids compared with captive belugas fed no Fish Analog. Feeding a DHA-enriched Fish Analog increased several n3 fatty acids, including eicosapentaenoic acid, but not DHA, compared with whales fed no Fish Analog or non-DHA-enriched Fish Analog. Fish Analog was shown to be a viable alternative to feeding fish at up to 50% of the dietary caloric density. Zoo Biol 30:32–51, 2011.

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Received 20 April 2009; Revised 21 January 2010; Accepted 23 February 2010

DOI 10.1002/zoo.20319
Published online 26 March 2010 in Wiley Online Library (wileyonlinelibrary.com).

Keywords: blood chemistry; body condition; cetacean; fatty acid; fish alternative

INTRODUCTION

Animals that have evolved to eat fish and other marine organisms are often challenging to feed in captivity. For many smaller aquatic species, such as fish, the use of commercially manufactured pellets, flaked food, and gel diets has provided alternatives to feeding fresh or frozen aquatic items. However, for larger aquatic species, such as elasmobranchs and marine mammals, commercially manufactured foods have either been unsuccessful or not evaluated. Therefore, freshly thawed previously frozen fish is the staple diet for the majority of large captive piscivores, including the belugas and all other marine mammal species housed at Mystic Aquarium and Institute for Exploration (MAIFE) and most other institutions.

Feeding a variety of fish to captive piscivores is very important to prevent an animal from becoming conditioned to eating only one particular type of fish that may in the future become less available, and to minimize selective feeding behaviors. However, using a variety of fish may be a challenge to institutions owing to cost and availability, as well as variability in nutritional content owing to sex, age, and reproductive status of the fish at the time of catch [Bernard and Allen, 2002], and loss of nutrients owing to postharvest decline [e.g., thiamin losses owing to activation of thiaminase postmortem [Rigdon and Drager, 1955] and vitamin E losses owing to oxidative rancidity [Dierenfeld et al., 1991]. Additionally, there are other concerns associated with feeding previously frozen fish, including the presence of toxins [Yasumoto and Murata, 1993] and environmental and microbial contaminants [Power et al., 2002; Rasmussen et al., 1990], and microbial concerns [e.g., mycobacterium; Leibovitz, 1980].

As a potential replacement for a portion of the frozen fish being fed to captive piscivores, a "Fish Analog" product was developed in the early 1990s (Mazuri® Exotic Animal Nutrition/PMI Nutrition Intl LLC, Gray Summit, MO). The palatability of the product has been demonstrated in seals, sea lions, bottlenose dolphins, and penguins [Edwards et al., 2001; Molitoris et al., 1998]. The purpose of this trial was to expand our knowledge on the use of Fish Analog to belugas, and based on earlier research, we hypothesized that there would be no difference in the response to feeding Fish Analog when compared with a diet of fish. In order to evaluate this hypothesis, we examined a variety of parameters encompassing measures of animal behavior, health status, and nutrient status over an 8-month period, and data were compared with similar data collected for each animal before the initiation of this feeding trial. A follow-up study was used to further examine observed changes, and to examine the ability of Fish Analog to serve as an o-3-enriched alternative to feeding frozen fish.

METHODS

Animals

This study was approved by MAIFE Institutional Animal Care and Use Committee (Project #06004) and Shedd Aquarium Research Committee. One male and two female belugas (*Delphinapterus leucas*) were maintained at MAIFE in an

outdoor 2,838,998 l (750,000 gal) saltwater exhibit, consisting of three interconnected pools which span more than 4,045 m^2 (1 acre). All animals were 26 years old at the time of the study. The two females, Naku and Kela, were both 3.4 m (11 ft) long and weighed approximately 726 kg and 544 kg (1,600 and 1,200 lbs), respectively. Inuk, the male, was 4 m (13 ft) long and weighed approximately 885 kg (1,950 lbs).

Study 1 Design

The initial study was designed to evaluate the addition of Fish Analog (Mazuri 5T8J, 1812641, St. Louis, MO) to the diet of belugas and monitor the animals for changes in behavior, motivation, gastrointestinal processes, body condition, GI transit time, hematological and serum chemistry variables, and immune function. This was accomplished by substituting 10–50% of the calculated caloric content of diet with Fish Analog (caloric content of frozen fish and Fish Analog calculated using Atwater values of 9 kcal/g fat, 4 kcal/g protein, and 4 kcal/g carbohydrate) over an 8-month period (December 2006–July 2007; Table 1). The Fish Analog product is a gel-based product (similar to gel-based diets provided to fish) made primarily from menhaden fish meal and oil. A complete nutritional analysis and list of ingredients is provided in Table 2. Fish Analog was received in frozen blocks, cut into smaller pieces at MAIFE, and fed thawed, similar to standard protocols for feeding frozen fish. The entire Fish Analog product is usable (no waste) and the product is flexible, easily broken into pieces, and holds together well.

Animal Behavior Monitoring

General behavior of each subject toward the Fish Analog product was observed during each session and recorded. Categories included (1) refusing product (spitting out, dropping, or swimming off during session), (2) sorting (the moving of other food fish around the product, and (3) holding product in mouth (for longer than 5 sec) before swallowing.

Animal motivation was based on an evaluation system that has been in place at MAIFE for many years. Each animal was given a score ranging from 0 to 5. A score of 5 (excellent) meant the animal performed all behaviors at or above established criteria, was attentive with quick behavioral responses, had no breaks from station, and showed no aggression. A score of 4 (very good) meant the animal carried out 80–99% of the behaviors at or above criteria, was attentive and responsive, and only had 1–2 breaks from station. A score of 3 (good) meant 60–79% of the behaviors were at or above criteria, the attention of the animal was decreased, the animal was distracted, and there were 3–4 short (<10 sec) or 1–2 long breaks (>10 sec) from station. A score of 2 (fair) meant only 40–59% of the behaviors were at or above

TABLE 1. Percentage of Fish Analog product fed over the study period (Study 1)

% dietary calories	Time fed	Start date	Season
10	1 month	December 1, 2006	Non-breeding
20	4 months	January 1, 2007	Non-breeding/breeding
30	2 weeks	May 1, 2007	Breeding
40	2 weeks	May 15, 2007	Breeding
50	2 weeks	June 1, 2007	Non-breeding
10	1 month	June 15, 2007	Non-breeding

TABLE 2. Ingredient and nutrient composition of Fish Analog products fed to belugas

	Study 1 and 3[c]		Study 2 and 3[d]	
Nutrients[a,b]	As fed	Dry matter	As fed	Dry matter
Moisture (%)[a]	77.72		74.31	
Crude protein (%)[a]	13.40	59.30	13.00	55.94
Lysine (%)	0.84	3.77	0.89	3.47
Methionine (%)	0.28	1.26	0.26	1.03
Methionine+Cystine (%)	0.39	1.75	0.37	1.43
Crude fat (%)	6.95	30.80	7.10	31.30
Saturated fatty acids (% of total fatty acids)[b]	0.41	1.83	0.55	2.14
Monounsaturated fatty acids (% of total fatty acids)[b]	0.30	1.34	0.31	1.20
18:2 n6 linoleic acid (% of total fatty acids)[b]	0.86	3.85	0.92	3.60
18:2 n3 linolenic acid (% of total fatty acids)[b]	0.41	1.83	0.33	1.28
20:4 n6 arachidonic acid (% of total fatty acids)[b]	0.21	0.94	0.25	0.96
20:5 n3 eicosapentaenoic acid (% of total fatty acids)[b]	2.45	11.00	2.10	8.17
22:6 n3 docosahexaenoic acid (% of total fatty acids)[b]	2.47	11.10	4.69	18.25
Crude fiber (%)	0.30	1.35	0.33	1.28
NDF (%)	0.73	3.28	0.66	2.57
ADF (%)	0.40	1.80	0.39	1.50
Ash (%)	2.67	11.98	2.67	11.98
Ca (%)[a]	0.35	1.53	0.56	1.72
P (%)[a]	0.26	1.16	0.36	1.19
Chloride (%)	0.29	1.30	0.30	1.17
Magnesium (%)[a]	0.03	0.14	0.04	0.13
Potassium (%)[a]	0.20	0.90	0.24	1.17
Sodium (%)[a]	0.11	0.68	0.20	0.78
Iron (ppm)[a]	74.00	325.50	49.46	192.40
Zinc (ppm)[a]	15.00	66.00	13.06	50.80
Manganese (ppm)[a]	10.50	47.00	28.64	111.40
Copper (ppm)[a]	13.00	55.00	6.30	24.53
Iodine (ppm)	4.94	22.17	15.50	60.32
Selenium (ppm)	0.25	1.03	0.03	0.13
Vitamin A (IU/kg)	2,956.00	13,266.53	4,015.00	15,623.00
Vitamin D3 (IU/kg)	590.00	2,630.00	590.00	2,630.00
Vitamin E (IU/kg)[b]	103.00	462.00	100.50	391.05
Vitamin K (IU/kg)	0.60	2.71	0.57	2.22
Vitamin B_{12} (μg/kg)	53.24	238.94	50.94	198.20
Choline (ppm)	533.28	2,393.36	504.00	1,962.00
Niacin (ppm)	30.24	135.72	22.51	87.61
Pantothenic acid (ppm)	26.16	117.41	7.31	28.45
Pyridoxine (ppm)	5.38	24.15	2.66	10.33
Riboflavin (ppm)	9.10	40.84	3.47	13.49
Thiamin (ppm)[b]	21.20	95.15	7.12	27.70
Folic acid (ppm)	1.39	6.23	1.39	5.40
Biotin (ppm)	0.07	0.03	0.10	0.39
Ascorbic acid (ppm)	36.00	161.57	56.00	217.90

[a,b]Nutrient variables denoted with a superscript represent analyzed values ([a]Dairy One Labs, Ithaca, NY; [b]NP Analytical Labs, St. Louis, MO). All nutrient variables without a superscript represent calculated values.

[c]Water, menhaden select fishmeal, menhaden select fish oil, gelatin, poultry meal, beet pulp, lecithin, potassium chloride, xanthan gum, salt, pyridoxine, spirulina, sodium selenite (0.06%), l-tryptophan, choline bitartrate salt, taurine, brewers dried yeast, calcium pantothenate, magnesium oxide, d-α tocopherol (60%), menadione, l-ascorbyl polyphosphate, thiamin mononitrate (10%), vitamin D3 (7500 IU/g), folacin (2%), mixed tocopherols, inositol, riboflavin, retinyl palmitate (60000 IU/g), dl-methionine, biotin (0.1%), vitamin B12 (300 μg/g), manganese oxide, niacin, copper sulfate (anhydrous), calcium iodate

[d]Water, menhaden select fishmeal, menhaden select fish oil, gelatin, docosahexanoic acid (algal source), poultry meal, beet pulp, lecithin, potassium chloride, xanthan gum, salt, pyridoxine, spirulina, sodium selenite (0.06%), l-tryptophan, choline bitartrate salt, taurine, brewers dried yeast, calcium pantothenate, magnesium oxide, d-α tocopherol (60%), menadione, l-ascorbyl polyphosphate, thiamin mononitrate (10%), vitamin D3 (7500 IU/g), folacin (2%), mixed tocopherols, inositol, riboflavin, retinyl palmitate (60000 IU/g), dl-methionine, biotin (0.1%), vitamin B12 (300 μg/g), manganese oxide, niacin, copper sulfate (anhydrous), calcium iodate.

criteria, the animal had poor attention, was not focused, and there were 5 or more breaks from station. A score of 1 (poor) meant 39% or less of the behaviors of the animal were at or above criteria, the animal was distracted, refused to cooperate, had poor stationing, and was not eating well.

Body Condition Monitoring

Girth and blubber thickness were used to monitor body condition [Richmond et al., in preparation). Axillary girth (at posterior insertion of pectoral flipper) was collected twice per month using a tape measure, and this data was compared with data collected for more than 2 years before the study and 1 year after completion of the study. One trainer was assigned to take girth measurements from each whale to reduce interoperator variability.

Blubber thickness was evaluated via ultrasound at a point dorsal to the anterior insertion of the pectoral flipper and 10 cm ventrolateral to mid-dorsum with the ultrasound probe parallel to the pectoral appendage. Measurements were performed twice per month and within 1 day of girth measurements by the same individual to avoid potential for interoperator variability.

Gastrointestinal Transit Time

Fecal color and consistency were monitored during training sessions and at any other time of day that a staff member observed an animal defecating. Initial gastrointestinal (GI) passage time (IPT) was monitored based on published methodology [Kastelein et al., 1997] 12 times for each whale, including 4 baseline time points, 2 time points at 10, 20, and 30% Fish Analog diet, and 1 time point at 40 and 50% Fish Analog diet by feeding an iron oxide (FeO_3) powder (0.2% of the diet incorporated into a gelatin capsule and placed into the body cavity of a squid during the first feeding of the day) with the morning meal. The percentage of iron oxide used was based on research conducted with carmine red as a marker for IPT in other cetaceans [Kastelein et al., 1997]. During the subsequent observation period, the animal was isolated into a smaller pool and a staff member was stationed at the pool to monitor defecation until the first signs of a rust color appeared which was considered the IPT. The observers recorded date and time of FeO_3 consumption and percentage of Fish Analog in the diet, as well as time, color and consistency (stringy, cloudy, or clumpy) of each defecation.

Animal Health Monitoring

Gastric samples

Fasted gastric samples were collected from the animals on a weekly or twice weekly basis under behavioral control. A piece of clear plastic tubing (5/8'' or 16 mm diameter) of the appropriate length was introduced into the mouth and guided down the esophagus into the animal's forestomach when the whale was stationed in a tail down position in the water column. A finger was placed over the bore of the tube to prevent loss of any sample and the tube was removed and the sample dispensed into a container. Samples were examined for clarity and content by the animal care staff and if a sample appeared abnormal it was examined further by a staff veterinarian. All the subjects were hand-fed their diets in 4–5 sessions each day allowing staff to easily monitor the animals for any signs of gastrointestinal discomfort that might be

related to feeding the Fish Analog product. Because most GI issues do not take place at the time of feeding but rather some period of time later, animals were also monitored for GI discomfort during exhibit cleaning and enrichment sessions (4–5 times daily). Gastrointestinal maladies are not uncommon in cetaceans and are most often accompanied by signs, such as abnormal swim patterns, cramping, excess logging (floating horizontally on the water surface), flatulence, air bubbles, abnormal body posturing, or regurgitation. All animals were monitored by experienced staff members for the appearance of any such signs as well as any other abnormal behavior.

Blood collection

Blood was collected every other week from a ventral fluke vein while the animals were positioned horizontally (under behavioral control) with a staff member holding the flukes. Using a 19 gauge 3/4" (1.10 × 19 mm) butterfly needle (Terumo, Tokyo, Japan), 22–32 ml of blood was collected; 2 ml was collected into an EDTA tube for hematology analysis, 10 ml into a thrombin tube (BD, Franklin Lakes, NJ) for serum chemistry and hormone analyses, and 10–20 ml into tubes containing sodium heparin for immune function assays. All samples were held at ambient temperature for 30–60 min before processing. The thrombin tube was centrifuged at room temperature for 15 min at 4,750 RPM and the serum was allocated into individual cryovials which were kept frozen at −10°C until analysis. Sodium heparin tubes were centrifuged at 2,900 RPM for 10 min at 10°C. The buffy coat was transferred to cryovials, mixed with freezing media (90% FBS + 10% DMSO) and placed overnight at −80°C. Cells were transferred and stored in liquid nitrogen until immune function tests were carried out.

Blood analyses

Hematological analysis was performed in the clinical laboratory at MAIFE (Heska, Vet ABC Hematology Analyzer, Fort Collins, CO) for total red and white blood cell counts (RBC and WBC, respectively), hemoglobin (Hb), mean corpuscular volume, mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration. Spun hematocrit, fibrinogen [Miller et al., 1971], and manual WBC differentials were also performed. Serum chemistries were analyzed by the Pfizer, Inc (Groton, CT) clinical laboratory using a Hitachi 917 (Roche Diagnostics Corporation, Indianapolis, IN). Serum concentrations of growth hormone (GH) and insulin-like growth factor (IGF)-1 were quantified using radioimmunoassay validated for use in belugas [Richmond and Zinn, 2009]. Immune function analyses were carried out in-house at MAIFE and included quantification of lymphocyte subsets and lymphocyte proliferation. Cells were thawed in a 37°C water bath, washed, counted, and resuspended at the desired concentration. Quantification of lymphocyte subsets was carried out [T, B, Class II+, and T helper cells according to the protocol of Romano et al., 2004]. For lymphocyte proliferation studies, 1,000 cells per well were plated in triplicate in a 96 well plate. One hundred microliters of Concanavalin A (ConA) and Lipopolysaccharide (LPS) were added to wells for 48 hr at a final concentration of 10 and 5 μg/ml. The cells were cultured for an additional 24 hr after the addition of bromodeoxyuridine. The plates were fixed, dried, and stored at 4°C until assay according to kit protocol (Chemicon® International, Inc., Germany).

Study 2

To further investigate the results of Study 1 (particularly changes in clinical chemistry), the same three whales were fed Fish Analog that was slightly modified to contain a greater concentration of docosahexaenoic acid (DHA, Table 2). The DHA-enriched Fish Analog was designed to provide similar DHA content as would be encountered with fresh fish, and as such may provide a fatty acid profile more similar to diets consumed in the wild. DHA is a long chain, highly unsaturated fatty acid that is concentrated in fish and other marine species, and is known to exert unique effects on cell membranes and thus impact cell signaling and cell function [reviewed by Chapkin et al., 2008].

These data not only provide information about the acceptance of the Fish Analog product, but also the ability to manipulate the fatty acid profile of the food to optimize nutrition of species which may have evolved with different fatty acid requirements (e.g., cold water vs. temperate water environments), and any associated changes in palatability with the addition of DHA to the Fish Analog.

Because the whales seemed to readily accept Fish Analog in Study 1, the DHA-enriched Fish Analog was introduced immediately at 50% of their diet (November 2007). This rapid introduction produced immediate behavioral and medical concerns (including sorting of fish, refusing to eat, abnormal feces and abnormal body posturing and arching) and the normal dietary regime was reintroduced the following day. One animal was administered simethicone. After 48 hours the product was reintroduced at 20% to the two animals not on gastric medications for one day then increased to 30% for the 6-week study. The third animal remained on a normal fish diet for 3 weeks and was then increased slowly up to 30% Fish Analog over the next 3 weeks and remained at 30% for the 6-week study. Spun hematocrit (Hct) and Hb were monitored weekly. Clinical blood sampling and analysis was performed as described above, as were behavioral analyses. Serum fatty acid analysis (by gas chromatography, NP Analytical Labs, St. Louis, MO) was performed and results were compared with that of Study 1, as well as from three samples collected from wild belugas. Wild beluga blood samples were provided by the National Marine Mammal Laboratory, Seattle.

Study 3

To further investigate the results of Study 2 (particularly changes in behavior), five belugas maintained at Shedd Aquarium, Chicago, IL, were fed the same two products fed in studies 1 and 2 in five one-day trials. The animals, 1 male (age 22) and 4 females (ages ranging from 9 to 27) were maintained in a 1 million gallon saltwater exhibit consisting of 3 pools. These animals had never before been offered either Fish Analog or DHA-enriched Fish Analog. On each trial day, the whales were randomly assigned to be offered one product at a morning feeding and the alternate product at an afternoon feeding. At each of these feedings, approximately 1,144 kcal of fish was replaced with 1 kg of the assigned Fish Analog product (equivalent calculated caloric value). The product was fed to the animals in exactly the same manner as fish. An individual who was not aware of which product was being fed (as they cannot be visually distinguished) observed each feeding and recorded behavior. The observation categories were (1) Animal accepts product and swallows immediately; (2) Animal accepts product but hold it in the mouth and

"sorts" or swallows other fish around the product before swallowing the product itself; and (3) Animal refuses the product, drops the product, or gives the product back to the trainer.

Data Analysis

Behavioral motivational scores at each percentage of Fish Analog were compared with pre-trial scores using a paired *t*-test. Blood chemistry variables were compared with each animal's normal reference range. To examine differences in caloric intake, pectoral girth, blubber depth, GH and IGF-I concentrations owing to season, year, and their interaction, a repeated measures ANOVA was used. Seasons were defined as winter (December–February), spring (March–May), summer (June–August), and fall (September–November). In addition, girth data from 2007 were compared with data from the 2 years before the study and 1 year after the study. A two-way ANOVA was used to examine the main effect of animal, Fish Analog inclusion, and their interactions on caloric intake, pectoral girth, blubber depth, IPT, GH, and IGF-1 concentrations, and immune function. Differences between means were determined using students *t*-test at significance level of $P < 0.05$. Additionally, a Pearson Correlation was calculated to examine the relationship between the percent of Fish Analog product in the diet and IPT as well as percent of Fish Analog product in the diet and immune function. Serum fatty acid data was analyzed using a one-way ANOVA examining the main effect of diet on serum fatty acids, and using a one-way ANOVA examining the effect of environment (e.g., captive vs. wild) on fatty acids. A nonparametric Sign Test [Siegel and Castellan, 1988] was used to evaluate the data from the palatability study performed at the Shedd Aquarium. The sign test was chosen because the behavioral assessment data was collected as discrete data (not continuous), and thus it does not meet the criteria for using a parametric test.

RESULTS

Study 1

In earlier years, the study animals were fed fewer calories in spring and summer than in fall and winter (Fig. 1; $P < 0.001$ for each comparison). At the start of the Fish Analog study (Study 1: 2007), all three whales consumed the Fish Analog product immediately, although two of the three animals held the product in their mouth longer than normal or "sorted" the fish in their mouths and swallowed the real fish before the Fish Analog for several (1–3) days. Throughout the 8-month study, there were very few (< 10) occasions that the whales dropped the product in the pool or refused to take the product from the trainer. One animal consistently treated the Fish Analog product identically to the real fish throughout the study. Motivational scores were consistent throughout the study and not significantly different from pre-trial scores ($P > 0.14$) (Fig. 2). The decreased score in one of the females at 20% coincided with a medical issue which was deemed by the veterinary staff to be unrelated to diet.

Axillary girth and blubber thickness via ultrasound were linearly correlated ($r = 0.91$, $P < 0.01$), indicating that the use of either measurement alone may be adequate to monitor body condition (Fig. 3A). Across season and year, body

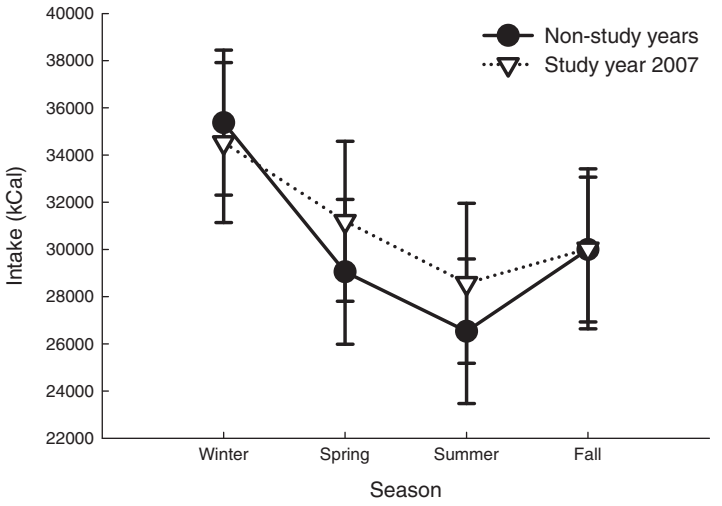

Fig. 1. Caloric intake for belugas varies owing to (**A**) season and year. Data represent mean ± SEM.

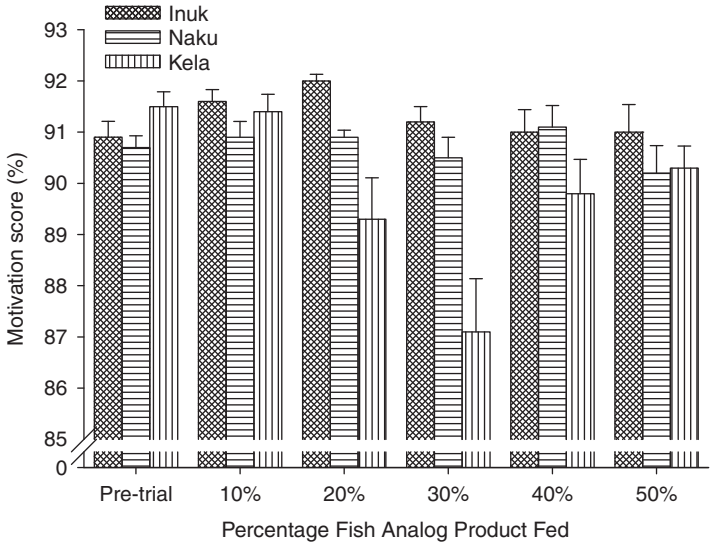

Fig. 2. Motivation scores for belugas ($n = 3$) fed 10–50% of caloric intake as Fish Analog (Study 1).

condition indices generally reflected intake from the earlier season. For example, girth and blubber measurements were greatest in spring, following the increased winter intake ($P < 0.001$ for each animal; Fig. 3B), and exhibited a similar seasonal pattern of change in all years ($P > 0.05$). Girth measurements in 2007 were lower than in other years ($P < 0.01$). Blubber depth measurements reflected a similar pattern with greater blubber stores observed in 2006 (6.1 ± 0.45 cm) compared with 2007 (5.6 ± 0.45 cm; $P = 0.001$). Differences in axillary girth and blubber thickness

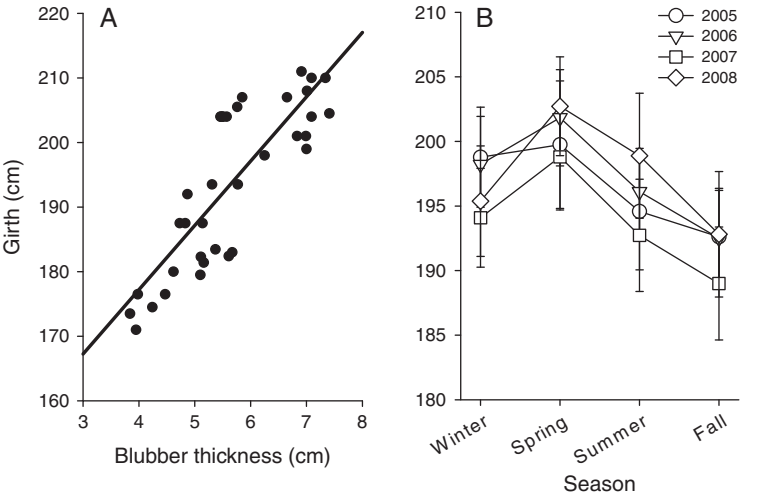

Fig. 3. Girth measurements of belugas are correlated to (**A**) blubber thickness, which is predicted by the equation: Girth (cm = 9.77 × Blubber thickness (cm)) + 137.38, $r = 0.82$, and **B**) season. Data represent mean ± SEM.

TABLE 3. Average initial passage time (IPT) at each percent of Fish Analog fed to belugas ($n = 3$; Study 1)

		IPT (min)		
Percent Fish Analog	*N*	Naku (♀)	Kela (♀)	Inuk (♂)
0	4	257 ± 22	166 ± 45	205 ± 26
10	2	217 ± 17	213 ± 51	196 ± 35
20	2	207 ± 1	258 ± 71	140 ± 44
30	2	210 ± 20	169 ± 28	195 ± 12
40	1	192	187	213
50	1	231	154	244

"*N*" represents number of observations of IPT per animal, and values with no standard deviation represent single trials.

between whales when fed Fish Analog vs. when fed whole fish were not statistically different ($P > 0.20$ for each).

Overall, there was no significant correlation between Fish Analog inclusion level and IPT ($P = 0.18$; Table 3); however, one animal had a negative correlation between these two variables ($r = -0.60$, $P = 0.03$). Mean IPT was 202.9 min. Fecal observations demonstrated that while whales were consuming the Fish Analog diet, both the color and consistency of the feces changed. The normal defecation in a beluga is a cloud of green feces that dissipates rapidly in the water column. When consuming Fish Analog, the feces were a darker shade of green and were more clumpy and stringy, and sank to the bottom or floated on the surface of the pool at the beginning of the study and at each increased percentage of Fish Analog. After a few days of acclimation to each increase in Fish Analog, the feces returned to the normal dissipating greenish cloud. During the IPT studies, when the animals were

observed continuously for several consecutive hours, more than 80% of the defecation events were normal.

Gastric samples did show some changes during the study. Gastric samples from two of the whales had an oily/gritty consistency with fish and skin tissue particles. Samples from one of the animals consistently had a reddish tinge. The third animal showed almost no change in the appearance of its gastric sample. There were no signs of gastrointestinal discomfort (cramping, vomiting, or logging at the surface) in any of the whales throughout the study.

Individual animals were variable in their immune function responses. The mean responses and standard errors for all animals from each diet regime (10–50% of caloric intake from Fish Analog) are shown in Table 4. The proliferative response to 5 μg/ml LPS was greater when animals were fed 20–40% Fish Analog as compared with those fed 0% Fish Analog ($P<0.01$), although this effect was not seen at higher levels of LPS, nor with either dose of ConA. Individual animals were also variable in their hematology and serum chemistry variables, but all variables fell within normal ranges for the individual animals throughout the study. Across all whales, only Hct and Hgb showed clinically significant changes over time. These variables had also manifested a seasonal decline in earlier years, although the decline was more dramatic in the study year (Fig. 4a, b). However, plasma Fe remained similar or increased over time on trial. GH concentrations were not affected by season ($P=0.69$), but were greater in 2005 and 2007 (both $4.2\pm0.55\,\mathrm{ng\,ml^{-1}}$) compared with 2006 and 2008 ($P<0.01$; 2.3 ± 0.46 and $2.1\pm0.97\,\mathrm{ng\,ml^{-1}}$, respectively). In contrast, IGF-I concentrations were greater in the summer and fall (301.6 ± 19.4 and $241.7\pm30.1\,\mathrm{ng\,ml^{-1}}$, respectively) compared with winter and spring ($P<0.01$; 176.9 ± 20.9 and $199.8\pm19.1\,\mathrm{ng\,ml^{-1}}$, respectively), but the seasonal pattern among years was similar (season*year, $P=0.09$). GH and IGF-I concentrations were measured at 0, 10, 20, or 40% Fish Analog fed. GH was greater in whales fed 20 or 40% Fish Analog compared with those fed 0% ($P<0.05$; Fig. 5A), and IGF-I concentrations were greatest for whales fed 0% Fish Analog vs. those fed 10–40% ($P<0.05$ for each; Fig. 5B).

Study 2

During Study 2, Hgb and Hct values were monitored while the whale consumed 30% of the slightly modified Fish Analog diet. Neither Hct ($P=0.74$; mean $=53.9\pm0.56$) nor Hgb ($P=0.55$; mean $=20.9\pm0.22$) were affected by the

TABLE 4. Immune function parameters of belugas ($n=3$) fed Fish Analog at 10–50% of their caloric intake (Study 1)

Parameter	Mean	SEM
CD19 (B cell marker; % gated)	1.06	0.055
CD2 (T cell marker; % gated)	61.91	1.684
CD4 (T helper cell marker; % gated)	41.49	0.775
Q5/13 (Class II marker; % gated)	82.25	1.609
Stimulation to ConA (10 ug/ml; stimulation index)	4.75	0.337
Stimulation to ConA (5 μg/ml; stimulation index)	4.35	0.289
Stimulation to LPS (10 μg/ml; stimulation index)	2.80	0.100
Stimulation to LPS (5 μg/ml; stimulation index)	2.22	0.079

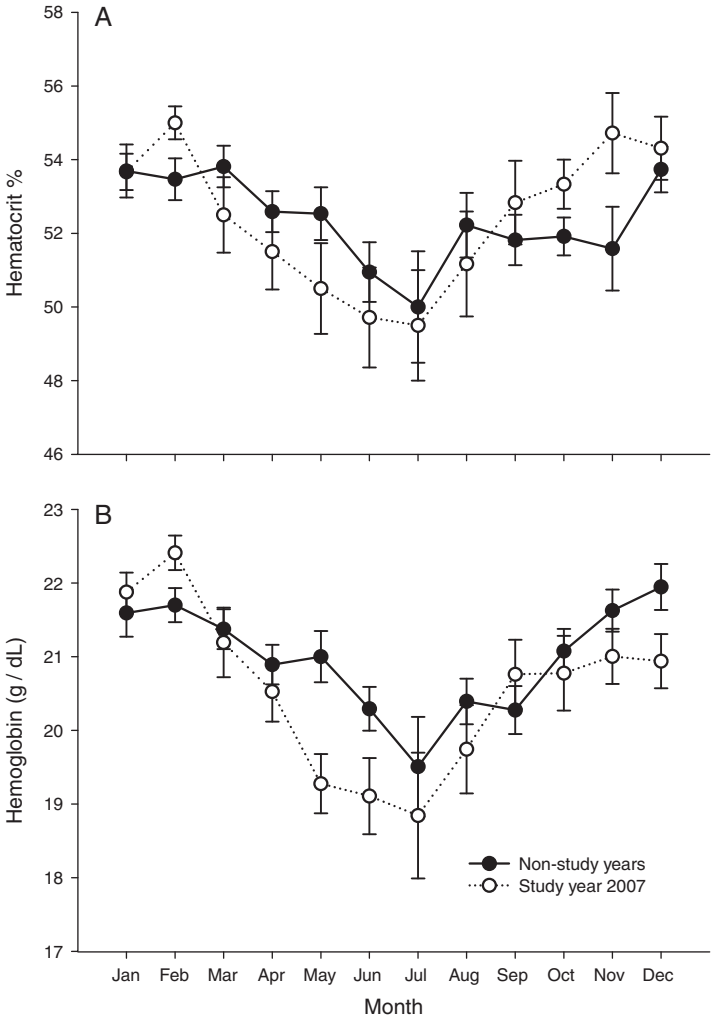

Fig. 4. Annual changes in (**A**) hemoglobin and (**B**) hematocrit in belugas with non-study years 2004, 2005, 2006 combined and study year 2007). Data represent mean ± SEM.

inclusion of DHA-enriched Fish Analog when compared with pre-study values. In Study 2, concentrations of GH were similar at both 0 ($4.8 \pm 0.66\,ng\,ml^{-1}$) and 30% ($3.6 \pm 0.55\,ng\,ml^{-1}$) inclusion rate ($P = 0.16$). Concentrations of IGF-I were slightly reduced ($P < 0.0001$) when animals were fed 30% ($187.8 \pm 13.1\,ng\,ml^{-1}$) Fish Analog compared with 0% ($288.9 \pm 15.4\,ng\,ml^{-1}$).

Blood fatty acid profiles in captive vs. wild belugas differed during this trial (Table 5). Compared with wild belugas, captive animals had reduced n9 fatty acids, but similar concentrations of n6 and n3 fatty acids. Individual fatty acids also varied between captive and wild belugas. Notably, captive animals had greater concentrations of lauric acid (12:0, ~2.5-fold), hexadecadienoic acid (16:2, ~2.5-fold), hexadecatrienoic acid (16:3n4, ~45-fold), linoleic acid (18:2n6, ~2-fold), linolenic acid (18:3n3, ~7.5-fold), eicosanoic acid (20:1n11, ~8.5-fold), homo-γ-linolenic acid (20:3n6,

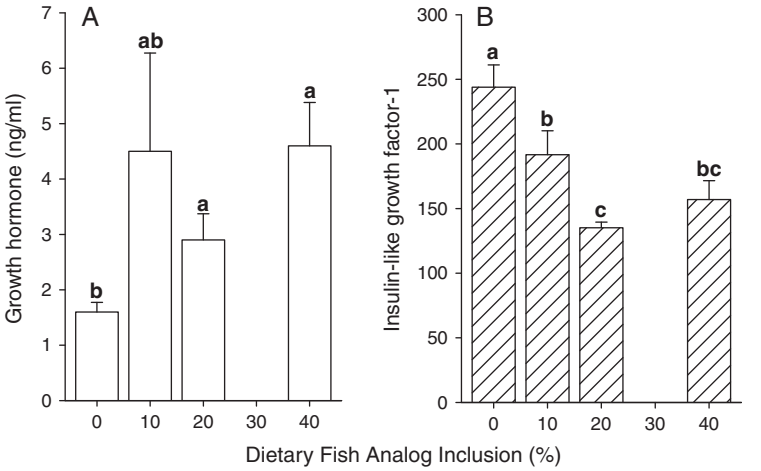

Fig. 5. Effect of percentage of Fish Analog inclusion on (**A**) growth hormone and (**B**) insulin-like growth factor-I concentrations in belugas. Data represent mean ± SEM.

~2-fold), eicosatetraenoic acid (20:4n3, ~2.5-fold), and erucic acid (22:1n9, ~24-fold) compared with wild belugas. Conversely, wild belugas had greater concentrations of some fatty acids including docosahexaenoic acid, 22:6 n3, 1.2-fold increase.

In captive belugas, there was significant variation in some fatty acids between individuals, as well as significant effects of diet (Table 6). After feeding Fish Analog or DHA-enriched Fish Analog, whales had reduced n9 fatty acids and increased n6 fatty acids compared with no Fish Analog. Whales fed DHA-enriched Fish Analog also had increased n3 fatty acids.

During this trial, a rapid transition to DHA-enriched Fish Analog was made, and animals subsequently had some feed refusal and signs of gastric upset including cramping and logging (horizontal and stationary floating) on the surface. Therefore, a small follow-up trial (Study 3) was conducted to determine whether changes in apparent palatability of the product were owing to DHA-enrichment or to a rapid transition to Fish Analog.

Study 3

The results of the sign test from this admittedly small experiment shows no preference between the two different Fish Analog products ($P = 1.0$). Two individuals (male and the oldest female) accepted both of the Fish Analog products very well, receiving scores of 1 or 2 (accepts product immediately or sorts product around other fish) for all observed feedings. A second female accepted the initial Fish Analog product (from Study 1) only 60% of the time it was offered, but ate the DHA-enriched Fish Analog (from Study 2) 80% of the time it was offered, sorting only during one trial. The remaining two females had a lower acceptance rate of both products (40% initial product and 20% DHA-enriched product).

DISCUSSION

Monitoring health status of individuals during the introduction of a new food product is extremely important in determining the suitability of this product for marine mammals. This trial carefully monitored health and nutritional status,

TABLE 5. Blood fatty acid profile of captive belugas fed Fish Analog or DHA-enriched Fish Analog vs. wild belugas

			P value		
Fatty acids	Captive	Wild	Environ-ment	Animal (captive only)	Diet (captive only)
Monounsaturated fatty acids (g/100 g)	0.09±0.01	0.08±0.01	0.8	0.22	0.51
Polyunsaturated fatty acids (g/100 g)	0.10±0.01	0.06±0.01	<0.01	0.23	0.49
Saturated fatty acids (g/100 g)	0.07±0.01	0.05±0.01	0.11	0.08	0.75
n9 fatty acids (%)	22.58±0.66	30.10±2.56	<0.01	0.04	<0.01
n6 fatty acids (%)	7.46±0.50	6.58±0.35	0.44	0.2	<0.01
n3 fatty acids (%)	26.93±0.81	30.57±0.70	0.21	0.32	0.03
C12:0 lauric (%)	0.14±0.01	0.05±0.01	<0.01	0.53	0.86
C14:0 myristic (%)	1.93±0.09	1.14±0.14	<0.01	0.2	0.02
C14:1n5 myristoleic (%)	0.06±0.01	0.05±0.01	0.4	0.55	0.68
C15:0 pentadecanoic (%)	0.25±0.03	0.23±0.02	0.82	0.43	0.1
C16:0 palmitic (%)	11.66±0.22	12.47±0.22	0.11	0.11	0.73
C16:1n7 palmitoleic (%)	4.29±0.22	5.36±0.69	0.06	0.06	<0.01
C16:2 hexadecadienoic (%)	0.21±0.02	0.08±0.03	0.02	0.05	<0.01
C16:3n4 hexadecatrienoic (%)	2.26±0.27	0.05±0.01	<0.01	0.34	0.02
C17:0 margaric (%)	0.42±0.01	0.56±0.09	0.01	<0.01	0.01
C18:0 stearic (%)	9.18±0.25	8.79±0.18	0.48	0.03	0.57
C18:1n7C vaccenic (%)	2.32±0.05	2.07±0.09	<0.01	0.58	0.02
C18:1n9C oleic (%)	20.59±0.59	29.23±2.46	<0.01	0.22	<0.01
C18:1n9T elaidic (%)	0.30±0.03	0.24±0.02	32	0.13	0.25
C18:2n6 linoleic (%)	1.99±0.10	0.91±0.23	<0.01	0.29	0.01
C18:3n3 linolenic (%)	2.68±0.29	0.35±0.14	<0.01	0.35	0.48

TABLE 5. Continued

Fatty acids	Captive	Wild	*P* value Environ-ment	Animal (captive only)	Diet (captive only)
C19:0 nonadecanoic (%)	0.19±0.02	0.11±0.01	0.04	0.08	0.07
C20:0 arachidic (%)	0.46±0.06	0.27±0.11	0.15	0.21	0.64
C20:1n11 cis eicosenoic (%)	2.71±0.21	0.31±0.13	0.01	0.22	<0.01
C20:2n6 eicosadienoic (%)	0.19±0.02	0.13±0.01	0.16	0.41	0.99
C20:3n3 eicosatrienoic (%)	0.13±0.02	0.05±0.01	0.06	0.28	0.99
C20:3n6 homo-γ-linolenic (%)	0.29±0.02	0.15±0.01	0.01	0.1	<0.01
C20:4n3 eicosatetraenoic (%)	0.62±0.05	0.22±0.04	<0.01	0.35	<0.01
C20:4n6 arachidonic (%)	4.61±0.34	5.07±0.34	0.55	0.3	<0.01
C20:5n3 eicosapentaenoic (%)	10.67±0.69	7.61±0.28	0.06	0.94	<0.01
C21:5 heneicosapentaenoic (%)	0.14±0.01	0.12±0.04	0.49	0.89	0.15
C22:0 behenic (%)	0.10±0.010	0.12±0.01	0.49	0.94	0.81
C22:1n9 erucic (%)	1.22±0.20	0.05±0.01	0.02	0.07	0.69
C22:5n3 docosapentaenoic (%)	3.48±0.18	4.81±0.44	<0.01	0.18	<0.01
C22:5n6 docosapentaenoic (%)	0.28±0.07	0.22±0.03	0.69	0.46	<0.01
C22:6n3 docosahexaenoic (%)	9.11±0.29	11.25±1.22	0.02	0.36	0.32
C24:0 lignoceric (%)	0.06±0.01	0.08±0.03	0.28	0.24	0.57
C24:1n9 nervonic (%)	0.41±0.05	0.53±0.09	0.32	0.21	0.61

Captive whales ($n = 3$) were sampled for blood fatty acid profile before the onset of the trial, and at the end of at least 6 weeks of feeding each of the Fish Analog products. Whales had at least 6 weeks of no Fish Analog in their diet before feeding the DHA-enriched Fish Analog. Wild belugas ($n = 3$) were sampled from Bristol Bay, Alaska. Data represent means±SEM. Captive belugas ($n = 3$) were fed no Fish Analog, Fish Analog, and DHA-enriched Fish Analog for at least 6 weeks, then blood fatty acid profiles were determined. Data represent means±SEM.

TABLE 6. Diet treatment affects beluga blood fatty acid profiles

Fatty acid	No Fish Analog	Fish Analog	DHA-enriched fish Analog
n9 fatty acids (%)	24.43 ± 0.52^{a}	20.95 ± 0.10^{b}	19.90 ± 0.61^{b}
n6 fatty acids (%)	6.13 ± 0.21^{b}	8.25 ± 0.17^{a}	9.74 ± 1.15^{a}
n3 fatty acids (%)	25.71 ± 0.82^{b}	26.12 ± 1.73^{b}	30.57 ± 0.69^{a}
C14:0 myristic (%)	1.77 ± 0.07^{b}	2.34 ± 0.24^{a}	1.89 ± 0.08^{b}
C16:1n7 palmitoleic (%)	3.80 ± 0.14^{b}	5.34 ± 0.40^{a}	4.40 ± 0.27^{b}
C16:2 hexadecadienoic (%)	0.16 ± 0.01^{c}	0.34 ± 0.04^{a}	0.21 ± 0.02^{b}
C16:3n4 hexadecatrienoic (%)	2.83 ± 0.25^{b}	2.12 ± 0.24^{ab}	1.07 ± 0.53^{a}
C17:0 margaric (%)	0.39 ± 0.02^{b}	0.46 ± 0.04^{a}	0.44 ± 0.02^{a}
C18:1n7C vaccenic (%)	2.38 ± 0.04^{a}	2.43 ± 0.07^{a}	2.07 ± 0.09^{b}
C18:1n9C oleic (%)	22.33 ± 0.23^{a}	19.2 ± 0.44^{a}	17.9 ± 0.45^{c}
C18:2n6 linoleic (%)	1.73 ± 0.09^{b}	2.34 ± 0.18^{a}	2.23 ± 0.15^{a}
C20:1n11 cis eicosenoic (%)	3.34 ± 0.10^{a}	2.14 ± 0.11^{b}	1.79 ± 0.24^{b}
C20:3n6 homo-ã-linolenic (%)	0.23 ± 0.02^{b}	0.38 ± 0.02^{a}	0.31 ± 0.05^{a}
C20:4n3 eicosatetraenoic (%)	0.48 ± 0.02^{c}	0.68 ± 0.06^{b}	0.89 ± 0.04^{a}
C20:4n6 arachidonic (%)	3.75 ± 0.13^{c}	5.01 ± 0.19^{b}	6.23 ± 0.77^{a}
C20:5n3 eicosapentaenoic (%)	8.96 ± 0.55^{c}	10.97 ± 0.45^{b}	14.33 ± 0.18^{a}
C22:5n3 docosapentaenoic (%)	3.74 ± 0.21^{a}	3.78 ± 0.07^{a}	2.57 ± 0.14^{b}
C22:5n6 docosapentaenoic (%)	0.14 ± 0.02^{b}	0.24 ± 0.01^{b}	0.65 ± 0.18^{a}

[a,b,c]Within a row, means with different superscripts are significantly different. Captive belugas ($n = 3$) were fed no Fish Analog, Fish Analog and DHA-enriched Fish Analog for at least 6 weeks, then blood fatty acid profiles were determined. Data represent means $\pm$ SEM.

including body condition, hematology, serum biochemistry, and other variables in response to feeding Fish Analog to belugas. These parameters were chosen to provide a comprehensive evaluation of the response to Fish Analog in terms of behavior, heath, and nutrition, and this study represents the first trial to examine an alternative to feeding fish in such depth.

The most common measure of body condition is mass; however, many field studies have successfully used other measurements, such as girth, length, and blubber thickness as alternatives or in addition to mass [Doidge, 1990; Koopman et al., 1996; Konishi, 2006]. Obtaining mass measurements on belugas is difficult; thus, axillary girth and blubber thickness via ultrasound were used, and data collected in these studies were compared with a retrospective database for each animal. Girth and blubber thickness were positively correlated, indicating a close association of girth and blubber thickness. Values of these condition measurements were generally greatest during the spring season, which most likely reflect greater caloric intake during the earlier two seasons (fall and winter). Girth measurements were reduced in 2007 (year of Study 1), but this is more likely related to 2006 caloric intake levels than Fish Analog intake, because the general pattern of girth change in 2007 was similar to the other years, and caloric intake was reduced in 2006 compared with other years.

Although bottlenose dolphins readily accepted the Fish Analog diet, they were reported to have a decreased gastrointestinal transit time when fed Fish Analog exclusively (Eric Jensen, U.S. Navy Marine Mammal Program, Personal Communication). This was likely owing to a lack of structural components (e.g., bone and scales) that would be present in whole fish prey items, and thus reduced time for digestion and absorption by animals fed with Fish Analog. Therefore, when fed at 100% of the diet,

there was a requirement for increased caloric intake in order to maintain body weight. Observed changes in GI transit time in earlier research suggested the necessity to measure GI transit time in this study. Measurement of IPT in this trial demonstrated that there is individual animal variation. One animal had a shorter IPT when fed increasing amounts of Fish Analog. For all three whales together, however, IPT was not significantly correlated with the proportion of Fish Analog fed.

Enrichment of Fish Analog with DHA did not affect palatability when compared with the standard Fish Analog product. We observed no signs of gastric discomfort, behavior, or motivation related to Fish Analog product when the dietary changes were transitioned slowly. Transient changes in fecal quality (approximately 1 week) may be expected when including Fish Analog into the diet, along with potential changes in gastric fluid. However, when dietary changes were transitioned rapidly, some signs of gastric discomfort were observed, suggesting that slow diet transition is critical for maintaining good health. In addition, there were differences in the acceptance of the product between animals. Although all three study animals at Mystic immediately accepted the product, the animals located at Shedd Aquarium had various responses to the product during Study 3. Differences in acceptance may be a result of palatability differences between individual animals or animal and trainer motivation. Palatability of this product has been demonstrated in other species, including seals, sea lions, bottlenose dolphins, and penguins [Edwards et al., 2001; Molitoris et al., 1998].

Examination of nutrition and health status of the animals was accomplished by measuring serum clinical chemistry variables and immune function. Based on the null hypothesis that there was no difference between responses to fish or Fish Analog, we did not expect to see differences in serum clinical chemistry or immune function. In Study 1, Hct, Hgb, and MCH dropped as the inclusion of Fish Analog increased, although blood iron concentrations increased. The decrease in these values, in both study and non-study years, coincided with breeding season (February–April). Nevertheless, to rule out any association with consumption of the Fish Analog product, a follow-up trial was conducted which did not include breeding season (Study 2), and there were no changes in these variables after incorporation of 30% Fish Analog in the diet of belugas. Immune function for both lymphocyte subset percentages and proliferative responses to T and B cell mitogens (Concanavilin A and LPS) were variable for individual animals. Variability in immune function for individual animals is expected owing to a number of factors, e.g., individual fitness, gender, genetics. There were no observed effects on immune function when whales were fed higher levels of Fish Analog, except for higher proliferative responses to LPS at 5 ìg/ml which also increased with increasing diet percentages. Although the number of B cells did not change significantly, the Fish Analog may have increased responsiveness of these cells. These effects were not seen at higher doses of mitogen (10 ìg/ml), which may have been masked owing to the higher dose response to the mitogen alone.

Blood fatty acids generally reflect short-term dietary intake, whereas adipose (e.g., blubber) fatty acid profiles generally reflect longer-term dietary intake [Katan et al., 1997]. There are several publications concerning beluga blubber fatty acid profiles [e.g., Dahl et al., 2000], but none documenting blood fatty acids. For this trial, in which Fish Analog was fed for a relatively short period of time, blood fatty acids were the most appropriate measure. Compared with their wild counterparts, aquarium belugas had reduced concentrations of blood n9 fatty acids, as well as

reduced concentrations of DHA. Feeding Fish Analog to aquarium belugas further reduced n9 fatty acids. This may suggest a need for n9 enrichment of this product or in the beluga diets to achieve profiles similar to that of wild animals. The sample size for wild animals is small and from only one season, so further research will be needed.

Enrichment of Fish Analog with DHA or other long chain n3 PUFA may be of value to marine species that have evolved to eat DHA-enriched prey items. It is unknown whether there is an essential dietary requirement for DHA in marine mammals, but it is clear that feeder fish contain DHA. However, there are substantial differences between the DHA concentration of feeder fish. For example, of the total fatty acids, herring from this trial contained 20.2% DHA, whereas mackerel contained 8.6% DHA. However, in Study 2, when Fish Analog was enriched with DHA, blood DHA was not increased, although its precursor EPA was enriched. Because the precursor for EPA (i.e., linolenic acid) was not enriched, these data suggest that DHA was converted to EPA, although this hypothesis remains to be tested.

Surprisingly, IGF-I concentrations increased in summer following a decrease in intake. In most species IGF-I is highly responsive to nutrient intake and generally decreases with reduced intake [Rausch et al., 2002]. These data may suggest that either there is an underlying seasonal response of IGF-I that is greater than the response typically observed with changes in intake or that the response of IGF-I lags changes in intake in belugas [Richmond et al., in preparation]. The greater GH and lower IGF-I concentration in 2007 compared with 2006 may account for the reduced blubber stores observed in 2007, because increased concentrations of GH are associated with increase lipolysis and decreased lipogenesis resulting in decreased stored energy in adipose [Kersten, 2001].

As inclusion rate of Fish Analog increased, GH concentration increased and IGF-I decreased. Typically, this pattern of response is indicative of reduced nutrient intake or nutritional stress [Thissen et al., 1994; Richmond et al., 2008]. Although the overall changes in GH and IGF-I observed with increasing inclusion may suggest that the animals metabolic needs were not met, alternatively, these data may reflect the normal pattern of hormonal change owing to seasonal changes in intake [Richmond et al., in preparation]. In support of the latter hypothesis in the second study when animals were abruptly fed an increased percentage of Fish Analog, GH concentration remained constant and IGF-I concentration decreased only slightly. This means that the ''nutritional stress'' observed during the first experiment was likely an artifact resulting from changes in nutrient intake with season [Richmond et al., in preparation] and not a result of metabolic stress owing to the Fish Analog diet. Because this study was designed to match the energetic and nutrient intake pattern (including seasonal change in nutrient intake) of earlier years, it is not possible to discriminate among these variables. Further research is needed to evaluate the confounding effect of season and intake on metabolic hormones.

In conclusion, the animals responded well to a slow transition to feeding the Fish Analog product (up to 50% diet) in terms of behavioral motivation, IPT, blood chemistry profiles, and immune function as the introduction/transition period was adequate. This study indicates that axillary girth measurements or blubber thickness may be used as an indicator of body condition in belugas, and are reflective of caloric intake in the months before the measurement. Blood fatty acid profiles varied between aquarium and wild belugas, and dietary DHA enrichment resulted in enrichment of blood n3 fatty acids, and in particular, enrichment of EPA.

ACKNOWLEDGMENTS

We acknowledge the efforts of Kent Lanter in assisting with preparation and feeding of Fish Analog and Edmund Kadyszewski, Pfizer Statistician, for his consultation on the appropriate statistical analyses. We thank the Mystic Aquarium whale team for their time and efforts in preparing, feeding, and recording the data for this study, the Mystic Aquarium Veterinary staff for collecting and processing blood samples, and the Shedd Aquarium Marine Mammal Team for collaborating on the palatability portion of the study. We thank Jeffrey Stott (University of CA, Davis) for the antibodies to cetacean CD2 and CD19 and Sandy Casinghino, Erin Hisrich, and Tracey Spoon, PhD (MAIFE) for carrying out the immunological analyses. Finally, the authors thank Rod Hobbs, PhD, Carrie Goertz, MS, DVM, and Teri Rowles, DVM, PhD, for providing the wild beluga samples (Marine mammal research permits 782-1719 (NMML, Dr. Rod Hobbs) and 932-1489 (Marine Mammal Health and Stranding Response Program, Dr Teri Rowles). This work constitutes scientific contribution No. 188 from the Sea Research Foundation.

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