Exxon Valdez Oil Spill
Restoration Project Final Report

Hormonal, Viral and Nutritional Studies on Captive Harlequin Ducks

Restoration Project 02423
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Habitat and Restoration Division
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Preface

In September 2001, twenty five (25) harlequin ducks (*Histrionicus histrionicus*) were collected from Montague Island, Prince William Sound for an *Exxon Valdez* Oil Spill (EVOS) funded study that was designed to address why this species has not recovered since the1989 oil spill. Shortly after the arrival of the ducks, several ducks presented with lameness and acute hemorrhage in the muscles and gastrointestinal tract and our veterinarian staff submitted sera samples for analysis, which indicated a possible reovirus. The original plan was to release these birds back to Montague Island, but after these findings, the birds were deemed unreleasable.

The reports contained herein are projects that arose from the Alaska SeaLife Center scientific staff that were facilitating the original project. The ideas for these projects were directly related to the ducks, and came from our knowledge of what challenges they presented us with as we husbanded free-ranging sea ducks. These studies have provided more insight than we could ever have imagined if we had the opportunity to forward plan. They have not only given us insight into captive sea ducks, but also into the free-ranging population and some of the problems that may have contributed to keeping the population at lower than optimal levels.

The format of this report is three manuscripts that are soon to be submitted to peer-reviewed international journals. Reprints from the published articles will be forwarded to the Trustee Council when they are ready.

Variables Affecting Corticosterone Levels in Captive Harlequin Ducks (*Histrionicus histrionicus)*

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Abstract

Little is known about baseline concentrations of adrenal hormones and hormonal responses to stress in sea ducks, although significant population declines documented in several species within the tribe Mergini suggest that sea ducks may be exposed to increased levels of environmental stress. Such declines also have been observed in geographically distinct harlequin duck populations, including those in eastern North America, and in areas oiled by the Exxon Valdez in Prince William Sound, Alaska. We characterized baseline corticosterone concentrations in captive harlequin duck females with the goal of developing methods to evaluate stress levels in individuals and populations in the wild. We determined natural circadian patterns of circulating corticosterone, performed an adrenocorticotropic hormone (ACTH) challenge to evaluate adrenal function, and investigated correlations between several variables (e.g. diet, weight, handling time etc) and serum hormone concentrations. Cosyntropin, a synthetic ACTH, was used in the challenge studies with an intramuscular dose of 0.25 mg per bird. Results of serial bleeds indicate that harlequin ducks exhibit circadian patterns of circulating corticosterone. Harlequin ducks responded to the ACTH challenge with an average of three-fold increase in serum corticosterone concentration approximately 90 min post injection. A negative correlation was found between body mass and corticosterone and between PCV values and corticosterone levels. No correlations between time since capture, restraint time, bumblefoot severity, diet, sampling order, amount of blood withdrawn and corticosterone levels were found.

Introduction

Adrenal glucocorticoid (GC) hormones, cortisol and corticosterone, are secreted by the adrenal cortex and stimulate gluconeogenesis in the liver, exert profound effects in the mobilization of energy, as well as play important roles in growth and development in birds and mammals (Cahill, 1971; Thompson and Lippman, 1974). GCs, popularly called "stress" hormones, also influence many physiological processes such that an animal can more readily adapt to and function in situations that the animal perceives as stressful. Of the two GCs listed, corticosterone has been established as the primary circulatory GC in birds (Holms & Phillips, 1976). Several factors have been found to increase corticosterone concentrations in birds, such as: insufficient food and water (Rees *et al.*, 1985; Harvey and Hall, 1990; Kontecka *et al.*, 1999), capture and handling (Wingfield *et al.*, 1992; Romero *et al.*, 1997; Gratto-Trevor *et al.*, 1998; Silverin, 1998), exercise (Harvey and Phillips, 1982), social rank (Schwabl *et al.*, 1988; Nunez-de La Mora *et al.*, 1996), and heat stress (Edens and Siegel, 1975). GC increases allow a shift to a physiologic state that favors energy diverted from processes that are not essential for immediate survival, and directs energy toward those processes that favor rapid response to the environmental change or stressor (Kacsoh 2000, St. Aubin, 2001). In birds, effects of chronic or prolonged increase in circulating GCs include reduction in size of the ovary (Petite and Etches, 1991) and testes (Gross *et al.* 1980), growth inhibition (Davison *et al.*, 1980), a reduction in antibody response to an antigen (Gross *et al.*, 1980), and increased salt gland activity (Phillips, 1968; Holmes, 1972).

Living organisms exhibit persistent rhythms with circadian (daily), tidal or lunar (monthly), and circannual (yearly) periods (Leland and Edmunds 1988). Studies have shown that basal plasma corticosteroid concentrations exhibit seasonal or circannual scales for several species of birds (Sato & George 1973; Wilson *et al.,* 1982; Silverin 1986; Westerhof *et al.*, 1994; Romero and Wingfield, 1998; Romero and Remage-Healey, 2000). Astheimer et al. (1994) showed that the circannual rhythm in one arctic bird species was strongly correlated with season. The main environmental cue or *zeitgeber* of GC rhythmicity in bird species studied to date is photoperiod (Daan & Gwinner, 1989).

In this study, we sought to investigate circadian GC rhythms in female harlequin ducks. While most birds and mammals are thought to have a circadian pattern of circulating GC concentrations, this pattern has not been demonstrated for sea ducks. Establishment of a typical circadian pattern for harlequin ducks allows investigators to better evaluate differences between GC concentrations in captive or free-ranging sea ducks. The objectives of the study were (1) to evaluate adrenal function in harlequin ducks by determining baseline circadian patterns of GCs, (2) to establish the maximum output of corticosterone in harlequin ducks by using an ACTH challenge, (3) to investigate the lag-time between a stressor (exogenous ACTH) and the maximum stress response (measured as the peak corticosterone concentration), and (4) to investigate the relationship between corticosterone levels and other variables (*i.e.* diet, body weight, severity of bumble foot, packed cell volume, heterophil/lymphocyte ratio, time since capture, restraint time (handling time), sampling order, bleeding site, and percent of blood volume withdrawn.

Materials and Methods

Birds and Housing

The harlequin ducks (n=12; 11 females, 1 male) were caught in Prince William Sound Alaska, in September of 2001. The average weight of the birds used in the study was 533 g (range 491.5 to 613.1 g). The ducks were housed in outdoor enclosures under ambient conditions at the Alaska SeaLife Center, located in Seward, Alaska (60° 69' N; 149° 26' W). Each enclosure had a floor space of 8.7 m^2 and a circular saltwater pool with an area of 4.7 m^2 with six animals per enclosure. Half of the birds were fed a diet of Atlantic silversides (*Menidia menidia*) and the other half received silversides plus Antarctic krill (*Euphausia superba*) with multivitamin supplementation (see manuscript by Tuomi et al).

Sampling Regime & Blood Collection

Blood sampling

All blood samples were drawn via jugular, right or left brachial, or tarsal veinipuncture utilizing a sterile 1 cc syringe and a 25 gauge needle. During each study, all samples were stored refrigerated prior to processing. At processing, blood samples were centrifuged for 10 minutes using a TRIAC centrifuge (Clay Adams Brand®, Becton Dickinson Company) at a maximum speed of 10,400 rpm. Serum was harvested from the samples and stored at -80° C until analysis.

Circadian Rhythms

Harlequin ducks were split into two groups of six and sampled concurrently every four hours over a 24-hour period. At the time of the study (mid-April), ambient light/dark was 14L/10D. 15 minutes prior to sampling, the birds were herded into a holding pen and loaded into individual veterinary transport kennels. An average of 3.9 ml (range: 3.5 to 4.1 ml) whole blood was collected per bird during the study period. The total amount of blood collected per bird was consistently below the recommended amount (Dein 1984) of < 1 % of body mass per 24 hours (range 0.43 to 0.85 %).

ACTH-Challenge

Six weeks after the circadian pattern blood samples were collected, 17 birds were used to evaluate adrenal function. Birds in the treatment group (n=12) received 0.5 ml (25 mg) I.M. synthetic adrenocotropic hormone (ACTH; Cortrosyn®, Organon Inc., West Orange, New Jersey, USA). Control animals (n=5) received an identical volume of sterile saline. For individual baseline ("time 0") samples, 0.2 - 0.7 ml was drawn just prior to, and serial samples drawn at 30, 60, 90, 120, 180, and 240 minutes following, ACTH administration.

Radioimmunoassays (RIA)

A double antibody RIA (ImmuChem™ Double Antibody Corticosterone ^{125}I RIA Kit, ICN Biomedicals, Inc., Costa Mesa, CA) was validated for use in harlequin ducks. Parallelism was tested by comparing a 50 µl pool of harlequin duck serum added to the kit standard calibrators to the log-logit standard curve (Rodbard 1974). The slopes differed by less than 10 % and were considered functionally identical. All serum samples were initially run at a dilution of 1:100 in the assay buffer provided in the kit. Inter-assay and intra-assay variations were 7.6 % and 4.7 % for the circadian rhythms study and 12.6 % and 5.3 % for the ACTH study, respectively.

Other Variables Affecting Corticosterone Levels in Harlequin Ducks

As part of the circadian rhythm trials, the correlation between serum corticosterone concentrations and the following variables were also investigated: (*a*) diet, (*b*) severity of the condition known as bumblefoot, (*c*) hematocrit/PCV (Packed Cell Volume), (*d*) heterophil/lymphocyte ratio, (*e*) time since capture, restraint duration, sampling order, bleeding site, and (*f*) percent of blood volume withdrawn. These variables were only investigated during the circadian rhythm study since the introduction of an exogenous stressor (e.g. artificial ACTH) negates naturally occurring fluctuations in serum corticosterone. The one exception was the heterophil/lymphocyte ratio to serum corticosterone because this ratio is independent of causal mechanisms for elevated stress hormones.

a. Diet

A simultaneous study at the ASLC investigated the effects of a mixed diet with multi-vitamin supplements versus a single-food item diet without supplemental vitamins on the health of harlequin ducks (see report by Tuomi et al.). In this study, we compared serum corticosterone concentrations in animals undergoing the two diet regimes.

b. Bumblefoot severity

Bumblefoot is a common affliction in captive seabirds and waterfowl, which manifests itself as inflammatory and infectious foci in the feet. If left untreated, bumblefoot may progress to a lethal systemic illness (Smith-Ruiz 1997). Causes of bumblefoot include injury to the foot, inactivity (inability to fly, Heidenrich 1995), inadequate diet, depressed immune system, or inadequate substrate in the holding facility (Smith-Ruiz 1997). We followed the classification scheme for bumblefoot severity in which the least severe case is assigned a "one" and the most severe a "seven" (WildCare, San Rafael, CA). Average corticosterone levels were compared among birds showing various levels of bumblefoot and birds with no symptoms (*i.e.* "0")

c. Packed Cell Volume (PCV)

Hematocrit or PCV (Packed Cell Volume) counts were performed periodically to ensure that the ducks did not become anemic. Ducks with PCV values below 30 % were withdrawn from the study. The correlation between PCV and corticosterone concentration was analyzed.

d. Heterophil/Lymphocyte Ratio

The ratio of heterophils to lymphocytes was compared with serum corticosterone concentration.

e. Time Since Capture, Restraint Duration, Sampling Order and Bleeding Site

The effects of capture, restraint time, sampling order and bleeding site on serum corticosterone concentration were analyzed to determine if manipulation of the animals contributed to data outcome. Time since capture was defined as the time lapse between first capture in the pens and the time of the blood draw. Restraint duration was defined as the time between the removal of the ducks from the individual kennels and the actual blood draw. Sampling order refers to the sampling order of each individual at each blood draw. Bleeding site refers to which vein was used to withdraw blood (e.g. jugular, brachial, or tarsal vein) or the site of venipuncture.

f. Total blood withdrawn –Absolute and Relative

The total amount of blood drawn (ml) over the 24 hour circadian study sampling period was compared to serum corticosterone concentration at the last sampling event for each individual. The relative total amount of blood drawn was defined as the total amount of blood sample (ml) over the 24 hour time period per gram body mass and the resultant value was compared to serum corticosterone concentration at the last sampling event for each study individual.

Data Analysis

Statistical analyses were performed using SAS® and SigmaPlot software packages. Outliers (values greater than two standard deviations from mean) were omitted from the comparisons, and nonparametric tests were applied to data sets that were not showing normal distributions. A repeated measures analysis was used to evaluate the variation of corticosterone levels in the circadian rhythm study. Pearson and Spearman correlation analyses were used to examine the relationships between corticosterone concentrations and body mass, PCV, total volume of blood withdrawn, and the heterophil:lymphocyte ratio. An analysis of variance (ANOVA) was used to compare corticosterone concentrations relative to time since capture, restraint time, blood collection site, sampling order, and severity of bumblefoot. Corticosterone

concentrations between diet groups were compared with a Student's *t*-test. All tests used a significance level of 0.05.

Results

Circadian Rhythms

For female harlequin ducks (n=11), the average corticosterone concentration did not differ significantly between the different sampling times (F= 1.079, P= 0.3837). However, a peak in serum corticosterone levels occurred for eight of the females and for the single male during hours of darkness (Table 1 and Figure 1). An example of the circadian pattern of corticosterone production for the male and one female is shown in figure 2.

ACTH – Challenge

Time 0 serum corticosterone level did not differ significantly (t = 0.266, P=0.217) between the experimental and control group. The control birds' average serum corticosterone values remained at the baseline levels (24.1 to 41.0 ng/ml) throughout the trial.

For female harlequin ducks, the average peak corticosterone concentration occurred 90 minutes post ACTH administration. Eight female ducks exhibited serum corticosterone peaks at 90 minutes post injection, while 3 females and one male exhibited peaks at 120 minutes (Figure 3). Mean peak corticosterone concentration was approximately 3 times higher than baseline, or time 0, serum values (mean: 146.2 ng/ml, range: 109.8 to 228.6 ng/ml). The final value (i.e. 240 minute sample) for the experimental birds did not differ significantly (t = -1.277, P = 0.217) from time 0, indicating that serum corticosterone had returned to baseline concentrations 240 minutes post-ACTH administration.

Other Variables Investigated

Mean bird handling time was 4 min 47 s (median = 4 min, SD = 3 min 28 s). On average 0.57 ml of blood (range 0.10 to 0.75 ml) were obtained per sample, for an average of 3.96 ml (range 2.05 to 4.10 ml) of blood withdrawn from each duck during the study. The relative amount of blood withdrawn was < 10 % of blood volume/24 hours (mean: 6.7 % of blood volume, range: 4.3 to 8.5 %).

Average time between capture and bleed was 16 minutes 18 seconds (median = 16 min, SD = 9 min 24 s, range 2 to 44 min). Mean pre-bleed handling time (*i.e.* time between when a bird was taken out of the kennel until the blood sample was obtained) was 3 min and 36 s (median = 3 min, SD = 2 min 36 s, range 1 to 12 min). The ducks exhibited bumblefoot stages ("grades") between zero and three (modal = 2) out of the maximal seven with the seventh grade being the most severe.

No correlation between time since capture, restraint time, bumblefoot severity, diet, sampling order, absolute and relative amount of blood volume withdrawn and corticosterone levels were found for the harlequin ducks in the study. There was no significant relationships between the heterophil/lymphocyte ratio and corticosterone concentration in female harlequin ducks. A negative correlation was found between body

mass and corticosterone (r_P = -0.2300, p = 0.0249; Figure 4) and between PCV values and corticosterone levels (r_S = – 0.4613, p = 0.0005; Figure 5).

Discussion

Circadian Rhythms

Circadian patterns in corticosterone production in birds have been shown to typically peak in the in the early morning prior to dawn with lowest levels observed at late afternoon to dusk (Beuving and Vonder 1978, Nelson 1997; Westerhof *et al.*,1994). The pattern observed in the harlequin ducks was similar with the lowest concentrations in mid to late afternoon. The male duck had a pattern that was slightly offset from the female ducks. The time of the year at which the study was conducted may have altered the circadian pattern. Studies on harbor seals (*Phoca vitulina*) have shown the presence of a circadian pattern in corticosterone levels during the summer, when sufficient sunlight is available to act as an efficient environmental cue or zeitgeber, and an absence of a pattern during the winter months, when insufficient sunlight failed to produce a clear cyclical pattern (Dan & Gwinner 1989; Oki and Atkinson 2003). The harlequin duck females exhibited peaks at different times, with most individuals peaking at 05:00, and the remaining individuals peaking earlier in the night. It may be that there are individual differences in sensitivity to sunlight as the *zeitgeber* or that spring months may be a period of adjustment, with resulting presence or absence of a clear daily pattern of hormone production in all ducks at the time of sampling (April). Alternatively, some individuals may have more rapidly adjusted to the upcoming long arctic summer in terms of regulating hormone production. It is therefore recommended to repeat this study in mid - summer and winter months.

The serum corticosterone concentrations found in this study were overall higher than what Perfito et al. (2002) found for the same species. Although the two studies were performed at roughly the same time of year (late March to May), the differences observed may be explained by latitudinal differences in the study sites (approximately 13 degrees latitude). The geographic differences result in shifts in light intensity, producing a disparity in stimuli of the entrainment mechanism. Other researchers have found variation in basal serum corticosterone levels as well as disparate intensity of stress responses for different populations and breeding sites during the same time of year (Romero et al., 1998). It may be that the differences observed are due to factors specific to populations, rather than environmental factors. Alternatively, the elevated levels could be caused by a slightly longer delay between the capture and bleeding of the animals in this study, thereby yielding higher serum corticosterone concentrations. Many studies have shown a rapid stress response in birds, suggesting that sampling time after handling may be an important factor (Harvey *et al.* 1980, Klingbeil 1985).

ACTH – Challenge

The observed 3-fold increase of basal corticosterone levels following the introduction of exogenous ACTH agrees with results from American black ducks (*Anas rubripes*) in a similar study by Spelman et al. (1995). Other avian ACTH studies have found an even greater relative increase, from a 5-fold increase in Sandhill cranes (*Grus canadensis pratensis*; Ludders et al., 1998) to a 9-fold increase in domestic ducks (*Anas*

platyrhynchos; Harvey et al., 1980). The time lag between the stressor and the peak concentrations in our study was also similar to that found in American black ducks (120 minutes), but differs from that found in some other avian taxa: 270 minutes for Moluccan Cockatoos (*Cacatua moluccensis*; Walsh et al. 1985) and 60-90 minutes for bald eagles (*Haliaeetus leucocephalus*; Zenoble et al. 1985). The sharp increase in and the subsequent gradual tapering off of corticosterone concentration observed in this study contrasts with the post ACTH-corticosterone profile for wild Gambells white-crowned sparrow (*Zonotrichia leucophrys gambelii*) observed by Astheimer *et al.* (1994), who found that a steep increase in corticosterone during the initial 10 minutes was followed by a 2 hour plateu at elevated levels (for breeding males).

The absence of increased serum corticosterone in the harlequin duck control group seems to indicate that these birds were not stressed by handling or blood-draws. These results differ from those from studies on other avian species, which observed increased corticosterone levels as a result of handling stress (Harvey et al. 1980, Klingbeil 1985, Beuving and Vonder 1986, Gratto-Trevor et al. 1991, Wingfield et al. 1992 (Harvey et al. 1980; Wingfield et al. 1982; Zenoble et al. 1985; Gratto-Trevor et al. 1991; Astheimer et al., 1994; Romero et al. 1997; Silverin, 1998, Perfito et al. 2002)). This could probably be explained by the habituation of our birds to the frequent handling which they were exposed to on a weekly basis for six months prior to the present study. El Halawani *et al.* (1973) observed habituation in the corticosterone response to other stressors in turkeys over several weeks.

Other Variables Investigated

The lack of a relationship between bumblefoot severity and corticosterone concentration may have been because our birds showed only mild degrees of infection which would not severely impact their health and, consequently, their basal corticosterone concentrations were not affected.

Kontecka et al. (1999) found a significant increase in hematocrits in only one (water restricted for two days) out of four groups of pekin ducks (*Anas platyrhynchos*) subjected to various stressors. The strong negative correlation observed in the present study suggests a fairly robust stress response as an individual becomes progressively more anemic. Similarly, the negative correlation between body weight and serum corticosterone level could be related to the general health status of the individual.

Other than increased corticosterone levels, changes in leukocyte relationships (*i.e.* an increase in heterophil count and decrease in lymphocyte count or heterophil/lymphocyte ratio have been used as a physiological indication of stress in birds (Mitchell et al., 1992, Maxwell, 1993), with some authors claiming the heterophil/lymphocyte ratio as a more reliable indicator of stress than serum corticosterone concentrations (Maxwell and Robertsson 1995). Kontecka et al. (1999) found a strong relationship between elevated corticosterone levels and an increased ratio of heterophils to lymphocytes in pekin ducks. Corticosterone has been shown to increase the heterophil/lymphocyte ratio in chickens (Gross et al. 1980). There was an absence of a significant statistical relationship between heterophil:lymphocyte ratio and serum corticosterone concentration in harlequin ducks in the present study, which may be a reflection of a relatively small sample size.

The absence of a relationship between sampling order and blood corticosterone concentration agree with previous findings for chickens (Lagadic et al., 1990; Littin and Cockrem, 2001). The absence of a relationship between total blood withdrawn is probably best explained by the relatively low volumes withdrawn. No physiological stress reaction due to blood-loss were expected at these low levels.

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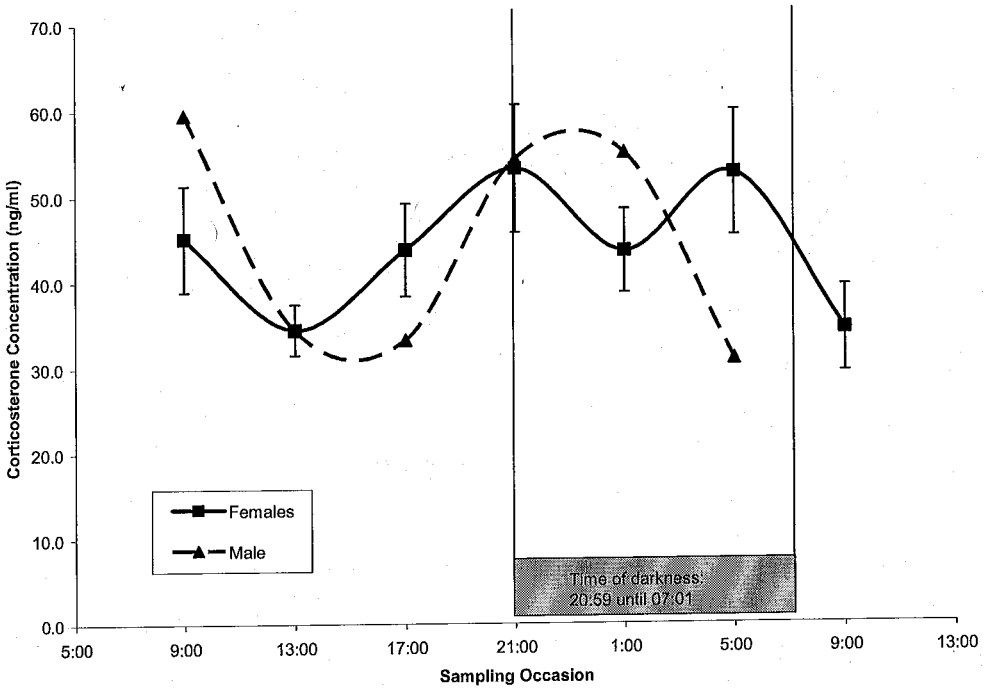

Fig. 1 Variation in corticosterone levels over a 24-hour period for eleven female and one male harlequin duck (*Histrionicus histrionicus*).

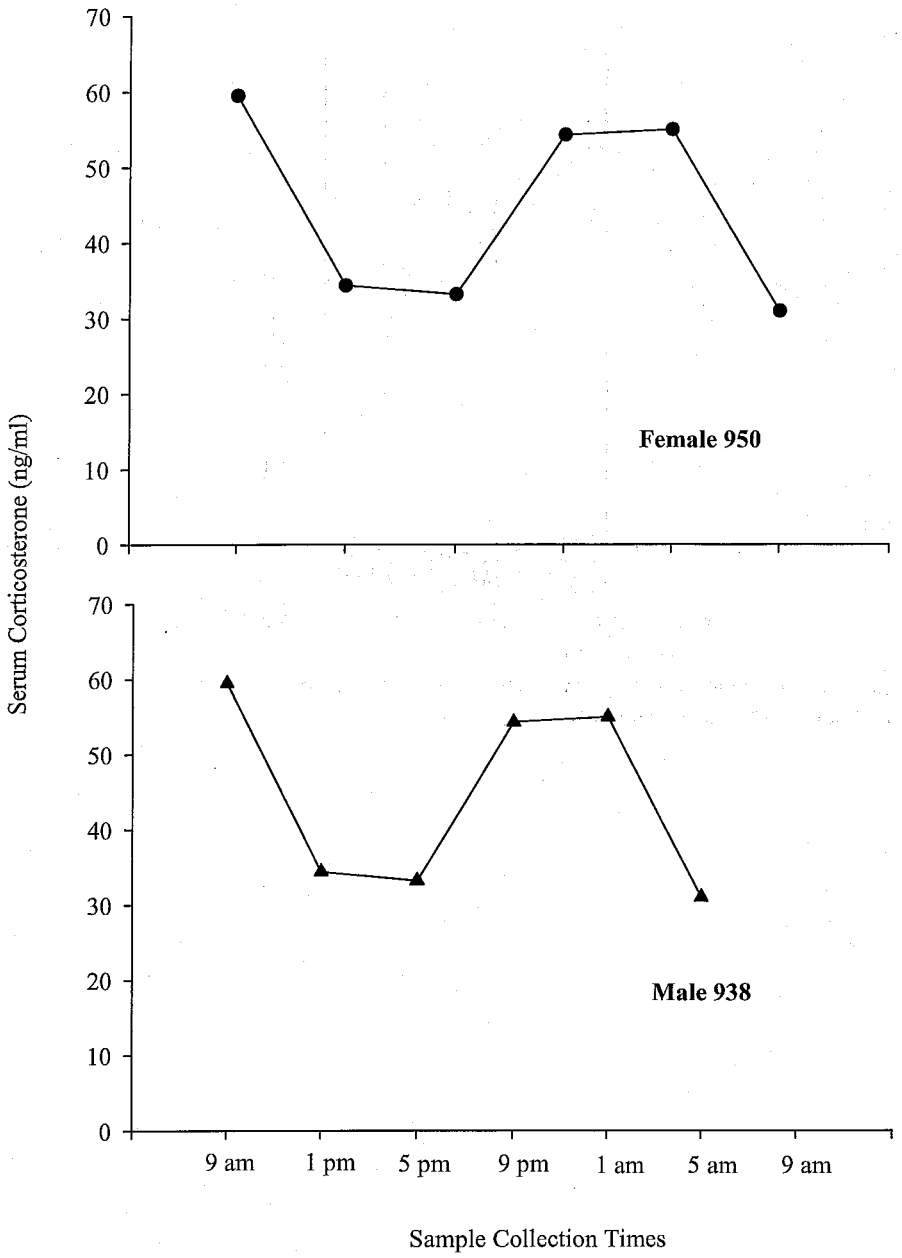

Fig 2. Comparison of the circadian pattern of corticosterone production in a male and a female harlequin duck

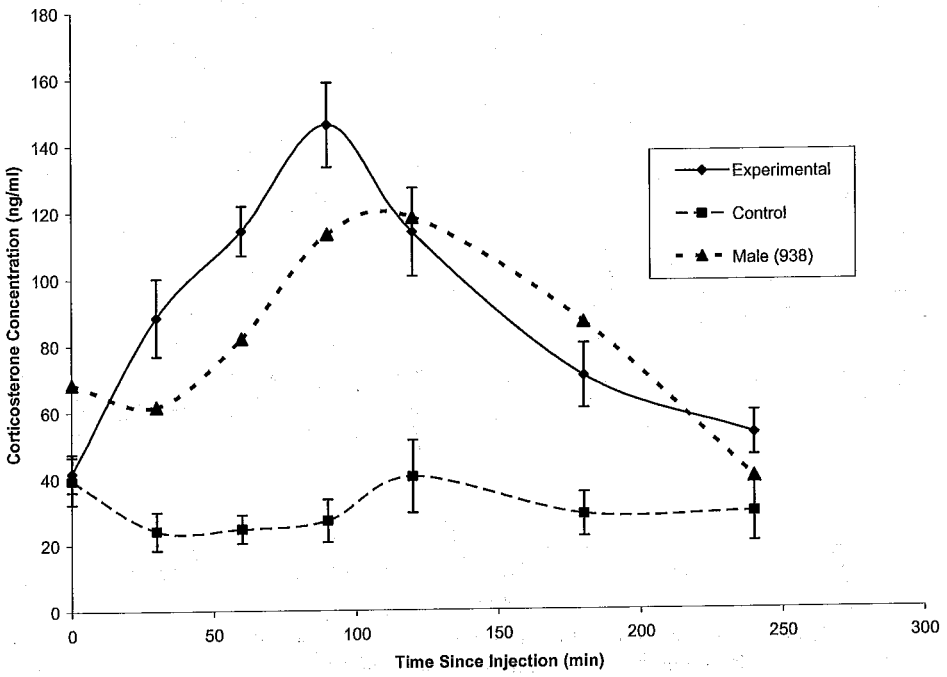

Fig. 3. Changes in corticosterone levels in Harlequin ducks, *Histrionicus histrionicus*, (16 female, 1 male) after injection of artificial ACTH or saline.

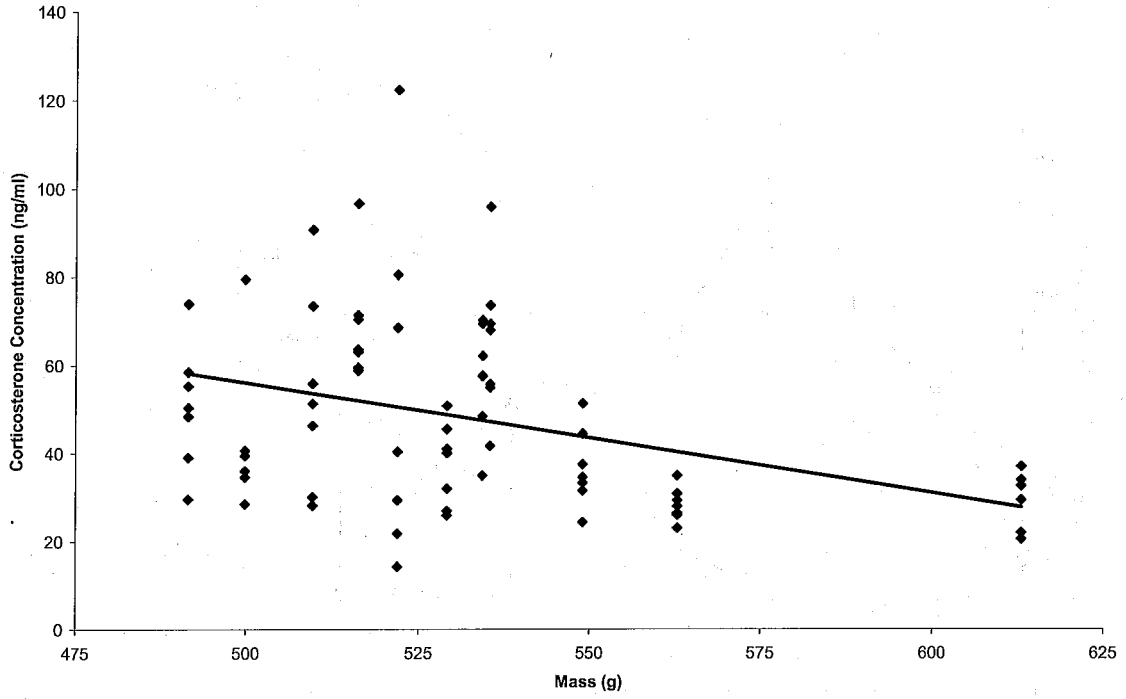

Fig 4. Corticosterone concentration vs mass in female Harlequin ducks (*Histrionicus histrionicus)*

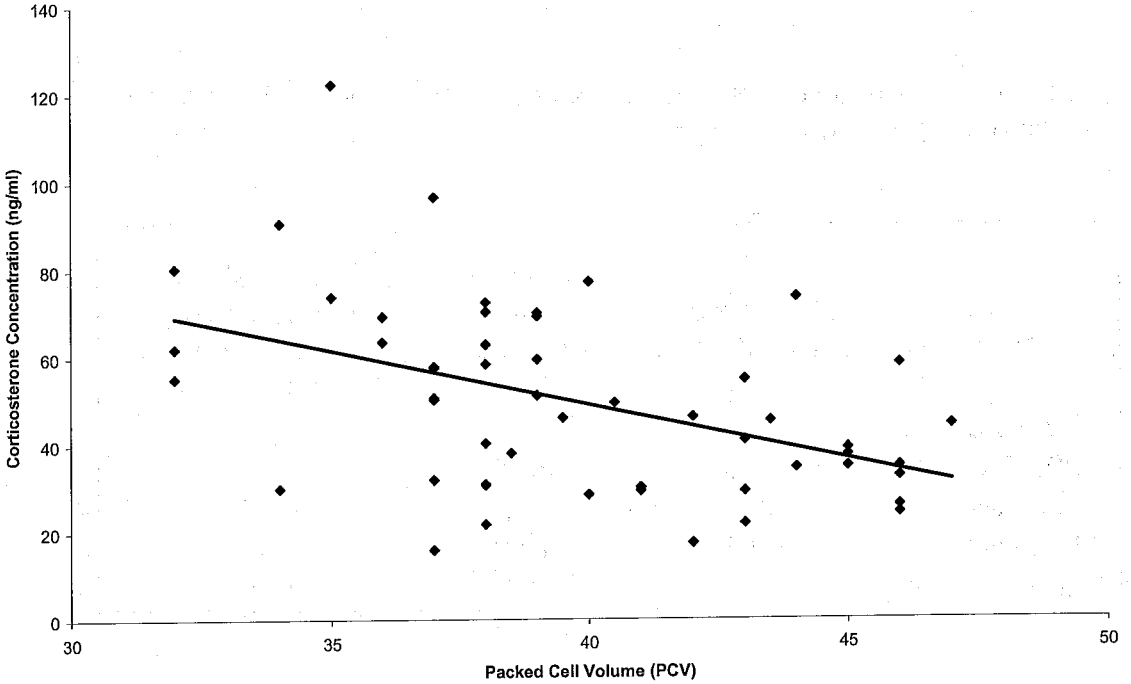

Fig. 5. Corticosterone concentration vs PCV values in female Harlequin ducks (*Histrionicus histrionicus*)

Table 1. Distribution of corticosterone peaks for 11 female harlequin ducks during a 24-hour period

No Pattern	n	Mean Peak Concentration	% of Total Group	% of Total that Peaked During Darkness
	3	N/A	27.3	N/A
Patterns				
5:00 AM	4	86.8	36.4	57.1
1:00 AM	1	51.3	9.1	14.3
9:00 PM	2	60.1	18.2	18.2
5:00 PM	1	73.4	9.1	N/A

Harlequin duck (*Histrionicus histrionicus*) reoviruses: lessons from captive studies and implications to wild populations in Alaska

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Abstract

Tenosynovitis and osteoarthritis were observed in 12 of 25 harlequin ducks (*Histrionicus histrionicus*) that were captured from the wild in Prince William Sound, Alaska, and held in captivity at the Alaska SeaLife Center, Seward, Alaska. Signs and lesions, closely resembling those described for primary reoviral arthritis/tenosynovitis in chickens, were first observed 3 weeks after the birds were captured and transported into captivity. Serum samples from ducks with clinical signs and lesions tested positive for reovirus antibodies, and a 4-fold increase in titer was observed in one individual that was bled multiple times during the course of illness. Histologic lesions consistent with primary reovirus infection were observed in the clinically affected captive ducks, and an experimental inoculation of five harlequin ducks with a reference arthritis reovirus (strain S1133) resulted in histologic lesions characteristic of primary reoviral arthritis. Reoviruses have not been reported as a disease agent in harlequin ducks before, and we tested serum samples collected from Prince William Sound to evaluate the presence of the virus in wild populations. Reovirus seroprevalence in wild harlequin ducks in Prince William Sound was found to be 38% during 1995-1996 (years of population decline), and 11% in 2002. Our findings indicate that reoviruses may be an endemic disease in harlequin ducks and that they associate with lesions similar to those described for reoviral arthritis in poultry. The significance of the higher seroprevalence found in Prince William Sound during the population declines in the mid 1990's remains unknown.

Key words: arthritis, harlequin duck, *Histrionicus histrionicus*, reovirus, tenosynovitis

Introduction

Harlequin ducks (*Histrionicus histrionicus*) inhabit a wide geographic range in the palearctic and nearctic regions and occur in four distinct populations (Bellrose 1980, Amirault 1997). The western North American population consists of an estimated 200,000 to 300,000 individuals and is dispersed widely along the Pacific coast from Alaska to California, with breeding areas ranging inland to the Rocky Mountain region of the northern United States. The long-term trend for the western population is unknown, but the endangered east coast population of North America has declined from historical numbers and now consists of fewer that 1,000 individuals.

The adult male harlequin duck has a striking appearance with a brightly colored blue and chestnut plumage with bold white markings on the head and body (Bellrose 1980). Females are uniformly brownish-grey. During most of the year, harlequin ducks are found in coastal marine environments, but unlike other northern see ducks, their nests are found inland near streams and are sheltered by shrubby vegetation, logs, trees, or river banks (Robertson and Goudie 1999). Feeding habits vary by season, but birds prefer shallow near-shore habitats even during the winter months. In the Prince William Sound, Alaska, harlequin ducks occur throughout the year in shallow near shore waters. In 1989, large numbers of harlequin ducks died in the western Prince William Sound after being exposed to oil spilled by the *Exxon Valdez* tanker (Piatt et al. 1990, Holland-

Bartels 2000). In the areas affected by the oil spill, numbers of harlequin ducks declined significantly during 1996 to 1998 and survival rates of females were lower as compared to the unoiled areas (Rosenberg and Petrula 1998, Esler et al. 2000). Recent results suggest that harlequin ducks continued to be exposed to oil at least through 1998 (Trust et al. 2000), and to date may not have fully recovered from the oil spill (*Exxon-Valdez* Oil Spill Trustee Council 1999).

Based on these results, controlled captive studies were designed and conducted during 2000-2002 to better understand the effects of long term chronic oil exposure to behavior and metabolism of female harlequin ducks, and ultimately, survival. In September 2001, 25 female harlequin ducks were captured from Prince William Sound and transported to the Alaska SeaLife Center to participate in studies evaluating potential mechanisms by which oil exposure can affect survival and establishing the relationship between oil exposure and cytochrome P450 induction, a hepatic enzyme associated with detoxification of oil. Approximately 3 weeks after arrival, three birds were observed with lameness and swelling of hock joints. Bacterial cultures were negative, and a primary viral etiology was suspected. Here, we describe findings from further laboratory studies conducted to characterize the lesions and evaluate viruses as the potential etiology for the observed disease syndrome in captive harlequin ducks. Furthermore, serologic screening of samples from harlequin ducks captured in Prince William Sound was conducted to determine if the potential etiologic agent was present in the wild population and therefore, would indicate that the newly discovered disease may be endemic for harlequin ducks in Alaska.

Case History

Bird capture and captive husbandry.

Harlequin ducks were captured on September 7 and 8, 2001, on Montague Island in an unoiled area in the western Prince William Sound. Twenty-five females determined to be in the after-second-year age class by bursal probing (Mather and Esler 1999) were transported to the Alaska SeaLife Center by a floatplane. During the trip, the birds were held in small animal kennels and released in three outdoor salt water pools (eight, eight, and seven bird each) immediately after arrival at the Alaska SeaLife Center. The birds were kept under ambient temperature and photoperiodic conditions, and left as undisturbed as possible to allow acclimation to the new environment. The birds were fed *ad libitum* during the daylight hours with a mixture of approximately 90% Antarctic krill (*Euphausia superba*) and 10% of Atlantic silversides (*Menidia menidia*). Food consumption per pen was estimated by weighing the food by type and pen before delivery and at the end of the day. Birds were dosed every other day with a multivitamin supplement (Sea Tab, Pacific Research Laboratories, Inc., El Cajon, CA) and inoculated IM once every two weeks with a vitamin K solution (Vitamin K_1 Injectable, 10 mg/ml, Neogen Corp., Lexington, KY). As part of the oil dosing study, birds were exposed to crude oil both externally and internally at various times between October 15, 2001 and February 8, 2002.

Clinical and postmortem findings in captive harlequin ducks.

Three ducks began to exhibit clinical signs approximately 3 weeks after arrival at the Alaska SeaLife Center. Signs included acute lameness and uni- or bilateral swelling of the hock and metatarsal joints. Aspirates from the swollen joints produced a clear, slightly hemorrhagic fluid that was negative in consequent bacterial cultures. Lameness and joint swellings resolved within a week but resulted in chronic osteomyelitis and/or fibrinous tenosynovitis of the gastrocnemius tendon. During the following months, nine additional birds exhibited similar clinical signs. Five of these birds died or were euthanized due to bacterial complications, and the rest recovered. Postmortem findings in the affected extremities included macroscopic and histologic lesions characteristic of chronic arthritis, chronic osteoarthritis, and pododermatitis, and were compatible with a primary reoviral disease (Figure 1).

Serology and experimental studies.

Serum samples were collected from seven birds (four clinically affected and three unaffected) between October 11 and November 28, 2001, during a period when clinical signs of lameness and swollen gastrocnemius tendon, hock joint, and metatarsal joint regions were observed. One individual was bled three times with an approximately 2 week and 1 month interval between the first and second and the second and third sample, respectively. Samples were tested with a standard microneutralization assay using a duck reovirus as a test antigen (Hollmén et al. 2002). All serum samples collected from the clinically affected birds and one sample from a bird that was not showing clinical sings tested positive for reovirus antibodies (titers $\geq$1:128) (Giambrone 1980). Seroconversion was documented in one individual that was bled three times with 2 week intervals. Two additional clinically unaffected birds were negative for antibodies.

In May 2002, eight ducks were transported to the National Wildlife Health Center (Madison, WI) for an experimental inoculation study. Birds were housed in 24" deep fresh water pools in BL-3 isolation units with a 12:12 hr light cycle and at ambient temperatures. The diet and vitamin supplementation was kept consistent with Alaska SeaLife Center procedures. Prior to the experiment, serum was collected and shipped to the Avian Health Laboratory at the Washington Sate University (Puyallup, WA) to be tested for antibodies against the avian arthritis reovirus using an ELISA assay. None of the samples showed titers and on June 5 five ducks were inoculated in right foot pad with 7×10^6 infectious units (determined by a $TCID_{50}$ calculation) of the avian arthritis virus (S1133, Spafas® Inc., Franklin, CT). Three ducks were kept as controls in a separate isolation room, and inoculated with saline injection in their right foot pad. All birds were monitored daily for food consumption and clinical signs of illness for a period of 18 days. Food consumption did not differ among the groups, and clinical signs were mild and consisted of soft tissue swelling in all of the inoculated footpads and around the hock joint in one individual. Serum samples were collected at 14 days and 18 days post inoculation and tested for antibodies against the avian arthritis virus at the Avian Health Laboratory, Washington State University. At 14 days post inoculation, two inoculated ducks had a moderate titer and three inoculated ducks had a low antibody titer by ELISA. At 18 days postinoculation, two inoculated ducks showed a moderate titer and two a low titer. One inoculated duck did not show a titer at 18 days postinoculation, and all samples

collected from control ducks were negative. At necropsy, mild thickening of foot pads was observed in the inoculated ducks, but not in the control ducks. Spleen was greatly enlarged (1×2 cm in size) in one duck. Samples from gastrocnemius, digital, and metatarsal tendons, footpad, tarsometatarsal and other affected joints, spleen, liver, heart, and intestine were collected for histopathologic evaluation and sent to be analyzed at the Northwest ZooPath (Snohomish, WA). Histological lesions observed in the inoculated ducks consisted of tenosynovitis, nonsuppurative pododermatitis, and nonsuppurative bursitis in three ducks. The enlarged spleen had moderately increased numbers of histiocytes and reactive plasma cells in the red pulp, reflecting antigenic stimulation and compatible with a viral infection. Two additional ducks inoculated with avian arthritis virus showed mild lesions pododermatitis and bursitis. Similar lesions were not seen in the control ducks.

Seroprevalence in wild harlequin ducks.

Serum samples collected from harlequin ducks in Prince William Sound, Alaska, in 1995-1996 (n=32) and 2002 (n=9) were tested for antibodies against a duck reovirus using a standard neutralization assay (Hollmén et al. 2002). Considering titers ≥1:128 as positive (Giambrone 1980), virus seroprevalence was greater (38%) in 1995-1996 and as compared to 2002 (11%) (Sokal and Rohlf 1995).

Discussion

Avian reoviruses are known to infect domestic chickens and turkeys with a worldwide distribution, and may also be prevalent in other avian species (McNulty 1993). In domesticated birds, reoviruses have been associated with a variety of disease syndromes including arthritis, tenosynovitis, malnutrition, enteritis, growth retardation, hepatitis, pericarditis, chronic respiratory disease, and sudden death syndrome (Rosenberger and Olson 1997). The wide variety of disease syndromes reflects the heterogeneity of viruses within the genus, and variation in virulence among strains may result in different disease manifestations even within the same host species. In recent years, reoviruses also have been linked to mortality events in wild avian species, common eiders (*Somateria mollissima*) (Hollmén et al. 2002)and American woodcock (*Scolopax minor*) (Docherty et al. 1994), but relatively little is known about pathogenicity and population effects of reoviruses in wild birds. Previous studies in wild birds have associated reoviruses with lymph tissue lesions, hepatitis, and emaciation, but characteristic arthritis/tenosynovitis lesions have not been previously reported in wild birds.

Viral arthritis was first detected in chickens in 1954 and reoviruses were identified as the etiological agent in 1957 (van der Heide 2000). Since then reoviral arthritis/tenosynovitis has been diagnosed in most areas of the world where poultry are raised and, of wild avian species, serologic evidence of arthritis/tenosynovitis virus has been found in pigeons in central Europe (Vindevogel et al. 1982).

We found clinical and serologic evidence of reoviral arthritis/tenosynovitis in wild and captive harlequin ducks in Alaska and, in addition, pathologic lesions characteristic of reoviral arthritis/tenosynovitis were reproduced in harlequin ducks inoculated with arthritis reovirus under controlled experimental conditions. The clinical

signs of lameness and arthritis were first noticed in the harlequin ducks at approximately 3 weeks after being transported from Prince William Sound into captivity. Our findings of serologic evidence of reoviral infections in wild harlequin ducks captured at Prince William Sound in the 1990's and in 2002 suggest that reoviruses may be an endemic disease in harlequin ducks and that the birds transported to the SeaLife Center were likely infected with latent viral infections at the time of capture. Previously, reoviral diseases have been linked increased stress associated with quarantine and translocation of birds, and the transport of harlequin ducks into captivity may have resulted in increased physiological stress and reactivation of latent infection (Rigby et al. 1981). The timing for the development of the clinical signs is compatible with previous reports describing manifestations of reovirus infections as a result of such transportation and quarantine stress.

In the Prince William Sound, the seroprevalence of reoviruses was higher in birds captured in 1995 and 1996 as compared to those captured in 2002. The potential links of reoviruses to population declines observed in the 1990's remain unknown. As increased environmental stress, concurrent infections, or other predisposing factors may play an important role in reoviral diseases by altering host susceptibility or viral pathogenicity, and virus prevalence and pathogenicity may have increased in the Prince William Sound during the time of the population declines in mid-1990s as a result of such factors. Our findings in captive and wild harlequin ducks emphasize the need to increase our understanding of infectious diseases in wild avian populations, especially those undergoing declines. Furthermore, diseases should continuously be considered as part of captive flock management programs and in translocation of animals between geographic locations.

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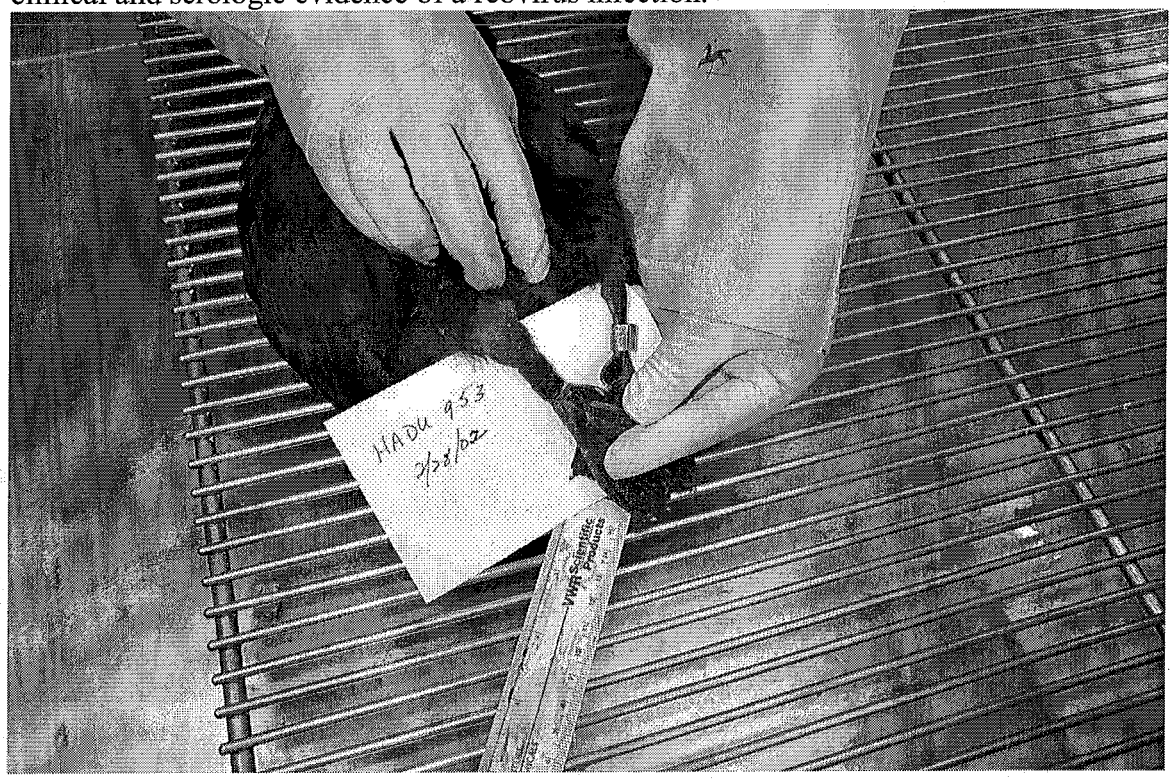
Figure 1. Chronic swelling around the hock joint in a captive harlequin duck exhibiting clinical and serologic evidence of a reovirus infection.



Effect of Diet on Hemostasis in Wild Caught Harlequin Ducks (*Histrionicus histrionicus)* during Captive Care

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Abstract

Acute onset of coagulopathy was observed in 7 of 46 wild caught Harlequin ducks held in captivity at the Alaska SeaLife Center (ASLC) for a study of the effects of low level crude oil exposure. Coagulopathy was seen in both control and oil exposed birds and was characterized by spontaneous intramuscular or gastrointestinal hemorrhage, epistaxis, hemoptosis and severe acute anemia. Four birds died acutely, and three additional birds treated with injectable vitamin K_1 recovered rapidly. No further bleeding tendencies were noted after intermittent vitamin K_1 supplementation was instituted. Previous reports have speculated on the role of vitamin K and E in blood clotting and their relationship to coagulopathies observed in captive piscivorous birds fed frozen fish diets (Nichols, 1989) and in humans ingesting high levels of vitamin E (Corrigan, 1974). To further study the associations between diet and the observed coagulopathies, vitamin K_1 supplementation was withdrawn from 17 birds after the completion of the oil exposure study, and the birds were randomly divided into two study groups. Each group was fed a study diet designed to mimic the nutritional conditions present at the time when the coagulopathy was first observed. The experimental group was offered a restricted diet of frozen Atlantic silversides (*Menidia menidia)* with no vitamin supplementation, and the control group was fed silversides and Antarctic krill (*Euphausia superba)* supplemented by multivitamin mineral tablets containing vitamin A and E.. Blood samples from each group were collected every 10 to 16 days and assayed for serum vitamin A (retinol), E (alpha-tocopherol) and K (phylloquinolone) levels. Levels of these vitamins were compared to those found in serum from wild harlequin ducks collected during the same time period and to samples collected from the captive birds prior to the diet experiments. Packed cell volumes (PCV), plasma prothrombin times and whole blood clotting times were also recorded for the captive birds. No mortalities or spontaneous bleeding occurred during the study period. Anemia (PCV <50% of pretrial values) occurred in 30% (5 of 17) of the birds with significantly lower values in the unsupplemented group and prolonged bleeding was observed at venipuncture sites. Whole blood clotting times were high compared to those reported for mammals and did not significantly vary between groups fed different diets. Plasma prothrombin times of all birds were variable and did not appear to be dependent on diet or vitamin K levels. Serum phylloquinolone concentrations in the wild harlequin ducks ranged from 0.30 to 3.64 ng/ml (mean ± SE = 1.59 ± 0.3 ng/ml) and serum phylloquinolone of all birds in captivity were 20 to 100 times higher than in the wild birds. Alpha-tocopherol levels in the wild birds ranged from 8.59 to 15.58 ng/ml (mean ± SE = 12.41 ± 0.7 ng/ml) and were similar to levels observed in pretrial, control animals, and post trial birds. However, alpha-tocopherol levels were significantly lower (7.89 to 10.97 ng/ml, mean ± SE = 9.12 ± 0.5 ng/ml) in the experimental birds after they had been held for 4 weeks on the unsupplemented silversides diet. Serum retinol concentrations in the wild ducks (0.91 to 1.60 ng/ml, mean ± SE = 1.20 ± 0.07 ng/ml) was not significantly different from that found in serum from the captive birds during or after the experiment, but the retinol levels in the pretrial group were significantly lower as compared to other samples.

Baseline data for the oil soluble vitamins have not previously been reported from wild harlequin ducks. These values should serve as a useful reference in establishing the need for and appropriate levels of supplementation of vitamin A, E and K in captive

waterfowl and in the analysis of suspected cases of vitamin deficiency or toxicity. Vitamin K and E supplementation should be provided at low levels in the form of commercially available multivitamin mineral tablets or in pelleted diets. If coagulopathy is observed, treatment with injectable vitamin K_1 (3 mg/kg intramuscularly) appears to be safe and effective. Incidence of observed coagulopathy appears to be reduced by prophylactic administration of vitamin K_1 at a rate of 1 mg/kg intramuscularly every 2 weeks, but lower doses and less frequent administration may be adequate and should be explored.

Key Words: alpha-tocopherol, coagulopathy, harlequin duck, phylloquinolone, retinol, vitamin A, vitamin E, vitamin K.

Introduction

Findings in captive birds in 2000-2002

Since September 2000, a total of 46 wild caught female harlequin ducks have been housed over winter at the Alaska SeaLife Center as part of a study of low level crude oil exposure (Esler,2000). Twenty-one of these birds were captured from Prince William Sound in September 2000, housed in outdoor pens with salt water pools and fed *ad libitum* a diet of commercially available frozen Atlantic silversides (*Menidia menidia)*. In the fall of 2000 the birds were supplemented with intramuscular injection of vitamin B complex (Vitamin B complex 150©, AMVET Scientific Products, Yaphank, NY) 0.05 ml once per week and vitamin E-selenium (Bo-Se, Schering, 1 mg selenium as sodium selenium and Vitamin E 68 IU/ ml) 0.02 ml once every two weeks. A severe coagulopathy was observed in 5 of these 21 birds in November and December 2000, resulting in the death of 2 birds and severe acute bleeding and anemia in 3 additional birds. The coagulopathy occurred in both oil-dosed and control birds. The anemia appeared to resolve rapidly after treatment with injectable phyltonadione (Vitamin K_1 Injectable, 10 mg/ml, Neogen Corporation, Lexington, KY) in the 3 anemic birds. Vitamin K_1 supplementation at ~ 1 mg/ml IM every 2 weeks was begun in all birds and the injectable vitamin E-selenium discontinued. No further bleeding problems were observed in this study group and they were released to the wild in March of 2001.

An additional group (24 females and one male) was captured in September 2001. These birds were again housed in the ASLC outdoor pens and fed a diet of the Atlantic silversides. The diet was supplemented by a hand fed multivitamin (Avian SeaTab©, Pacific Research Labs, Inc., El Cajon, CA) 1/4 tablet every other day (Appendix 1). Coagulopathy was again observed in October, approximately 6 weeks after capture, when one bird died as a result of acute gastrointestinal hemorrhage and a second bird was noted acutely anemic. Vitamin K_1 supplementation was reinstituted for all birds at a dose of ~1-3 mg/kg body weight administered IM once every 2 weeks for the 6 month duration of the study. Beginning November 29, 2001, frozen cooked Antarctic krill (*Euphausia superba)* was introduced into the diet at 5 grams per bird and gradually increased until December 5, when they were eating 85-90% krill and 10-15% silversides. Morbidity and mortality were not observed after the addition of the vitamin K_1 injections but many of the birds still exhibited delayed (≥30 min.) whole blood clotting time.

The basic physiology of avian blood clotting has not been well described but is thought to be similar to that of mammals (Powers, 2000). Two pathways exist for

initiation of blood clotting. Extrinsic clotting mechanisms are triggered when blood is exposed to tissue thromboplastin released by vascular injury and utilize factors III, VII and calcium. Intrinsic clotting, which is initiated by contact with subendothelial collagen and basement membrane within vasculature, causes activation of factors XII, XI, and IX in mammals but is thought to be weak or absent in avian species (Archer, 1971). Vitamin K_1 is essential for the synthesis of clotting factors II, VII, IX, and X in the liver and is thought to be involved in the carboxylation of the inactive precursors of these factors to form active compounds (Plumb, 1995). Vitamin K_1 is normally obtained from food or formed by bacteria in the intestinal tract. Studies suggest that bacterial synthesis alone will not meet the needs of poultry (Almquist and Stoksad, 1936) but reports of vitamin K deficiency or clinical bleeding disorders are rare in avian species (Powers, 2000) and no published data exists for wild sea ducks. Excessive consumption of vitamin E has been reported to interfer with vitamin K function causing coagulopathy in pink backed pelicans (*Pelecanus rufescens*) (Nichols et al. 1989) and in humans (Corrigan and Frank, 1974). Studies have also indicated that excessive vitamin A supplementation may cause decreased levels of vitamin E in serum due to antagonism of vitamin E absorption from the gastrointestinal tract (Crissy, et al, 1998; Dierenfeld, 1989).

We hypothesized that 1) vitamin K levels would drop to deficient levels in serum of harlequin ducks fed a silverside diet without vitamin supplementation, resulting in coagulopathy and anemia, 2) oil soluble vitamin (A and E) concentrations in captive birds exclusively fed frozen fish for a period of 4 weeks would differ significantly from those fed a mixed diet with vitamin supplementation and from levels found in harlequin ducks in the wild, and 3) supplementation of the frozen fish diet with a commercially available multivitamin-mineral tablet would result in serum concentrations of vitamins A and E higher than those found in wild harlequin ducks.

To test these hypotheses, we manipulated the diet of 17 female harlequin ducks under controlled experimental conditions over a five week period and collected blood samples to monitor vitamin levels and blood clotting times.

Methods

Experimental study

Seventeen birds remained at the at the ASLC after the completion of the oil exposure study were housed in three adjacent outdoor aviaries with natural salt water pools and allowed to recover from oral and topical crude oil exposure and surgical liver biopsies performed during the winter of 2001-2002 (Appendix 2). All birds were continued on the mixed diet of silversides and krill *ad libitum* supplemented by hand fed multivitamin-mineral and vitamin K injections as described above. In March of 2002, the birds were randomly assigned to one of two dietary study groups so that each oil-dosing treatment was equally represented in each group. Ten birds (experimental Group A) were held in two of the pools and fed frozen Atlantic silversides *ad libitum* with no further supplementation of vitamins. The seven remaining birds (control Group B) were placed in the third pool and continued on the mixed diet plus ¼ multivitamin mineral tablet every other day. Vitamin K_1 injections were discontinued 10 days prior to the start of the diet experiment (Day 0) and were not repeated in any of the ducks until completion of the diet restriction portion of the trial on day 46. Every other day, the birds were herded

from the pools into a capture cage and handled briefly to obtain body weight measurements, to monitor for health problems, and to administer the multivitamin-mineral tablet to the Group B birds.

Blood samples were collected with a 25 gauge needle and 5 ml syringe from the jugular vein on the first day of diet restrictions (Day 10) and again on Days 22, 38, 46 or 47 and 77. A blood sample equal or less than approximately 1% of body weight (4.0 to 4.5 ml) was collected from each bird at each sampling. Venipuncture sites were closely monitored for delayed blood clotting and any unusual bleeding was noted. Direct pressure was applied with clean gauze sponges held over the venipuncture site for 30 to 60 seconds after the needle was withdrawn. Any appearance of blood from the skin after the pressure was released was noted and additional direct pressure applied. If the site continued to bleed for more than 5 minutes, ice was applied to the feet of the bird and to the skin at the venipuncture site along with the direct pressure. The skin was checked every 3 to 5 minutes until no further bleeding was noted.

A portion of each blood sample was placed in sodium citrate (1 part sodium citrate to 9 parts whole blood) and mixed gently for 20-30 seconds. Two microhematocrit capillary tubes (10µl with EDTA, Drummond Scientific Co) were filled and spun at 11,500 RPM for 3.3 minutes in a Compur battery operated microcentrifuge (Compur M1100, Bayer/Zeiss Munich. Germany) to determine PCV and to estimate the buffy coat layer. The remaining sample was placed in serum separator vials and held for 30 to 60 min in a covered container at room temperature. Tubes were gently inverted every 1 to 5 minutes until a visible blood clot was formed, then refrigerated. Serum separator tubes and citrated blood vials were spun at 3500 RPM for 10 minutes (Triac centrifuge, Benton, Dickinson Co) within 2 hours. Serum and citrated plasma were transferred to sterile 1 ml cryovials and stored away from light at -70C until shipment for vitamin assays and measurement of prothrombin time.

The diet restriction portion of the experiment was ended when birds in both groups (Table 1) exhibited significantly low PCVs (less than 25% in seven individuals) or prolonged venipuncture bleeding times on Day 46. Severely anemic birds (PCV < 18.5%) were treated with intramuscular vitamin K_1 (~3mg/kg IM once daily for 5 days), provided with an oral hydrating solution (Pedialyte©, Abbott Laboratories, Columbus, OH) at a dose of 60 ml/kg via stomach tube once daily until observed eating well, and provided with supplemental warmth (radiant heat lamps on a haulout adjoining a small indoor saltwater pool). PCV in all of these birds increased rapidly and all were returned to the outdoor pens at the end of the 5 day treatment.

The remaining 13 birds were given a single IM dose of vitamin K_1 (~3mg/kg) after their blood draw on Day 46 or 47 and all birds had a booster dose of ~ 1mg/kg vitamin K_1 administered IM on Day 54. All birds were also treated with iron dextran (Iron Dextran, 100mg/ml, The Butler Company, Columbus, OH) at ~10 mg/kg IM as a hematinic on Days 43 and 50. The pretrial diet (mixed silversides and krill with multivitamin-mineral tablets) was resumed for all birds on Day 49. A blood sample was obtained from all birds for PCV and plasma prothrombin time determination prior to vitamin K_1 resumption on Day 46 or 47 and again 4 weeks after completion of the diet experiment (Day 77) for final vitamin assays and prothrombin time measurement.

All animal housing and treatments were approved by the ASLC Institutional Animal Care and Use Committee (ASLC # 02-003).

Collection of samples in the field

Blood samples were collected by jugular venipuncture from 22 wild harlequin ducks (male and female) in Prince William Sound in mid-March 2002. Samples were centrifuged in the field and serum frozen in sterile cryovials at -20 C for one week and then at -70C until vitamin analyses.

Laboratory analyses

Whole blood clotting time

Whole blood was placed in nonheparinized glass microhematocrit tubes and small sections broken off every 1 to 2 minutes. Whole blood clotting time was recorded as the time when a distinct clot was observed in the tube (Powers, 2000).

Prothrombin time/clotting time

Two parts Simplastin (Organon Teknika Corporation, Durham, NC) was added to 1 part of freshly thawed citrated plasma at 37C in a fibrometer (Becton Dickinson, Franklin Lakes, NJ) and clotting time is determined.

Venipuncture bleeding time

Direct pressure was applied to the skin at venipuncture sites with clean gauze sponges for 30 to 60 seconds after the needle was withdrawn. Any appearance of blood from the skin after the pressure was released was noted and additional direct pressure applied. The skin was checked every 3 to 5 minutes until no further bleeding was noted and total elapsed time recorded.

Phylloquinolone (Vitamin K)

Aliquots of serum were denatured with 1 ml ethanol and extracted with 6 ml hexane. Dihydro-K was used as an internal standard to adjust for extraction recovery. The hexane extract was dried under a stream of filtered air. Dried extracts were dissolved in 1 ml hexane and filtered through silica Sep-Pak cartridge (Waters Corporation, Milford, MA) using 3% ethyl ether in hexane. The filtrate was dried under a stream of filtered air. Dried samples were dissolved in 250 µl of methanol and measured using reverse-phase HPLC (high performance liquid chromatography) with post column zinc reduction and fluorometric detection by a modification of the method of Haroon et al (1986). The sample was injected onto a Zorbax ODS 4.6 mm I.D. X 25 cm analytical column (Mac-Mod Analytical, Chadds Ford, PA) using a 1 ml/min flow rate with mobile phase of 15% methylene chloride in methanol. Sample values were determined by comparison of peak height data with those of known standards.

Retinol (Vitamin A) and alpha-tocopherol (Vitamin E)

On the day of analysis samples were thawed and 100 µl of serum was mixed with 100 µl methanol and 50 µl delta-tocopherol (internal standard). Samples were extracted 3 times with 0.5 ml hexane. The hexane layer was removed and evaporated under a gentle stream of nitrogen. The residue was dissolved in 100 µl propanol and injection amount was 20 µl. Retinol (vitamin A) and alpha-tocopherol (vitamin E) were determined by reverse phase isocratic high performance liquid chromatography. Vitamins were

separated on a microsorb C18 column (Varian, Walnut Creek, CA) protected with an Upchruch C18 guard column (Upchurch Scientific, Oak Harbor, WA). A mobile phase of methanol/water (98/2) at a flow rate of 1 ml per minute was used to elute the vitamins. Detection for both vitamins was by absorbance (retinol 325 nm, tocopherols 290 nm). All solvents were HPLC grade (VWR Scientific, Rochester, NY). Minimum detection levels were at or below 0.62 ng (2.1 pmol) for retinol and 27 ng (62 pmol) for alpha-tocopherol:

Statistical Analysis

Correlation between serum vitamin K, A and E levels was examined and compared between captive and wild sampled birds. Packed cell volume and plasma prothrombin time were compared between and within captive study groups at various times during the trial and changes were examined in relation to vitamin levels in the captive birds. All values are given as means ± standard error unless stated otherwise. Differences within treatment groups were tested with repeated measures one-way analysis of variance, with Tukey test to determine specific differences. Differences between treatments were tested with either student's t-test or Wilcoxon signed rank sum for small sample sizes or unequal variation. Relationships between variables were tested with linear regression analysis. All statistical tests were performed with SigmaStat 2.03.

Food analysis

Samples of the Atlantic silversides, Antarctic krill and multivitamin mineral tablets used in this study were wrapped in aluminum foil and commercial self-sealing plastic freezer bags and held frozen at -70C. Several samples of whole invertebrates reported as natural diet items for wild harlequin ducks were also collected from Resurrection and Kachemak Bay in the spring of 2002 and preserved in an identical manner. Vitamin levels will be assayed in these food items in 2003.

Results

Bleeding Time

Several birds were noted to have difficulty achieving hemostasis after venipuncture on Day 45 and 46. In one case, bleeding was not controlled despite application of digital direct pressure and ice packs until 35 min after sampling of a tarsal vein. The vessel then began bleeding again after the bird was returned to a holding cage and an additional 30 minutes of pressure and ice was required before bleeding was controlled

Whole blood clotting time

Formation of blood clots in whole blood was prolonged in the majority of samples from all birds at all times. On Day 38, visibly observable clotting time ranged from 8 minutes to > 60 minutes and 70% (12 of 17) samples were in the >60 min category.

Packed cell volume

Packed cell volume (PCV) decreased between pretrial and the Day 47 (end of nutrition restriction) samples in both diet groups (Fig. 1). Birds in Group A (silversides

only) had a significantly greater decline compared to birds in Group B. Forty percent (4 of 10) of the Group A had severe anemia (PCV < 18.5%) and an additional 30% had moderate anemia (PCV < 28.5%). Thirty-three percent of Group B had moderate anemia and none were severely anemic. End of trial PCV collected four weeks (Day 77) after resumption of mixed diet and vitamin K administrations had returned to pretrial levels in both groups. PCV values were not determined for the blood samples from the wild ducks collected in the field.

At the end of the experiment (Day 47) we found suggestive, but inconclusive evidence that the mean PCV of Group A was significantly less than the mean PCV of Group B (one-sided $p = 0.0614$, 95% CI of the difference -18.83 to 2.50). A comparison of birds classified as not anemic, moderately anemic and severely anemic in each group also suggests a treatment effect, although comparison of the proportions with a Fischer's exact test is not significant, most likely due to the small sample size.

Prothrombin Time

Plasma harvested from citrated whole blood was collected by jugular venipuncture on Days 10, 22, 38 and 47 and at the end of the trial (~30 days after resumption of vitamin K_1 supplementation). Values ranged from 4 to >250 seconds (Table 2) and were highly variable both between groups and between dates on individual birds. There was no correlation between prolonged prothrombin times and observed anemia or increased venipuncture bleeding times.

Vitamin K

Phylloquinolone concentrations (Table 3) in serum collected from 15 wild harlequin ducks immediately after capture ranged from 0.30 to 3.64 ng/ml. Values found in males and females were similar and values combined for statistical analysis (mean ±SE = 1.59 ± 0.3 ng/ml).

Average phylloquinolone values from the pre-trial samples, drawn 14 days after the most recent vitamin K_1 administration, were 100 times higher than those obtained from wild harlequin ducks. A progressive decline was observed in all birds following cessation of injectable vitamin K_1 (Fig. 2). The decline was similar (~50%) in both diet groups on Day 22 (3 weeks after vitamin K_1 administration), but birds in Group B continued to decline over the next 2 weeks to approximately 25% of pretrial levels while birds in Group A remained at 50% of pretrial level. On Day 38, the mean (±SE) vitamin K concentration of Group A (87 ± 18.4 ng/ml) was significantly higher than that of Group B (36 ± 3.3 ng/ml) (two-sided $p = 0.0452$, 95% CI -100.5 to -1.3). All birds again had greatly increased serum phylloquinolone on Day 77 (1 month after resumption of injectable supplementation). The highest levels were seen in 4 birds that had been given serial injections to treat severe anemia 25 to 30 days earlier but, even when the values for these birds were excluded, the mean concentration of serum phylloquinolone in the post trial samples were higher than the pretrial values.

Vitamin E

Alpha-tocopherol levels (Table 4) in serum from 9 wild ducks (8.59 – 15.85 ng/ml, mean ±SE = 12.41 ± 0.7 ng/ml) were similar to those found in captive birds receiving the multivitamin mineral supplement (Table 5). The January 2002 baseline

samples (7.22- 23.99 ng/ml , mean 15.20 ng/ml), Group B captive birds on Day 38 of the diet study (12.08-20.38 ng/ml, mean ± SE = 15.85 ± 1.2 ng/ml) and all captive birds at the end of trial (Day 77) (11.88-15.59, mean ± SE = 13.54 ± 0.6 ng/ml) were not statistically different from levels in the wild birds. However, the mean (± SE) serum concentration of alpha-tocopherol was significantly lower (Fig. 3) in the non-supplemented Group A birds on Day 38 (9.12 ± 0.5 ng/ml) when compared to the any of the other sample groups ($p < 0.001$).

Vitamin A

Serum retinol (Fig. 4) was assayed in each of the samples submitted for serum alpha-tocopherol analysis. Retinol levels in serum from the wild ducks (Table 4) ranged from 0.91 to 1.60 ng/ml (mean ±SE = 1.20 ± 0.1 ng/ml). These values did not differ significantly from those found in Group A or Group B on Day 38 or from the entire captive group on Day 77 (Table 5). Pretrial values were only obtained from 3 birds but were significantly lower than for any of the other sample groups (0.62-1.30 ng/ml, mean ±SE = 0.86 ng/ml ± 0.3 ng/ml).

Discussion

Vitamin K

Oral vitamin K can be supplied by utilizing commercially available pelleted feed (Sea Duck Diet, Mazuri). This diet was not introduced during the original harlequin duck oil exposure study because provision of small fish allowed food intake to be easily monitored and was expected to more accurately reflect the natural diet of oil exposed birds feeding in Prince William Sound. It was also suggested that because the birds were to be released back into the wild at the end of each 6 month study period, a major shift to pelleted diets might adversely affect their resumption of normal feeding after release.

Consumption of frozen fish diets has been linked to fatal coagulopathy in captive piscivirous birds (Nichols, 1989; Zollinger, 2002). Nichols suggested that excessive levels of vitamin E supplementation caused interference with the function of vitamin K in the clotting cascade. Excessive bleeding tendencies are well documented in humans on high levels of vitamin E (Corrigan, 1974) and treatment in these cases includes vitamin K_1 administration.

Vitamin K_1 supplementation is recommended for treatment of hemorrhagic disorders in avian medicine at doses ranging from 0.2 to 2.5 mg/kg IM as needed (Ritchie, 1994) to 2.5 to 5 mg/kg every other day (Papich, 2000). A dose of 3 mg/kg administered intramuscularly appeared to rapidly reverse hemoptosis and severe anemia in 4 captive wild caught harlequin ducks during oil exposure studies between October 2000 and February 2002. Supplemental doses of vitamin K_1 (0.25 to 5 mg/kg /day, divided into two or three oral doses daily for 4-6 weeks) are recommended to prevent bleeding in small animals after ingestion of coumarin rodenticides (Plumb, 1995). An empirical dose of ~1mg/kg was administered intramuscularly once every 2 weeks to the captive harlequin ducks from October through February in each of the study years and no further bleeding episodes occurred. All of these ducks had surgical liver biopsy at the end of the oil ingestion phase of the study and excessive bleeding was not observed during or after these procedures.

We had hypothestized that vitamin K would decline to deficient levels in the ducks fed a piscivorous diet after cessation of vitamin K_1 supplementation. However, serum levels at the beginning of the diet restriction were much higher than expected and never dropped below those found in wild birds. Serum phylloquinolone concentrations were nearly 100 times higher than in wild ducks in the baseline samples from harlequin ducks after receiving 1 mg/kg doses of vitamin K_1 every 14 days. Values dropped to 50% of baseline level within 3 weeks after the last supplement administration but were still 20 times that of wild ducks in the Group B birds 38 days after the last vitamin K_1 dose. These results suggest that much smaller and less frequent doses may be adequate and should be evaluated if therapeutic or prophylactic administration of injectable vitamin K_1 is indicated in the future.

We had also hypothesized that vitamin K deficiency or interference with vitamin K function would result in decreased ability to limit blood loss through defective intrinsic blood clotting with resulting anemia.

Although severe acute anemia and some evidence of increased bleeding time were observed in the harlequin ducks in this study, overt coagulopathy was not clearly demonstrated. High serum levels persisting after cessation of supplementation were unexpected and no clear causal relationship between vitamin K levels and anemia could be demonstrated. Further studies using an unsupplemented frozen fish diet and birds which have never received vitamin K_1 injections would be needed to determine if blood levels do eventually fall low enough to interfer with blood clotting.

Vitamin E

Vitamin E deficiencies have been reported in most taxa of captive wildlife (Liu,1985; Dierenfeld, 1989). This deficiency has occurred most frequently in captive and wild animals consuming fish based diets (Geraci and St. Aubin, 1979; Citino *et al.*, 1985; Nichols *et al.*,1989). Clinical signs include accumulation of peroxides, steatitis, liver necrosis, anemia, and muscular degeneration. High concentrations of PUFAs in marine fishes, frozen storage of fish for several months before use, and evisceration of fish all contribute to vitamin E deficiency.

Vitamin E deficiency has been reported in captive pinnipeds. Citino *et al.* (1985) reported the death of a yearling California sea lion thought to have died perhaps of vitamin E deficiency. Clinical signs included myopathy and steatitis. Englehardt and Geraci (1978) studied the effect of experimental vitamin E deprivation in harp seals (*Phoca groenlandica*) by feeding headless, eviscerated herring for 18 months. Electrolyte imbalance characterized by low plasma sodium levels, decreased plasma tocopherol (from 20 to 42 *u*g/mL to 15 to 27 *u*g/mL), and irregular molting patterns were observed. The suggested level of vitamin E needed to prevent deficiency disease in piscivorous species is 100 IU vitamin E/kg fish (equivalent to 200 to 300 IU/kg dry matter; Englehardt and Geraci, 1978; Calle *et al.*, 1989).

Reports of vitamin E toxicity in animals are rare Increasing the vitamin E administered to humans on anticoagulants caused depressed levels of vitamin K dependent coagulation factors and increased bleeding (Corrigan, 1974). The mechanism of vitamin E synergism in anticoagulant therapy may involve blocking of the oxidation of vitamin K, thus altering the balance between the inactive and active forms by increasing the inactive form.

Vitamin E is absorbed in the gastrointestinal tract and transported in the circulatory system via β-lipoproteins. It is distributed to all tissues and is stored in adipose tissue. Vitamin E is metabolized in the liver and excreted primarily in the bile. Vitamin E is indicated in the treatment of capture myopathy and is thought to be important in oxidation of potentially toxic metabolites at a cellular level. Hypovitaminosis E has been reported in birds and mammals fed fish based diets resulting in cardiomyopathy, skeletal muscle necrosis, fat necrosis and steatitis (Lowenstine, 1986; Nichols, 1987)

Low level oral vitamin E supplementation (25 IU/kg D1-alpha-tocopherol acetate every other day) was provided to harlequin ducks in the oil exposure study from September 2001 through March 2002. This supplement was continued in Group B birds in the diet study. Serum alpha- tocopherol levels were similar to those in wild ducks for Group B and post trial samples. In Group A, alpha-tocopherol levels declined significantly over the first 4 weeks after withdrawal of the oral vitamin E. This would suggest that supplementation with oral vitamin E at the empirical rate of 25 IU/kg body weight every other day should be provided to captive birds receiving a frozen silverside diet. It is unclear whether similar declines would be encountered with feeding of other fish but previous publications have recommended vitamin E supplementation to all captive piscivorous animals (Englehart and Geraci, 1978; Geraci. 1972).

It is interesting to note that continued decline in vitamin K over time was greater in birds provided vitamin E, but our results did not clearly identify the vitamin E as the cause for this decline, and differences in vitamin E levels were not associated with coagulopathy in this experiment.

Vitamin A

The liver is the major storage organ for vitamin A, and carnivores fed eviscerated fish or meat exclusively are prone to signs of deficiency (Lowenstine, 1986). In addition, for species receiving frozen and stored meat or fish products, there is the additional problem of potential vitamin loss during storage or thawing prior to their use. Geraci and St. Aubin (1979) suggested that the diets of piscivores (fish eating species) be supplemented with vitamins A and E because deficiencies have been linked to fish-based diets.Among carnivores, the family Hyaenidae (hyaenas) and suborder Pinnipedia are exceptions having at the most only trace amounts of retinyl esters in the blood. Hypervitaminosis A has not been documented in captive pinnipeds, but the possibility exists considering the wide range of supplements being dispensed at some institutions. Vitamin A toxicity has been reported in captive marine birds maintained on fish diets supplemented with vitamin A (Dierenfeld, 1994).

Serum retinol concentrations were opportunistically provided by one of the co-investigators during analysis of alpha-tocopherol. Vitamin A supplementation (~500 IU/kg vitamin A acetate every other day) had been provided to the captive harlequin ducks during the September 2001 to March 2002 oil exposure studying the form of hand fed oral multivitamin mineral tablets. This supplement was continued for Group B birds during the diet study and again to all birds for the 30 days prior to the post-trial blood sample. Serum retinol concentrations were slightly higher in Group B on Day 38 and in all of the post-trial samples than that seen in wild harlequin ducks in March of 2002. Group A had significantly lower levels of serum retinol on Day 38 than in the post trial

samples but were not statistically different from the wild birds. Longer duration of feeding trials without vitamin A supplementation would be needed to determine if retinol levels would eventually fall significantly below those found in wild birds.

Retinol levels reported for the 3 birds tested in the pretrial blood sample drawn in January 2002 appeared lower than in the wild birds or all other study samples tested. Possible causes for the lower levels in the January sample include 1) seasonal variation in retinol levels associated with photoperiod, 2) a metabolic effect associated with oral oil ingestion in the pretrial birds, or 3) an aberration attributable to small sample size.

Conclusions

Baseline data for the oil soluble vitamins A, E and K have not previously been reported from wild harlequin ducks. The values reported here should serve as a useful reference in establishing the need for and appropriate levels of supplementation of these vitamins in captive waterfowl and in the analysis of suspected cases of vitamin deficiency or toxicity.

Vitamin K and E supplementation should be provided to captive harlequin ducks at low levels in the form of commercially available multivitamin mineral tablets or in pelleted diets. If coagulopathy is observed, treatment with injectable vitamin K_1 (3 mg/kg intramuscularly) appears to be safe and effective. Incidence of observed coagulopathy appears to be reduced by prophylactic administration of vitamin K_1 at a rate of 1 mg/kg intramuscularly every 2 weeks, but lower doses and less frequent administration may be adequate and should be explored.

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Table 1.. Packed cell volumes of captive Harlequin ducks.

ID	Pre-trial Baseline 1/29/02	Day 10 3/20/02	Day 22 4/1/02	Day 38 4/17/02	Day 47 4/26/02	Post-trial Day 77 5/27/02
Group A (silversides)						
936	46	42	45	40	15	45
938*	49	48	49	44	39	46
939	42	34	37	34	15	36
950	40	41	40	36	18	36
960	44	45		38	19	44
963	44	46		43	28	47
968	42	40	40	37	25	37
969	46	37	35	37	29	-
971	45	40	38	39	33	40
973	42	41	41	38	39	45
mean	**44**	**41**			**26**	**42**
standard error	**0.8**	**1.3**			**2.9**	**1.5**
Group B (mixed diet)						
933	43	43	44	40	22	40
941	45	43	38	40	38	40
944	38	34	44	33	23	38
946	48	37		43	40	47
947	44	41	45	39	33	37
972	43	39	40	40	49	44
mean	**44**	**40**	**42**	**39**	**34**	**41**
standard error	**1.3**	**1.5**	**1.4**	**1.4**	**4.3**	**1.5**
p	**0.741**	**0.369**	**0.507**	**0.733**	**0.123**	**0.734**

*Male.

**Please note: There was a significant difference within the Group A treatment during the trial period ($p < 0.001$) as well as between the trial period and the pre-trial sample ($p < 0.001$). There was no significant difference during the trial for Group B ($p = 0.127$), however, the trial period was significantly different from the pre-trial values ($p = 0.038$). There was no correlation between packed cell volumes and vitamin K values on Day 30 ($r^2 = 0.055$, $p = 0.400$).

Table 2. Prothrombin times of captive Harlequin ducks (seconds).

ID	Day 10 3/20/02	Day 22 4/01/02	Day 38 4/17/02	Day 47 4/26/02	Day 77 5/27/02
Group A (silversides)					
936	137	41	1	>250	125
938*	37	56	>250	178	>250
939	58	56	>250	-	214
950	48	71	>250	-	43
960	219	95	54	67	42
963	6	-	4	>250	>250
968	32	40	60	87	38
969	>250	>250	>250	>250	-
971	48	73	50	224	>250
973	>250	45	51	-	108
mean	**109**	**81**	**122**	**187**	**147**
standard error	**30.6**	**22.0**	**35.4**	**30.0**	**31.6**
Group B (mixed diet)					
933	28	30	32	61	34
941	32	-	42	65	46
944	>250	>250	>250	>250	>250
946	165	-	36	172	>250
947	>250	>250	>250	>250	70
967	>250	-	96	122	249
972	>250	200	174	-	>250
mean	**163**	**183**	**131**	**162**	**150**
standard error	**44.0**	**52.2**	**43.6**	**34.3**	**45.0**
p	**0.318**	**0.054**	**0.871**	**0.599**	**0.951**

*Male.

Please note there were no significant differences within treatments over time ($p = 0.131$ and $p = 0.839$, respectively).

Table 3. Serum phylloquinone (Vitamin K_1) from field samples of harlequin ducks in Prince William Sound, Alaska (1/21/02 to 3/26/02).

Female Bird ID	ng K_1/ml	Male Bird ID	ng K_1/ml
785-01762	1.07	0965-66903	1.35
755-74176	0.74	0965-66927	2.26
0965-66907	1.69	0965-66934	0.30
0965-66910	3.64		
0965-66912	1.81		
0965-66917	2.67		
0965-66918	2.88		
0965-66923	1.09		
0965-66924	0.41		
0965-66928	2.73		
0965-66930	0.66		
0965-66933	0.52		
mean	**1.66**		**1.30**
standard error	**0.314**		**0.566**
p	**0.615**		

*Please note there is no significant difference between the two sexes. Combined mean 1.59 ± 0.269 ng K_1/ml.

Table 4. Serum retinol (Vitamin A) and alpha-tocopherol (Vitamin E) of harlequin ducks in Prince William Sound, Alaska (3/21/02 to 3/26/02).

Bird ID	**Retinol** (μg/ml)	**Alpha-tocopherol** (μg/ml)
965-66906	1.21	8.59
965-66913	1.11	12.39
965-66915	1.26	13.86
965-66919	0.91	13.38
965-66920	1.26	15.85
965-66926	1.04	11.16
965-66930	1.06	13.05
965-66932	1.60	11.53
785-01903	1.34	11.91
mean	**1.20**	**12.41**
standard error	**0.067**	**0.672**

Table 5. Serum retinol (Vitamin A) and alpha-tocopherol (Vitamin E) of captive harlequin ducks (μg/ml).

	Retinol		Alpha-tocopherol	
		Post-trial		Post-trial
	Day 38	Day 77	Day 38	Day 77
ID	4/17/02	5/27/02	4/17/02	5/27/02
Group A (silversides)				
938*	1.08	1.29	10.97	17.24
950	1.40	1.82	9.73	15.14
960	0.89	1.41	10.31	16.39
968	1.32	1.40	8.30	13.38
969	0.67		7.90	
971	1.66	1.41	7.89	11.63
973	1.07		8.75	
mean	**1.16**	**1.47**	**9.12**	**14.75**
standard error	**0.125**	**0.091**	**0.463**	**1.016**
Group B (mixed diet)				
933	1.35	1.53	14.76	13.70
941	1.64		14.27	
944	0.84	0.98	12.30	12.69
946	1.79	1.91	20.38	13.86
947	1.26	1.28	18.43	15.59
963		1.33	15.01	
972		1.37		11.88
mean	**1.38**	**1.40**	**15.85**	**13.54**
standard error	**0.165**	**0.126**	**1.213**	**0.625**
p	**0.304**	**0.692**	**<0.001**	**0.339**
Pre trial (1-29-02)	**(not included)**			
934	0.67		7.22	
969	0.62		14.38	
972	1.30		23.99	
mean	**0.86**		**15.19**	
standard error	**0.219**			

*Male.

Retinol: There was no difference within treatment groups ($p = 0.914$ and $p = 0.909$, respectively).

Alpha-tocopherol: There was a significant difference within Group A, ($p < 0.001$), but not Group B ($p = 0.146$).

Appendix 1. Composition of Commercial Multivitamin-Mineral Supplement (SeaTabs© for Birds, Turtles, Fish and Sharks, per tablet)[1]

Vitamin A (acetate)	1000 I.U.
Vitamin D3	20 I.U.
Vitamin E (D1-alpha-tocopherol acetate)	50 I.U.
Vitamin C (ascorbate)	10 mg
Vutamin B1 (thiamine mononitrate)	50 mg
Vitamib B2 (riboflavin)	.25 mg
Vitamin B6 (pyridoxine)	.15 mg
Vitamin B12 (cyanocobalamin)	2.0 mcg
Niacin	.15 mg
Pantothenic Acid	1.5 mg
Folic Acid	.1 mg
Biotin	2.0 mcg
Choline	5.0 mcg
Inositol	5.0 mcg
Taurine	5.0 mcg
Iodine (KI)	7.0 mcg
Iron (FeSO4)	1.0 mg
Copper (CuSO4)	.1 mg
Magnesium (MgO)	5 mg
Zinc (ZnO)	.05 mg
Manganese (MnSO4)	Trace
Kelp	1.0 mg

[1]Courtesy of: Pacific Research Laboratories, El Cajon CA 92019, 1998

Appendix 2. Timeline for Captive Harlequin Duck Diet: Hemostasis study –ASLC-2002-2003

Fall 2002
9/8-10 Capture Prince William Sound AK, transport to Alaska SeaLife Center
10/21 Mortality 1 – hemorrhagic into gastrointestinal tract
10/16 Acute anemia (#969), PCV 16%, Vit K x 5 days, rapid return of red blood cells
10/30 Coagulopathy (#967), Vit K
11/4 Coagulopathy (#936), Vit K
Start Vit K supplement to all birds ~1-3 mg/kg every 2 weeks

2003
1/29 Health monitor/blood sample all birds; PCV, "Pre-trial" serum archived
2/8-9 Liver biopsies, end of oral oil dose trial
2/23 Acute bleeding from mouth and nose (#969), PCV 16%, Vit K daily for 7 days
3/? End of external oil exposure study

Diet:Hemostasis Trial
Day 0 (3/10) Last dose Vit K (~1 mg/kg IM) for all study birds (n=16)
Day 10 (3/20) Divide into Groups A and B, start diet restriction in Group A
Blood sample all birds (~4.2 ml each)
Day 22 (4/1) Blood sample all birds, (~4.2 ml each)
Day 29-31 (4/8-10) Serial cortisol blood samples x 8 birds (3.55-4.1 ml ea over 24 hr).
Day 38 (4/17) Blood sample (~4.2 ml each) for PCV, vitamin levels, prothrombin assay
Day 43 (4 /22) Iron dextran injection (all birds)
Day 45-46 (4/24-25) ACTH trials x 8 birds (2.05 -4.1 ml each total over 4 hours)
Day 47 (4/26) PCV<50 pretrial value in 5 birds, prolonged bleeding (#933)
Blood sample all birds for PCV, prothrombin assay
Resume vit k injections once every 2 weeks
Day 49 (4/28) Resume mixed diet for Group A (all birds now back on pre-trial diet/vits)
Day 50 (4/29) Iron dextran injection (all birds)
Day 51 (4/30) End vit K x 5 days (for severely anemic birds #936, 939, 950, 960)

Day 64 (5/13) Vit K injection – all birds (~ 1 mg/kg IM)
Day 71 (5/20) Repeat ACTH trials x 12 birds
Day 77 5/26) "Post-trial blood samples all birds (~4.2ml each); PCV, vit, prothrombin

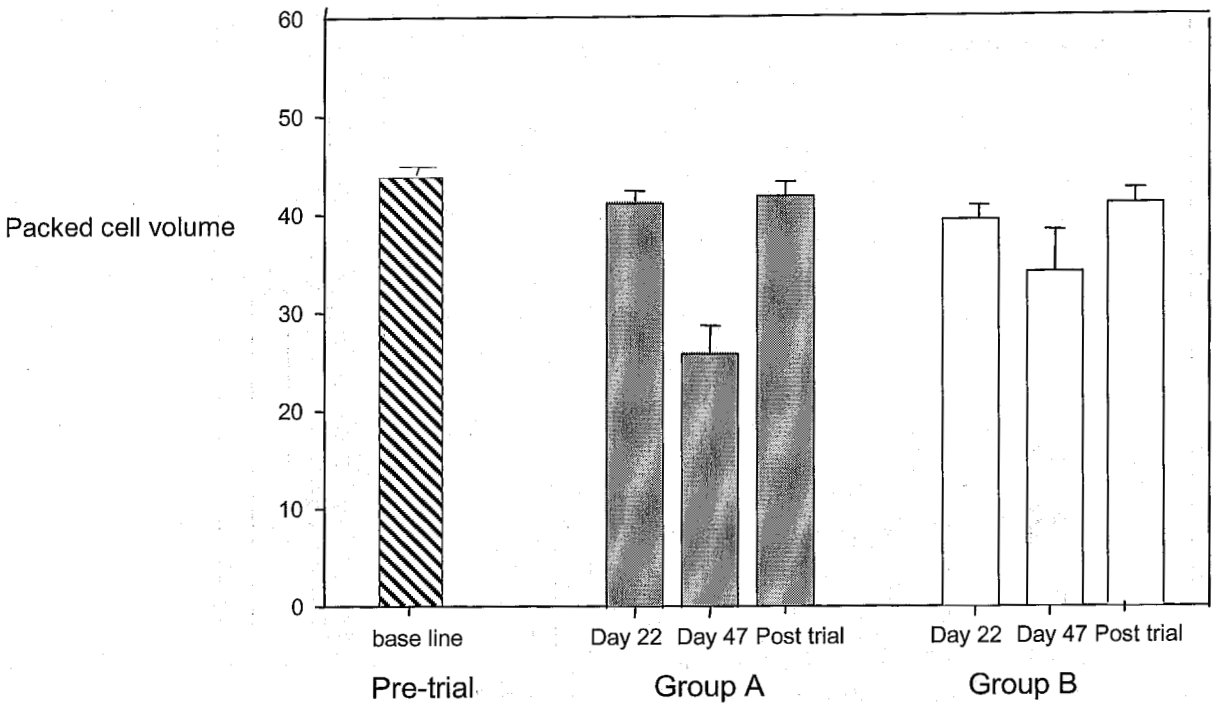

Figure 1. Packed cell volume of captive Harlequin ducks. Group A were fed silversides ad libitum with no vitamin supplement whereas group B were fed a mixed diet with oral multivitamin mineral supplementation. *Please note: both groups were combined for pre-trial value

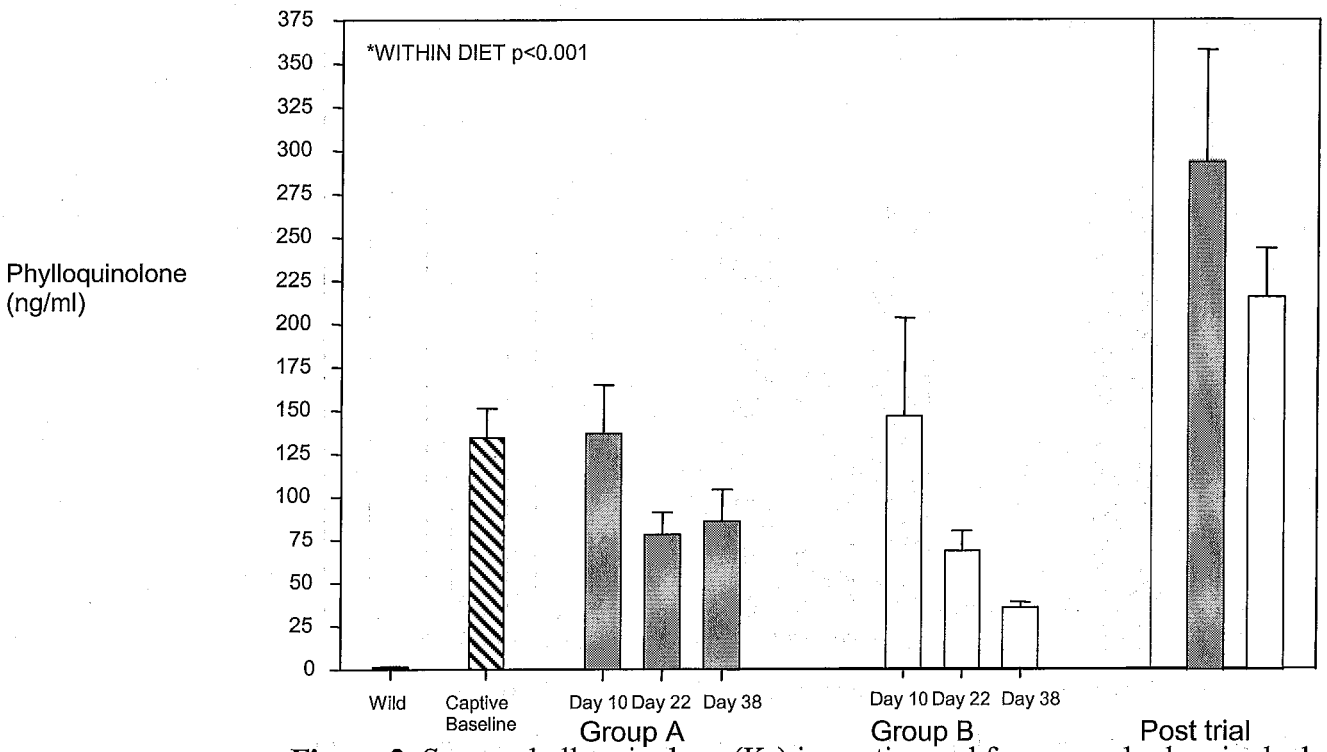

Figure 2. Serum phylloquinolone (K_1) in captive and free-range harlequin ducks. Group A were fed silversides ad libitum with no vitamin supplement whereas group B were fed a mixed diet with oral multivitamin and mineral supplementation.

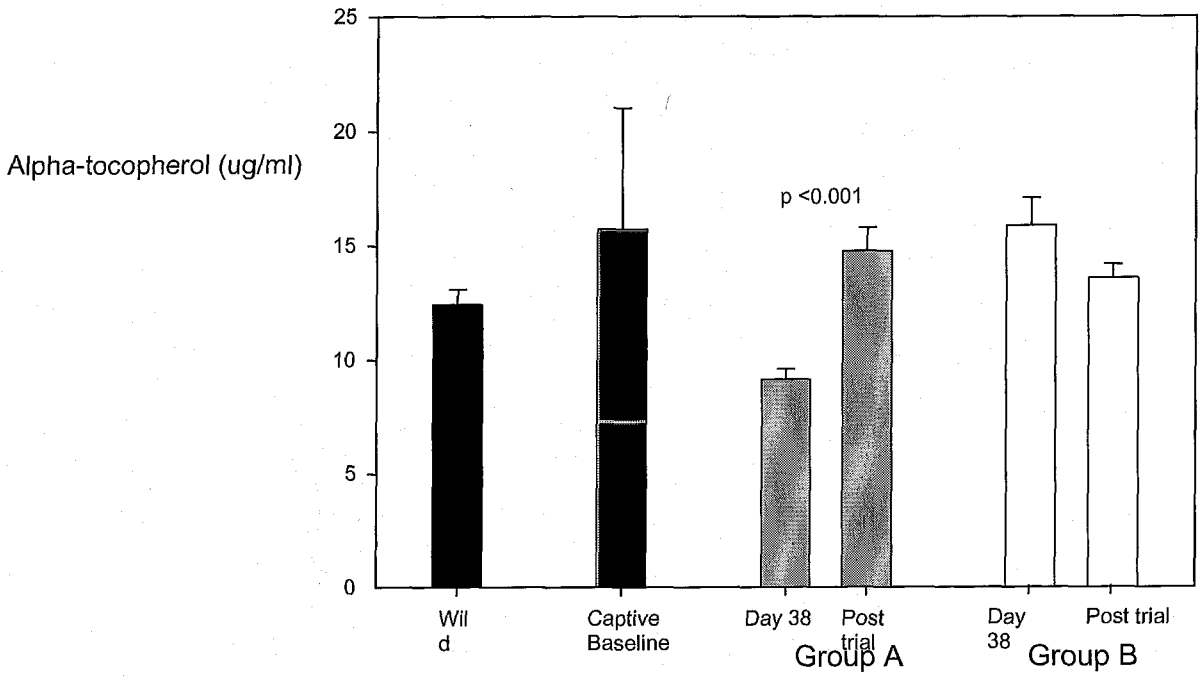

Figure 3. Serum alpha-tocopherol (Vitamin E) in free-range and captive Harlequin ducks. Group A were fed silversides ad libitum with no vitamin supplement whereas group B were fed a mixed diet with oral multivitamin mineral supplementation including vitamin E. There was a significant difference between treatment groups on Day 38 ($p < 0.001$) but not at the post trial sample ($p = 0.339$) 30 days after Group A resumed oral multivitamin mineral supplementation.