Aquatic Mammals 2004, *30*(2), 251-256, DOI 10.1578/AM.30.2.2004.251

Blood Spots in Pinnipedia Hormone Studies: Measure of Cortisol Levels in Southern Elephant Seals (*Mirounga leonina*)

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Abstract

The collection of blood spots on filter paper for hormone analysis has become quite popular in human and primate studies, mostly because of the ease of handling, storage, and transportation of samples, but has never been tested in wild marine mammals. In this paper, we describe a protocol for the collection of blood spots and the analysis of cortisol in southern elephant seal (*Mirounga leonina*) weanlings. We demonstrated that cortisol measured in blood spots is very well correlated to cortisol measured in serum samples, and we calculated equations to convert between the two. We describe the possible pitfalls of the blood spot analysis protocol, and suggest solutions. The use of blood spots for hormone analysis presents many advantages for field research, may open interesting opportunities like the serial sampling of unrestrained adult individuals, and represents a step towards the reduction of invasiveness of hormone studies.

Key Words: Cortisol, blood spots, filter paper, southern elephant seal, *Mirounga leonina*, Falkland Islands

Introduction

The use of filter paper to collect blood for hormone analysis presents many advantages for field-based studies (Whitten et al., 1998) and is becoming popular in primate and human research (Shideler et al., 1995; Worthman & Stallings, 1997). Samples collected on filter paper (blood spots) do not require much special post-collection processing and do not require freezing. They can be stored at room temperature for quite a long time and are easily shipped by mail (Worthman & Stallings, 1997). Moreover, this method can represent a less invasive alternative to conventional blood sampling methods. Notwithstanding these potential advantages, we are unaware of any published account using this method in hormone studies of seals and sea lions, and there are very few studies conducted on other animal species, and almost never in the wild.

Cortisol plays a crucial role in both applied and theoretical studies of elephant seal biology. Release of cortisol is related to stress and can be used as an objective measure of invasiveness of the handling procedures involved in research activities (Engelhard et al., 2002). Moreover, the relationship between blood levels of cortisol and stress can be used to study the physiological effects of social phenomena such as aggression and dominance (Bartsh et al., 1992; Serrano, 2000). Lastly, blood cortisol concentrations are related to one of the most striking aspects of seal biology—the prolonged fasting observed during the land phases of the yearly cycle (Ortiz et al., 2001). In this paper, we tested the use of filter paper for cortisol analysis in a sample of southern elephant seal (*Mirounga leonina*) weanlings.

Materials and Methods

We collected 75 blood samples from 69 southern elephant seal weanlings of the population of Sea Lion Island, Falkland Islands (Galimberti & Boitani, 1999). All weanlings of the population were individually recognizable (double tagging by Jumbo Rototags, Dalton Supplies Ltd) and of known sex. A total of 75 blood samples were collected—37 (49.3%) from undisturbed resting weanlings and 38 (50.7%) from restrained weanlings after weighing. Mean handling time was 466 ± 132 s (n = 28) for restrained weanlings and 49 ± 14 s (n = 36) for resting ones.

Blood was collected from the rear flippers using Vacutainers (5 ml, red top, no additive) and 20-gauge, 1.5-inch needles (Becton Dickinson). Each sample was subjected to three different handling procedures immediately after collection.

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First, four drops of blood were spotted on a filter paper blood collection card (Sigma Diagnostics, Inc, Catalog No. 160-C). Cards were placed in individual, sealable plastic bags. Second, a subsample of unclotted blood was transferred to a siliconized microcentrifuge tube (Costar Brand, Catalog No. 3207). The remainder was left in the original Vacutainer. All samples were kept at a temperature between 15º and 25º C for a few hours (2 to 8) after collection. Samples were then processed as per the description below.

Blood Spots

The spotted filter paper was removed from the plastic bag and dried overnight in an airtight container with silica gel. The dried filter paper was transferred into a paper coin envelope to avoid heat build-up or moisture accumulation that has been reported for samples stored in plastic bags (Knudsen et al., 1993). Each envelope was stored at temperatures ranging from 4º to 15º C for 15 to 60 days in an airtight container with silica gel. After transportation from the field, samples were frozen at –20º C.

Liquid Blood

The blood in the microcentrifuge tube was centrifuged for 20 min at 6,000 rpm (VWR Mini Centrifuge). The serum was pipetted into a new microcentrifuge tube and then stored frozen at –20º C within 24 h.

Blood left in the original Vacutainer sat undisturbed overnight (temperature 10º-15º C). The next day, and with no centrifugation, the serum was pipetted into a new microcentrifuge tube. The serum was frozen at –20º C within 24 h of separation. All the samples were then transported from the Falklands to Canada in thermal resistant containers (with ice packs for the frozen serum samples) and stored at –20º C upon arrival. Samples were analyzed six to seven months after blood collection.

Cortisol concentrations were determined using Coat-a-Count radioimmunoassay kits (Catalog No. TKCO1, Diagnostics Products Corporation), in which ^{125}I-labeled cortisol competes with cortisol in the samples for antibody immobilized on the wall of a polypropylene tube. The sera were analyzed in duplicate following the kit manufacturer's procedure. Blood spots were analyzed with the following modified protocol. Blood spot assay standards, ranging from 1 to 50 µg/dL, were prepared by pipetting 25 µl of each kit standard onto the filter paper card. Additional standards of 20, 50, 100, 400, and 1000 µg/dL were prepared from cortisol (Hydrocortisone, Sigma, H-0888) initially dissolved in ethanol and then diluted with "0" standard buffer, and finally spotted onto the cards. The cards dried overnight at room temperature and then were stored at –20º before use.

All filter paper cards were allowed to reach room temperature. From each unknown sample/card, a $\frac{7}{16}$ inch (11.1 mm) diameter filter paper disk was cut using a custom-made high carbon steel punch. This size was chosen to make optimal use of the blood spot's surface. Each filter paper disk was cut exactly in half. Each of the two resultant semicircles was cut again into four smaller pieces of roughly the same size, which were all transferred into the same assay tube. The smaller wedges of filter paper provided better duplicates and increased specific binding.

To gain accuracy in the estimation of cortisol concentrations, two different sets of standards were used to assay the blood spots, hereinafter referred as the "big" and "small" protocols (Table 1). For the "big" protocol, we used the standards provided with the kit, with concentrations from 1 to 50 µg/dL; for each standard, we used one $\frac{7}{16}$ inch semicircle, as described above, for the samples. For the "small" protocol, we used one $\frac{1}{8}$ inch (mm) disk cut from our custom-made standards, with concentrations ranging from 20 to 1,000 µg/dL. Hence, despite the difference in the size of the filter disks used for the standard curve, the disks for the unknowns were all the larger semicircles as described above.

As per the kit instructions, 1.0 ml of I^{125}-labeled cortisol was then added to all tubes. Tubes were gently swirled using a multi-tube vortexer (SMI Multi-Tube Vortexer) then incubated at 27º C in a water bath for 18 h. Approximately halfway

Table 1. Summary of the "small" and "big" protocols; SC = serum cortisol concentration; BSC = blood spot cortisol concentration

	"Small" protocol	"Big" protocol
Disk size per replicate, samples	One $\frac{7}{16}$ inch diameter semicircle	One $\frac{7}{16}$ inch diameter semicircle
Disk size per replicate, STDs	One $\frac{1}{8}$ inch diameter disk	One $\frac{7}{16}$ inch diameter semicircle
STDs concentrations	0, 20, 50, 100, 400, 1,000 µg/dL	0.5, 10, 20, 50 µg/dL
Linear regression	SC = -1.231 + 0.098 BSC	SC = - 0.505 + 0.347 BSC
R^2	0.937	0.934

through the incubation period, samples were again gently swirled. The contents of each tube (excluding the total count tubes) were decanted and discarded, and the lip of each tube was blotted on absorbent gauze to remove residual droplets. The inverted tubes were allowed to drain thoroughly for about half an hour. The lip of each tube was again blotted against gauze and then counted for one min in a gamma counter (LKB Wallac Gammamaster 1277 automatic gamma counter). Based on the standard curves for each size of filter disk, cortisol concentrations were automatically calculated using a spline function algorithm (*RiaCalc* software).

Assay results showed that cortisol values were not normally distributed either in serum samples (Shapiro-Wilk test; W = 0.776, $p < 0.0001$) or in blood spot samples (W = 0.81896, $p < 0.0001$). Thus, randomization tests (Manly, 1997) were applied using 10,000 replicates. Descriptive statistics (mean ± standard deviation) and normality tests were calculated using *Stata 7* (Stata Corporation). Randomization tests were run on *RT 2.0* software (Manly, 1996).

Results

Cortisol levels were significantly higher in sera from centrifuged samples than in sera that were not centrifuged (n = 36, mean difference = 1.226 µg/dL; paired *t*-test, with randomization: t = 8.250, $p = 0.0000$), but results from both serum handling methods were highly correlated (centrifuged serum cortisol = 0.675 + 1.142 noncentrifuged serum cortisol, $R^2 = 0.932$, RMS residual = 0.821). Cortisol levels were, on average, 25% lower in sera from noncentrifuged samples than in sera that were centrifuged. In field situations where centrifugation is impractical, serum collected from noncentrifuged blood samples may be adequate to determine relative serum cortisol concentrations, in particular when the goal is the comparison of levels between individuals. The lower concentration of noncentrifuged samples, however, should be taken into account when comparing cortisol values from different studies.

We analyzed the relationship between cortisol concentrations in serum (centrifuged samples) and blood spots, using both the "small" and "big" methods, respectively. Serum cortisol (SC) concentration was effectively predicted by a linear regression on blood spot cortisol (BSC) measured in both cases. The predictive equations for the two protocols were SC = –1.231 + 0.098 BSC (n = 33, $R^2 = 0.937$, RMS residual = 0.737) for the "small" method, and SC = –0.505 + 0.347 BSC (n = 33, $R^2 = 0.934$, RMS residual = 0.756) for the "big" method (Figure 1). The significance of the regression coefficient, as tested by randomization, was very high in both cases ($p = 0.0000$), and its parametric confidence limits were small (0.089-0.108 for the "small" and 0.313-0.381 for the "big" protocols). The relationship between cortisol concentration in serum and blood spot samples was not affected by the weanling's sex (ANCOVA on SC concentration versus BSC concentration and sex, test on BSC x SEX interaction, with randomization: F = 1.493, $p = 0.24$), nor by the handling time during sampling (multivariate linear regression, test on time of handling regression coefficient, with randomization: $p = 0.20$). "Small" and "big" protocols BSC levels were strongly correlated (n = 36; Pearson's correlation coefficient, with randomization test: r = 0.998, $p = 0.0000$). Therefore, the "big" protocol is preferred for the processing of blood spots because it uses pre-made standards included in the kit.

For the assay using the "big" protocol, sensitivity was 0.66 µg/dL. The sensitivity was calculated from the mean counts per min of 18 replicates of the "0" standard minus 2 times the standard deviation of these counts. The average recovery (calculated as percent of expected values) was 97.3%; intra assay and interassay CV were 8.8% and 12.9%, respectively.

Decanting of assay tubes is a critical step of the protocol. We tested two different methods of decanting tubes. In the first method, the coated tubes were inverted and repeatedly rapped on the absorbent gauze to remove the residual moisture. In the second method, inverted tubes were blotted gently, left inverted to dry, and reblotted approximately 30 min later. In both tests, SC levels were predicted effectively by a linear regression on "small" protocol BSC levels, and the slope of the regression line was almost equal in the two tests (slope = 0.097 vs 0.098; homogeneity of slopes test, with randomization: $p = 0.93$); however, the fit was better using the results from the "gentle blotting" method (n = 33, $R^2 = 0.937$, RMS residual = 0.737), rather than from the "sharp rapping" method (n = 26, $R^2 = 0.860$, RMS residual = 1.271).

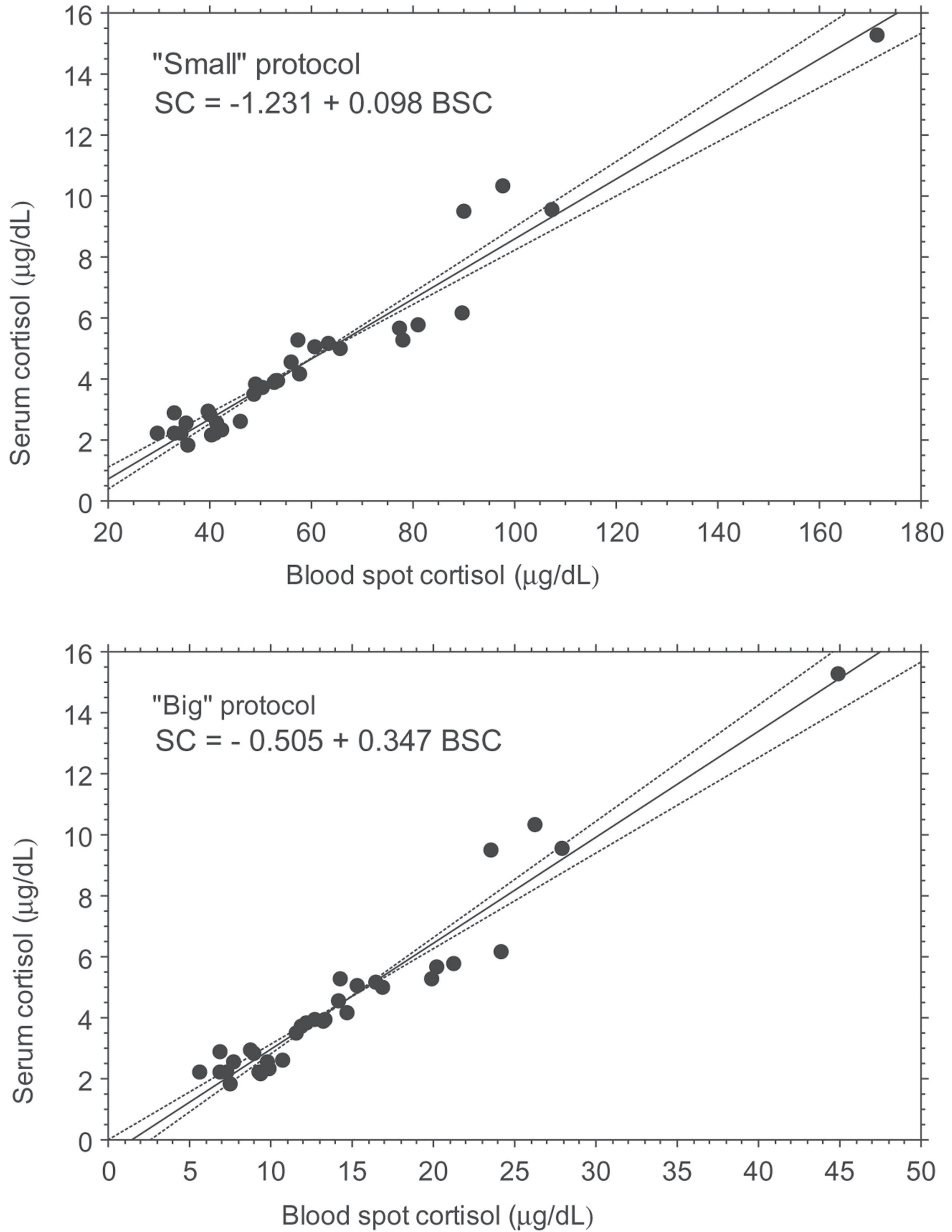

Figure 1. Scatterplot and normal/standard regression line (with 95% parametric confidence limits of the slope) of serum cortisol concentration versus blood spots cortisol concentration; upper panel: blood spots concentration obtained with the "small" protocol; lower panel: blood spots concentration obtained with the "big" protocol.

Discussion

Hormone analyses are important in both theoretical (Boyd, 1991) and applied (Engelhard et al., 2002) studies of seals and sea lions. Results of our study demonstrated that blood collected on filter paper is a good alternative to serum collection for cortisol determination. This result is consistent with those obtained in human studies using finger-pricks (Kraiem et al., 1980). In humans, the reliability of filter paper collection has been demonstrated for various hormones, including cortisol (Worthman & Stallings, 1997).

The use of blood spotted on filter paper is a practical method to store blood for cortisol analysis in elephant seals. Both assay protocols described, "small" and "big," are valid, and the reported equations may be used to predict SC from blood spot samples that were handled and stored as reported. Clinical assayers of blood spot hormone levels and other field scientists (Worthman & Stallings, 1997) commonly use a 1/8 inch diameter hole punch to prepare all filter paper samples and standards. When unknown values fall in the lower range of the standard curve where the fit is not precise, one solution is to increase the amount of blood analyzed in the unknown samples by increasing the number of disks used per assay tube. This is not always practical in cases where the amount of sample is limited. The cortisol levels for blood spot samples in our study were relatively low, and to increase the quantity of blood for assay, we used a larger-sized disk. One 7/16 inch diameter disk is equivalent to approximately 12.5 small disks. The use of a larger disk instead of many smaller ones maximizes the available sample. In fact, 7/16 inch is roughly the diameter of a single blood spot. Depending on the range in which unknown samples fall, the size of the disk used can be modified, provided that appropriate equations to relate this to the serum values are calculated. When opportunistically sampling blood from unrestrained adults (e.g., sampling of blood from wounds of adult elephant seal males; unpublished data), it may be difficult if not impossible to obtain enough blood to use the larger-sized disks. In such cases, the use of smaller-sized disks may then be considered, taking into account the potential loss of precision. Other experiences in our laboratory combined with the additional evidence gained in this study suggested that the coated assay tubes can be affected by the abrasion the filter paper disks might produce on the wall of the tubes. Thus, care should be taken when agitating the tubes prior to and during incubation as well as when blotting the tubes after decanting.

The use of filter paper sampling instead of serum has obvious advantages. We showed that blood spots stored for a long time (up to two months) without refrigeration, at variable temperatures, and transported from field sites without special packaging procedures, contain levels of cortisol that highly correlate with those found in sera. Some human hormones measured from blood spots are stable over many weeks even when stored at higher temperatures (Howe & Handelsman, 1997; Worthman & Stallings, 1997). Blood spot samples are routinely shipped by surface mail from sampling sites (Howe & Handelsman, 1997; Knudsen et al., 1993; Worthman & Stallings, 1997). Our results suggested that mail may be an effective way to ship samples for elephant seals; however, further analysis is required to determine the exact trend of degradation of various hormones and in different species.

The validity of the blood spot method opens interesting opportunities for hormone analysis in free-ranging seals. The small amount of blood needed to measure hormone levels in blood spots may be obtained using modifications of the finger-prick method used for humans. Additionally, blood may be obtained from fresh natural wounds or small cuts. The use of blood spots may enable sampling of adult individuals without any form of physical or chemical restraint. In large wild animals, such as male elephant seals, traditional blood sampling is impractical. With blood spot sampling, even serial samples can be obtained from these animals (unpublished data). This type of approach may minimize any carryover effects of stress due to sample collection and generally reduce the invasiveness of traditional hormone sampling (Theodorou & Atkinson, 1998). Moreover, this method, due to its low invasiveness and speed, can be very useful to determine hormone baseline level, in particular for hormones that are highly and rapidly affected by stress, as cortisol is. Frequently, it is almost impossible to estimate baseline levels with traditional blood sampling methods because handling itself produces stress, and this affects the measured hormone levels. In these cases, blood spot collection can be a valid alternative.

In the current study, we obtained both sera and blood spots from which we measured and correlated hormone concentrations. Even when it is not possible to collect serum from a sample of animals to establish this relationship, the blood spot value alone allows for comparison between individuals. This may be a useful method when interest is focused on relative seasonal profiles or on the correlation of relative hormone levels with behavioral traits.

Acknowledgments

We thank Maria Luisa and Roberto Sanvito and Carla and Alberto Galimberti for their support of our research on elephant seals; the Falkland Islands Government for the granting of the research license; and the Falkland Islands Development Corporation and Strachan Visick Ltd for allowing us to do the fieldwork on Sea Lion Island. This research project has been funded by the Lerner Gray Memorial Fund of the American Museum of Natural History. The research project on the Falklands elephant seals is supported by Strachan Visick Ltd and EarthWatch Institute. We thank the Editor, Dr. Georg Engelhard, and Dr. Lawrence Dunn for their comments on the manuscript that greatly improved it.

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