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THE "SEAL PRICK": A LOW INVASIVE METHOD FOR BLOOD SAMPLING IN MALE ELEPHANT SEALS

SIMONA SANVITO
Department of Biology, Memorial University of Newfoundland,
St. John's, Newfoundland A1B 3X9, Canada
and
Elephant Seal Research Group, Sea Lion Island, Falkland Islands

FILIPPO GALIMBERTI
ROBERTO SANVITO
Elephant Seal Research Group, Sea Lion Island, Falkland Islands

CHIARA BRASCHI[1]
Elephant Seal Research Group, Sea Lion Island, Falkland Islands
and
Department of Animal Biology, University of Roma "La Sapienza",
Viale dell' Università 32, 00185 Roma, Italy

Hormone studies are becoming a core aspect of the investigation about the physiology and behavior of wild animals (Whitten *et al.* 1998*a*, Altmann and Altmann

[1]Address all correspondence to: Dr. Chiara Braschi, Borgo Vittorio 27/A, 00193 Roma, Italy; e-mail: chiara.braschi@uniroma1.it.

2003). A limitation to the application of hormone analysis is the invasiveness of the procedures required to obtain the samples to be analyzed. The collection of blood samples from large animals in the field usually requires physical or chemical restrain. This may produce biased results because the stress induced by the handling procedures may strongly affect the hormone levels themselves (Morton *et al.* 1995, Engelhard *et al.* 2002, Whitten *et al.* 1998*b*, Sakkinen *et al.* 2004). These problems are particularly relevant in longitudinal studies.

In a previous paper (Sanvito *et al.* 2004) we demonstrated the effectiveness of blood spot collected on filter paper to measure cortisol levels in elephant seal weanlings. Here we present a low invasive method to get blood spot samples from adult elephant seals without any form of restraint, that permits serial sampling, and we show that this method is effective to estimate the total testosterone and cortisol concentrations in adult males during the breeding season without affecting their behavior.

In 2002 and 2003 we collected blood samples from elephant seal males at Sea Lion Island (Falkland Islands) using a modified "finger-prick" method. Our modification, the "seal prick," uses a hardened carbon-steel pointed blade (78 × 25 × 3 mm) held by a nylon support which is mounted on a short pole with a handle. A nylon disk was placed transverse to the blade and through it. The disk prevents the blade from cutting too deep and holds the filter paper used to collect the blood. The nylon disk was loose on the blade. Two holes on the disk enable investigators to determine when bleeding has occurred. Two small hooks (1 mm in diameter and leaning 1.5 cm from the disk) keep the disk on the body of the seal during the sampling. A long string (5 m) attached to the other end of the hooks allows the recovery of the disk and the sample (Fig. 1). A piece of filter paper blood collection card, approximately 60 × 70 mm, is placed on the nylon disk, on the side pointing towards the tip of the blade, held by the two hooks. The seal is hit on its back by a sure blow, the blade penetrates in the skin to a maximum depth of 20 mm, and then it is immediately removed, while the nylon disk with the filter paper, remains on the body, held by the two hooks. Through the holes in the disk, bleeding can be detected immediately. As soon as the filter paper turns red, the nylon disk and the sample are retrieved by the string to avoid over-saturation of the filter paper and clotting of the blood.

After an initial blood spot sample is collected directly from the "seal prick," additional samples can be collected by hand, when bleeding is sufficient and the animal is calm and resting. Those extra samples are collected from the fresh wound, using 20 × 50 mm filter paper strips, approaching again the animal from behind. Attaching the strips to an extendable pole (up to 2 m long), allowed collection from a distance. Blood spots were also collected from natural wounds (often produced during males fights), with the same methodology described above for the hand collection of samples from the "seal prick" wounds.

Sometimes it was possible to aspirate a small quantity of blood directly from the wound made by the "seal prick" using a pipette. These samples were used to validate the blood spot method and to calculate the relationship between serum hormone levels and blood spot hormone levels. Collected blood was transferred to

a microcentrifuge tube and centrifuged. Serum was removed and stored frozen at −20°C (see Sanvito *et al.* 2004).

Blood spot samples were left to dry in air for approximately 15 min and then temporarily placed in individual, sealable plastic bags for 2–8 h. Later on, the spotted filter paper was removed from the plastic bag and dried overnight in an airtight container containing silica gel. The dried filter paper was then transferred into a paper coin envelope and stored at temperatures ranging from 4° to 15°C for 15–60 d in an airtight container with silica gel.

The samples were analyzed to measure total testosterone and cortisol concentrations, using the RIA technique described in Sanvito *et al.* (2004). The reliability of cortisol concentration measure in elephant seal blood spots was confirmed in a previous paper (Sanvito *et al.* 2004). To check the reliability for total testosterone, we measured its concentrations in sera and blood spots using Coat-a-Count radioimmunoassay kits (Catalog No. TKTT1, Diagnostics Products Corporation, Los Angeles, CA). Serum samples were analyzed in duplicate following the kit manufacturer's procedure, including 4 min centrifugation before assaying at 12,700 rpm (Fisher Microcentrifuge Model 235A) to clear lipemic samples (TKTT1 Coat-A-Count Total Testosterone package insert). Blood spots where analyzed in duplicate with the modified protocol described in Sanvito *et al.*, 2004 as the "big protocol," using two ⅛ in. (3.18 mm) card disks for both samples and standards. Statistics calculations and tests were run in StatView (SPSS Inc.) and StatXact (Cytel Software Inc.).

We used the "seal prick" on free-ranging breeding elephant seal males of various age classes during 184 trials. In 14 (7.6%) of those trials, we were unable to collect any blood spot sample, either due to insufficient bleeding and too small a cut (about 60% of cases), or the animal appeared too nervous to be approached or went to sea immediately after being pricked (40%). The 170 successful trials were carried out on 34 males during the whole breeding season, with some animals sampled almost weekly. In 74% of trials, we were able to collect at least one sample directly from the "seal prick," while in 84% of the cases we collected at least one sample by hand. In 58% of the trials we collected samples both from the "seal prick" and by hand. In 17% of the trials the "seal prick" was crucial in getting the sample, because we were not able to approach the animal after the first sampling to collect further samples by hand. The quality of the samples obtained was generally high, and 79% of the successful "seal prick" trials resulted in at least one optimal sample, whereas the percentage was 85% for hand samples.

The time elapsed between the cut and the start of bleeding varied from immediate bleeding to 11 min (mean = 153 s ± 130, $n = 76$) and was affected by

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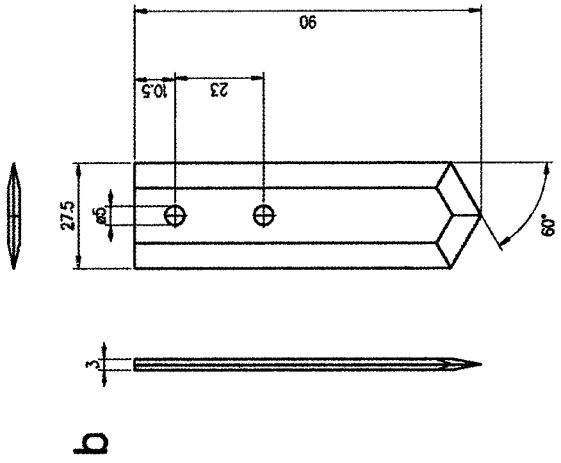
Figure 1. The "seal prick." (a) overall view, with handle partially omitted. The filter paper, omitted here to better show the instrument, is placed on top of the nylon disk, and through the blade, and held in place by the hooks. (b–d): technical designs, with dimensions, for the three main components of the "seal prick": the blade (b), the nylon disk (c), and the nylon blade support (d). Measures in mm.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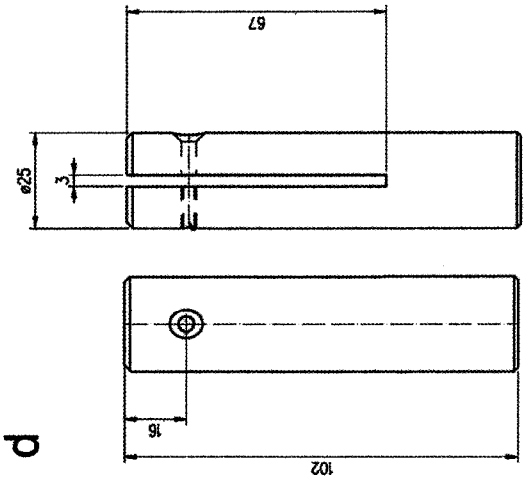
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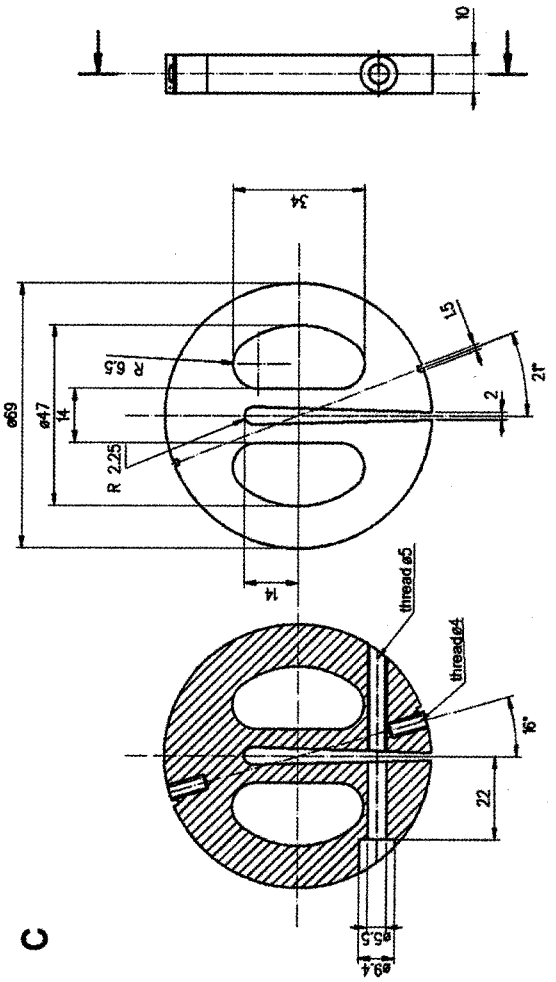
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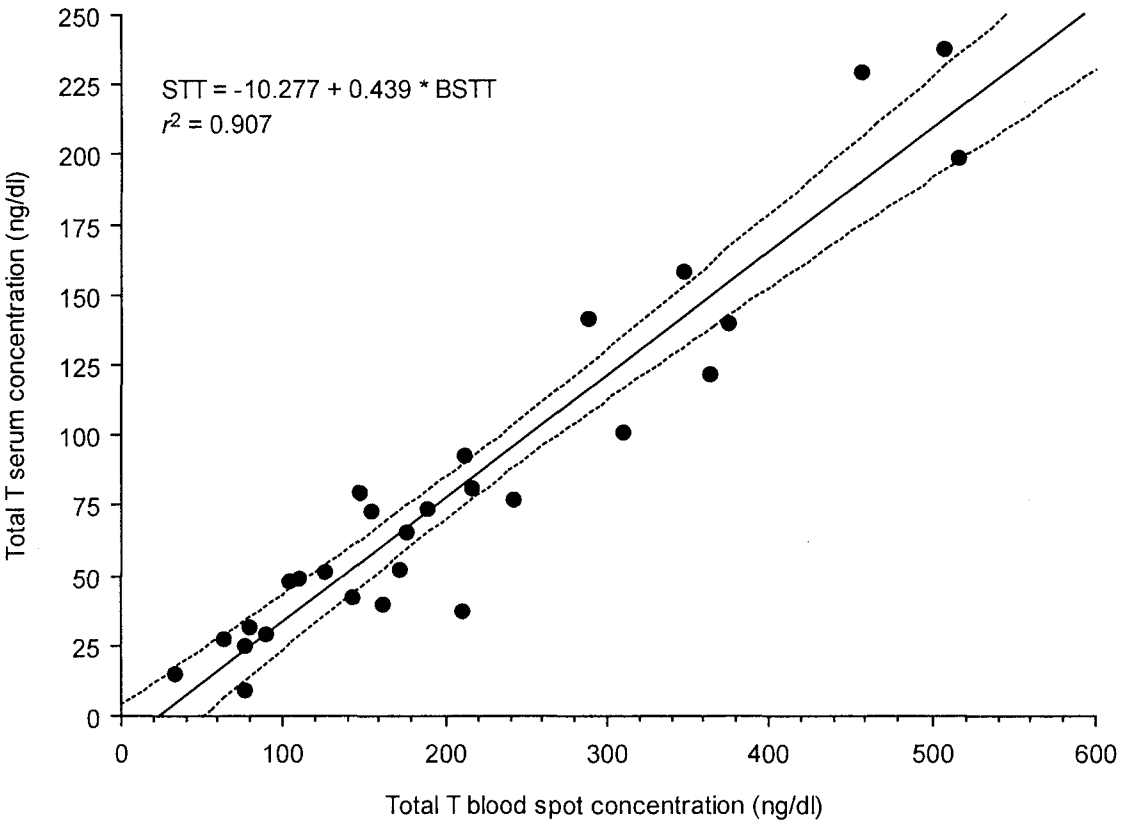

Figure 2. Scatterplot and regression line (with 95% parametric confidence limits of the mean) of serum versus blood spot total testosterone concentrations.

the local weather, in warm days bleeding started faster than in colder days. The total sampling time (from the cut to the retrieval of the sample) with the "seal prick" varied from 20 s to 8 min (mean = 155 s ± 97, $n = 168$). Sampling time for the hand collected samples and for liquid blood samples was not measured, but it was always higher than for the seal prick samples.

To check the reliability of total testosterone concentration measurement in blood spot, we collected both liquid blood samples and blood spots from 28 breeding males. Serum total testosterone concentration (STT) was effectively predicted by a linear regression on blood spot total testosterone concentration (BSTT). The equation was STT = −10.286 + 0.4395 × BSTT ($n = 28$, $r^2 = 0.91$, Fig. 2). A similar relationship was already calculated for cortisol and presented in Sanvito *et al.* 2004.

For the sera assay, the limit of detection was 3.60 ng/dl, as calculated from the mean counts per min of 13 replicates of the "0" standard minus two standard deviations of these counts. The average recovery and dilution (calculated as percent of expected values) were 97.9% and 97%, respectively, and the intraassay CV (calculated as average CV for duplicate samples) was 6.9%. For the blood spot, the limit of detection was 36.82 ng/dl (18 replicates of the "0" standard). The average recovery was 103.9%, the average dilution was 97.2%, and the intraassay CV was 10.3%.

From the 2002 collection, a total of 166 blood spot pricked samples were

analyzed to estimate total testosterone and cortisol. For testosterone, five samples (3.0%) gave non-reliable results (completely out of the range or non-measurable). For cortisol, 16 (9.6%) were non-reliable. Overall, 70% of the samples that were non-reliable after assay, had already been classified as non-optimal during the collection in the field as either the sample was insufficient or already clotted at time of collection. In 10 cases we measured total testosterone in matched "seal prick" and hand-collected samples, and found no statistically significant difference in concentration (Exact Wilcoxon signed rank test: $P = 0.43$).

We also collected blood spots from naturally bleeding wounds produced during 26 male fights (collecting from one or both males). In 88.8% of the male fights sampled, we collected at least one usable sample. After assaying 33 of these samples (from independent male fights) for total testosterone and cortisol, 24 (73.7%) samples gave reliable results for testosterone and 29 (87.9%) for cortisol.

Our blood collection methodology permits the study of blood hormone levels in free-ranging animals within a natural setting and with minimal effects on their behavior. Practical difficulties have often hampered the possibility of collecting simultaneous blood samples and behavioral data in the field without affecting the reliability of hormone level estimates, and without disturbing the natural flow of behavior. The collection of blood on filter paper using our sampling tool permits serial sampling of large individuals, including elephant seal males. We noticed that during colder days the bleeding started later, but lasted longer than in warm days (temperature $>10°C$ approximately). In colder weather the peripheral circulation may be reduced, hence delaying the start of bleeding, while blood clotting is delayed due to the low temperature (Dreher and Sutor 1978, Valeri *et al.* 1995). Notwithstanding this variability, the "seal prick" method was very effective, as demonstrated by the high success rate of both sampling and assaying, and by the good correlation found between hormone concentrations in blood spots and sera. The reduction of the total sampling time reduces the risk of biased results for hormones that are quickly affected by stress (Pollard 1995, Cash et al 1997, Whitten *et al.* 1998*a*, Engelhard *et al.* 2002).

Another advantage of using blood spots on filter paper is the reduction in the time needed to process the samples and prepare them for storage. Blood spots only need to be dried overnight and stored in airtight containers, and they may be preserved at room temperature for a relatively long time (Worthman and Stallings 1997, Sanvito *et al.* 2004). Blood spots are also easier to ship, than liquid blood samples, because they do not require refrigeration.

Although our method is much less invasive than traditional blood collection involving restraint and anesthesia, it does affect the animal. At each sampling, a small wound is produced. We checked sampled males for signs of infection and found none. The cut produced by our sampling device is small and superficial, much smaller than natural wounds that are often observed on elephant seals. The reaction of the subject to sampling was variable, but always limited in intensity and time. Typically the animal became alert and turned around when hit by the sampling tool, but generally calmed down quickly. We observed no medium or long-term effect on subject behavior. We sampled both peripheral males and harem

holders, and they followed the usual behavioral pattern observed during a long term study of the reproductive behavior of the study population, none abandoned the breeding areas or changed its social status.

Several non-invasive methods have been used to measure hormone levels, from feces, urine, and saliva. These methods were found to be reliable in some cases (Whitten *et al.* 1998*a*, Kendall and Shannon 2004), but not in others (Gulland *et al.* 1999). The main advantage of blood spots over fecal and urine sampling is that hormone concentrations in urine and feces reflect the cumulative secretion and elimination of hormones over a number of hours, whereas blood concentrations reflect the current state of the animal (Whitten *et al.* 1998*a*). Sampling of urine and feces is usually opportunistic, and, therefore, the results may be biased. Saliva collection also presents some drawbacks. Salivary hormone concentrations are difficult to relate to serum concentrations, because, for most hormones, saliva is thought to reflect not the circulating concentration but the free fraction (Theodorou and Atkinson 1998), making comparisons with serum concentrations already published in the literature difficult. Moreover, blood contamination in saliva poses strong limitations to the use of this method (Worthman and Stallings 1997), in particular when working with wild animals, where the mouth is often dirty and full of small wounds. Finally, the collection of saliva samples is a challenging task when the study subjects are wild, non-trained, animals (Theodorou and Atkinson 1998, Whitten *et al.* 1998).

In conclusion, the low level of invasiveness and the effectiveness of the "seal prick" makes it competitive with both liquid blood collection of restrained animals and totally non-invasive methods. It is a practical method to obtain hormone profiles of specific individuals, reaching a good balance between the needing to get accurate hormone concentration estimates and reduction of disturbance of individuals.

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