Development of an animal-borne blood sample collection device and its deployment for the determination of cardiovascular and stress hormones in phocid seals

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BIO-LOGGING SCIENCE IS A GROWING research field that enables an animal’s behavior in the wild to be tracked across various habitats. Thanks to the recent progress in electronic engineering, such as downsized microprocessors, sensors, and long-life batteries, we have entered into a new era of behavioral ecology using animal-borne data loggers. We can observe the diving behaviors of marine mammals and seabirds using compact and high-performance data loggers and cameras (5, 6, 26, 37, 38, 45, 46). Bio-logging science enables the integration of functional and behavioral ecology in wild animals (4, 34). Heart rate and plasma gas tension have been measured during diving in Weddell seals, Leptonychotes weddellii (10, 22, 36) and emperor penguins, Aptenodytes forsteri (35), which return to ice holes for breathing after diving in Antarctica. Heart rate was also measured in wild grey seals, Halichoerus grypus, using ultrasonic and radio telemetry (42). In addition, muscle blood flow and deoxygenation rates were measured after forced submersion in naïve and trained harbor seals, Phoca vitulina (19). However, changes in plasma hormone levels have not been investigated in relation to diving in marine mammals and seabirds. For these studies, a compact blood sampler that causes little stress to the animals is required. Some devices have been developed previously, including a microcomputer-driven blood sampler for free-diving Weddell seals (16) and a blood sampler for diving emperor penguins (35), but these had limited applicability. Because of the rapid progress in the development of miniaturized electronics in the intervening years, it has been possible to develop and test a new, remote blood sampler for use in captive and free-ranging phocid seals.

Endocrinological studies have been carried out on seals in relation to the prolonged periods of aphagia in lactating females and postweaned pups, particularly in grey seals and northern elephant seals, Mirounga angustirostris. In these studies, either stress hormones, such as adrenocorticotropic hormones (ACTH) and cortisol, or metabolic hormones, such as thyroid hormones (T3 or T4), IGF-1, leptin, insulin, and glucagon have been measured (3, 7, 44). As fasting is associated with adipsia and resulting disturbances in body fluid balance, osmoregulatory hormones, such as arginine vasopressin (AVP), atrial natriuretic peptide (ANP), and aldosterone, have also been measured (31–33, 47, 48). Thus, while it is possible to collect blood samples from seals when they are on land, such as during the breeding season or the molt, or after capture in the water followed by sampling on land, it is much more challenging to collect blood from swimming and diving seals.

Land is a harsh environment for cardiovascular regulation due to the impact of gravity, particularly in erect humans. By contrast, the effect of gravity is almost nullified in water by the increased pressure acting on the lower limbs. Therefore, the mechanisms for cardiovascular regulation are quite different between terrestrial and aquatic animals (40). Hormones play important roles in cardiovascular regulation; for example, vasopressor hormones such as ANG II and AVP are more important than vasodepressor hormones such as ANP and adrenomedullins for high blood pressure maintenance in ter-
restrial mammals, but the relationship is reversed in low-pressure aquatic fishes (41). In humans, head-out water immersion, which decreases the gravitational effect and produces a prompt redistribution of circulating blood to increase venous return to the heart, has been shown to increase ANP secretion and decrease renin and AVP secretion (9). In captive bottlenose dolphins (Tursiops truncatus), however, plasma ANP, BNP, and ANG II concentrations did not change after standing (27). The complete adaptation of cetaceans to a fully aquatic lifestyle may explain the loss of a response to gravity. Therefore, it is very interesting to learn how semi-aquatic pinnipeds respond to gravity and how their plasma cardiovascular hormone levels change between land and water.

This study was aimed primarily at developing a new animal-borne blood sampler for application in future endocrinological studies of free-ranging marine mammals during diving or during swimming. The sampler was tested on captive grey and harbor seals to assess its performance. Plasma stress hormones (ACTH and cortisol) were measured in blood obtained manually from seals and using the remote sampler from freely moving animals. Plasma glucocorticoids, using a combination of enzyme immunoassay (EIA) and HPLC, were also characterized. Finally, an initial study to compare plasma concentrations of ANP, ANG II, and AVP when seals were on land or in the water (where the effect of gravity was lessened by buoyancy), was carried out using the blood sampler. These hormones were measured by RIA developed in our laboratory, and the major molecular form of ANP and ANG II circulating in seal blood was determined by the elution position of HPLC.

MATERIALS AND METHODS

Animal-borne blood sampler. A custom-made blood sampler (1.2 kg in air and 160 g in water, 18 × 8.6 cm OD) was designed to obtain two blood samples from an animal at one deployment, while it was under different physiological conditions (Fig. 1A). The sampler contained two 5-ml syringes for blood (sample syringes) and one 10-ml syringe for discard between samples to prevent cross contamination (waste syringe). Each syringe was connected with silicon tubes (ID: 1 mm; OD: 3 mm, Tigers Polymer, Osaka, Japan) to the inlet of the device (Fig. 1B). The access to each syringe was regulated by a valve (PSK-1015NC, Takasago Electric, Aichi, Japan), which could be activated by preprogrammed timing using elapsed time, depth, and body angle. The body angle was calculated from the low-frequency component of the longitudinal acceleration, as described in a previous study (39). These parameters were determined by a timer, pressure sensor, and accelerometer on the circuit board (Fig. 2). Water temperature was also monitored by a thermosensor, and the swimming efforts of the animals were assessed from the magnitude and frequency of rear flipper movements recorded by the accelerometer (12). The sampler could also be triggered manually by a 5-kHz light using a photosensor. The sampling rate was set to 1 Hz for time and depth, and 8–32 Hz for the accelerometer (Fig. 2). The internal pressure of the sampler was kept negative by vacuuming the air from the case to allow smooth aspiration of blood into the syringes.

After the first activation of the valve by a trigger signal, blood was sucked into the waste syringe until it reached 5 ml, which was detected by an optic sensor with a phototransistor (SFH3710, OSRAM Opto Semiconductors, Regensburg, Germany) placed at the middle of the syringe (Fig. 1A). Then, the valve to the first sample syringe was open until it was filled by 5 ml blood. The maximum deployment time after device setting was 72 h, and the timing of blood sampling could be regulated by the preset timer for up to 3 h. Before blood sampling, the whole tubing system was filled with heparinized saline. The dead volume of tubing, including joints that contaminated the first and second sample, was 43 μl and 39 μl, respectively, and the other dead space (150 μl) was cleared by a blood collection to the waste syringe (Fig. 1B). Thus, contamination of heparinized saline to 5-ml samples was 0.86% and 0.78% in sample 1 and sample 2, respectively. The picture of the sampler after blood collections is shown in Fig. 3. The sampler was operated by four lithium batteries (CR123A). The device was connected to a vascular catheter (Intech Solomon CBAS C707 French heparin-coated PU round tip catheter, Linton Instrumentation, Norfolk, UK), which was also cleared by the waste sampling. After the experiment, all data recorded in the microcomputer were downloaded into a laptop computer using custom-made software (Fig. 2B), and the data were analyzed by Igor Pro (ver. 6.22, Wave-Metrics, Lake Oswego, OR).

Animals

Captive juvenile grey seals (one female with a mass of 45.2 kg and one male with a mass of 43.6 kg) and a harbor seal (one male with a mass of 62.2 kg), temporarily housed at the Sea Mammal Research Unit’s Home Office licensed captive seal facility, were used for the experiments to measure cardiovascular and stress hormones. They were anesthetized using a combination of midazolam (Hypnovel, Roche Products, UK; 5 mg/ml solution, 0.03 ml/kg im as a premedication sedative and 0.01 ml/kg iv to control tremors) and ketamine (Ketaset, Zoetis, UK 100 mg/ml solution, 0.01 ml/kg iv). Prior to attachment of the sampler, background blood was collected from the
ODS-120T column. Concerning angiotensin peptides, the relative amounts of ANG II and its NH$_2$-terminal truncated forms (ANG III and ANG IV) were determined in HPLC by a 15–35% linear gradient of acetonitrile in 10 mM ammonium acetate at pH 7.0 for 40 min at 1 ml/min. The antibody for ANG II was raised against human [Ile$^5$] ANG II, but seal ANG II was [Val$^5$] ANG II, as mentioned, and the cross-reactivities to [Val$^5$] ANG II, [Val$^5$] ANG III [ANG II-(2–8)], and [Val$^5$] ANG IV [ANG II-(3–8)] were 81.5%, 61.5%, and 50.6%, respectively. The cross-reactivities were used to correct the amount eluted at each peak.

Measurement of Stress and Cardiovascular Hormones

Blood collected from a harbor seal was used for the measurement of stress hormones (ACTH and cortisol), and those from grey seals were used for cardiovascular hormones (ANG, ANG II, and AVP). For the stress hormone analyses, the two blood samples were collected 2–7 h after the catheterization, and both samples were collected when the seals were on land. For the cardiovascular hormone analyses, the first blood was collected when the seal was on the land area, and the second blood was collected 15 min after the animal entered the water. The plasma samples were extracted using acidic acetone, freeze dried, and subsequently measured by RIA for ANP (24), ANG II (49), and AVP (18) established after iodination of each peptide. EIA kits were used for measurement of cortisol (IBL International, Hamburg, Germany) and ACTH (DI Bioproducts, St. Paul, MN), according to the manufacturer’s instruction. The cross-reactivity of this cortisol EIA for cortisone was low (4.2%). The antisera used for the ACTH EIA was directed to the NH$_2$-terminal 23 amino acids, which are identical in all mammals thus far examined.

Characterization of Seal ANP and Angiotensins in Plasma

Initially, the identity of seal ANP to human [Met$^1$] or rat [Ile$^{12}$] ANP was determined by the elution position in HPLC. The HPLC condition was a linear gradient of acetonitrile concentration in 0.01% trifluoroacetic acid from 15% to 45% for 40 min at 1 ml/min in the ODS-120T column. Concerning angiotensin peptides, the relative amounts of ANG II and its NH$_2$-terminal truncated forms (ANG III and ANG IV) were determined in HPLC by a 15–35% linear gradient of acetonitrile in 10 mM ammonium acetate at pH 7.0 for 40 min at 1 ml/min. The antibody for ANG II was raised against human [Ile$^5$] ANG II, but seal ANG II was [Val$^5$] ANG II, as mentioned, and the cross-reactivities to [Val$^5$] ANG II, [Val$^5$] ANG III [ANG II-(2–8)], and [Val$^5$] ANG IV [ANG II-(3–8)] were 81.5%, 61.5%, and 50.6%, respectively. The cross-reactivities were used to correct the amount eluted at each peak.

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Characterization of Glucocorticoids by HPLC

Plasma collected from a female or a male grey seal (1 ml each) was treated with the same volume of acetic acid (acetone:water:1 M HCl = 40:5:1), centrifuged at 12,000 rpm for 5 min in a microrefrigerated centrifuge (model 3700; Kubota, Tokyo, Japan), and the supernatant was freeze-dried. The residue was reconstituted in 1 ml of 40% acetonitrile and was subjected to a reverse-phase ODS-120T column (4.6 × 250 mm, Tosoh, Tokyo, Japan) with a linear gradient

of acetonitrile concentrations in water from 40% to 70% for 30 min at 0.7 ml/min, as described previously (23). The elution position of cortisol, cortisone, corticosterone, or 11-deoxycortisol, which has cross-reactivity by 100%, 15.8%, 4.8%, and 15.0%, respectively, in the cortisol EIA used for this experiment (Cortisol EIA kit, Oxford Biomedical Research, Oxford, MI), was determined in this HPLC condition using authentic steroids as standard.

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Table 1. Sampling periods in seconds (mean) to fill each syringe with blood from two grey seals and a harbor seal

<table>
<thead>
<tr>
<th>Animal</th>
<th>First Sampling</th>
<th>Second Sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Waste</td>
</tr>
<tr>
<td>HG1</td>
<td>6</td>
<td>0.7 ± 0.4</td>
</tr>
<tr>
<td>HG2</td>
<td>7</td>
<td>9.9 ± 3.3</td>
</tr>
<tr>
<td>PV</td>
<td>6</td>
<td>0.5 ± 0.5</td>
</tr>
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</table>

Values are expressed as means ± SE. HG, grey seal; PV, harbor seal. Only the samples that were collected with an accurate volume (5 ml) in 2 min are shown (HG1 and PV catheter OD = 2.3 mm; HG2 catheter OD = 1.2 mm).

Statistical Analyses

All values are expressed as means ± SE. Changes in plasma hormone concentrations between different conditions (Captured vs. Free or Land vs. Water) were compared by Student’s t-test. The Aspin-Welch method was used when homogeneity of variance was rejected. Nonparametric Mann-Whitney U-test was also used when normal distribution of data was rejected. Paired t-test was applied where appropriate. P < 0.05 was considered as significantly different between the two groups.

RESULTS

Development of New Blood Sampler

A total of 32 attempts were conducted to obtain blood samples from grey seals using the blood sampler. Comparing the time to achieve blood sampling using different size vascular catheters showed the sampling period was shorter with the larger inner diameter than with the smaller one (Table 1). Only 1 attempt out of 14 failed using the larger catheter, while 3 out of 18 attempts failed using the smaller catheter. These failures occurred when the sampler was activated more than 5 h after the deployment. As no blood could be withdrawn from the first sampling, the failure may have been due to clotting at the tip of the catheter in the sinus. In the case of larger vascular catheter, the second blood sampling was successful, even after the failure of the first sampling. Because of the rapid sampling time and potential to solve the clotting problem, the larger catheter (ID: 2.05 mm) was used for subsequent experiments.

In a harbor seal, 7/18 deployments failed to obtain blood samples. However, there was no clear evidence of clotting in both vascular catheter and device tubing in these failures. The data from the sampler occasionally showed that the first blood withdrawal to the waste syringe was successful, but no blood had been collected into the first sample syringe during 2 min of valve opening (Fig. 4). This indicated that the inlet of the vascular catheter was closed by the vessel wall due to excessive suction just after the opening of the valve for the sample syringe, even though the sucking-release sequences were frequently repeated to prevent closure (Fig. 4). This may have been due to the increased negative pressure inside the device, to ~101.3 kPa in the harbor seal experiments, to obtain blood samples even with clotting in the catheter as it was lower, at ~74.0 kPa, in the grey seal experiments. The period to fill up the waste syringe in the initial samplings was consistently within a second (Table 1), which was too short for it to be recorded in the memory of microcomputer (Fig. 4). The blood sampler was set for 23 h consecutively after deployment in each sampling protocol, and no clotting in the circuit, including heparin-coated vascular catheter, occurred during the experiments.

Identification of Homologous Hormones in Seal Plasma

Corticosteroids. After HPLC separation, using one sample from the female grey seal, it was found that cortisol was the dominant glucocorticoid and that cortisone, corticosterone, and 11-deoxycortisol concentrations were negligible. The second sample from the male grey seal had a higher level of cortisol and also cortisone in the plasma (Fig. 5, A and B). Thus, the major glucocorticoid that responds to stress (ACTH) in phocid seals is cortisol, not corticosterone.

Angiotensins and ANP. Elution positions of ANG III and ANG IV were very close even after the best separable conditions using HPLC (Fig. 5C). [Val⁵] ANG II and ANG III/IV were identified in the plasma of the male grey seal, but ANG III/IV was the major form in that of the female seal. Therefore, it is apparent that significant amounts of ANG III and ANG IV were circulating in the seal blood in addition to ANG II. Seal ANP was eluted at the position of [Met¹²] ANP (1–28) but not that of [Ile¹⁰] ANP after HPLC separation (data not shown). Thus, the seal has [Met¹²] ANP as in other carnivores.
Effect of Blood Sampling on Plasma Stress Hormones

To determine the degree of stress after deploying the sampler on the animal, plasma ACTH and cortisol concentrations were compared in a male harbor seal between the blood collected directly from animal following capture (Captured) and by the sampler when it was on land (Free). Plasma ACTH concentrations were significantly lower in Free samples than Captured samples (Fig. 6A). Plasma cortisol concentration was also low in Free samples (Fig. 6B), but the difference was not statistically significant ($P = 0.078$).

Effect of Gravity on Plasma Cardiovascular and Stress Hormones

Plasma ANP and AVP concentrations tended to be higher and lower, respectively, when grey seals were in the shallow pool (Water) than when they were on the haulout land area (Land), but the difference was not statistically significant ($P = 0.069$ for ANP and $P = 0.074$ for AVP; Fig. 7, A and B). Plasma ANG II concentrations exhibited large variations and did not show any difference between the two conditions (Fig. 7C). By contrast, in the harbor seal, plasma ACTH concentrations were highly variable and showed no difference between Land and Water (Fig. 7D). Plasma cortisol concentration was lower when this seal was in Water than on Land (Fig. 7E). The blood was collected by the sampler in this series of experiments in both seal species.

DISCUSSION

Development of an Automated, Animal-Borne Blood Sampler

The cardiovascular physiology of marine mammals has attracted the attention of researchers for many years, as they live in an aquatic environment where cardiovascular regulation requirements are quite different from those in terrestrial environments (4, 21). Blood gathers in the central part of the body when terrestrial animals are submerged in the water, as exemplified by the head-out water immersion in humans (9). In particular, the cardiovascular regulation changes dramatically when semi-aquatic pinnipeds and seabirds dive to the...
depth (4, 34). However, cardiovascular responses, bradycardia, for example, differ considerably between voluntary diving in animals in the wild and forced submersion under water in animals in captivity (19, 21). Therefore, studies in free-ranging animals under minimal stress are required to understand the true nature of the cardiovascular response, particularly to various forms of external and environmental stress in marine mammals.

Previous efforts have been dedicated to collecting blood automatically from free-ranging animals under water without stress. For example, Hill (16) was the first to develop a microcomputer-assisted, back-pack blood sampler, which allowed blood to be collected at depth in Weddell seals (300–400 kg body mass). The blood was withdrawn by a pressure-resistant peristaltic pump that was reversible to flush the blood in the circuit (i.e., in the tubing and dead space) in the case of multiple blood samplings. The blood collection could be triggered by pressure and/or time during either the ascending or descending phase of the dive through a microcomputer monitor. The size and weight of the sampler were not described, but it was probably quite large, judging from the assembly of a computer monitor and, thus, was only applicable to large pinnipeds. Subsequently, Ponganis et al. (34) produced a more compact blood sampler (1.25 kg, 24 cm × 8.5 cm OD) for emperor penguins. The sampler collected one blood sample using two samplings; one for waste and the second for the sample, and was programmed to commence sampling at a specified depth or after a prefixed time interval. However, changes in plasma hormone concentrations were not measured in the samples collected using these devices.

In the present study, we have developed a downsized blood sampler, which reliably collects blood samples from free-diving, small seals by utilizing rapidly developing electronics technology. The sampling can be triggered by preset parameters that are detectable with the electronic sensors (currently using hydrostatic pressure, posture, temperature, and time), and additional parameters can be added if suitable sensors are available (e.g., salinity). Furthermore, two test samples of 5 ml can be collected by the three syringes (two samples and a waste) with <1% contamination of heparinized saline or the previous blood sample, most of which are removed from the vascular catheter and sampler tubing by the larger waste syringe. Thus, we expect this sampler to be applicable to various species in the wild, although several improvements are currently being developed, such as the use of a pressure-resistant electromagnetic valve for deeper-diving animals, as discussed below.

The success rate of blood collection by the sampler was 78% in 50 trials. As sampling was carried out not only in water but also on land in this study, we set a negative pressure inside the device for smooth suction from the vein within 2 min of the maximum sampling time. We found that most of the failures in blood sampling were not due to blood clotting in the sampling circuit but probably due to the closure of the catheter at its tip by contact with the vascular wall. Thus, it is important to regulate the negative pressure generated by evacuation before deploying the sampler. Indeed, negative pressure may not be necessary when blood is collected from the diving seals. Triggering of sampling at depth during diving is another important feature for future versions of the device. As the current experiment was carried out in a pool of <1.6 m depth, we used a valve that is resistant to <10 m depth. As phocid seals can dive to >100 m in the wild, a valve that can withstand these depths must be substituted when applying it to free-swimming seals. The aluminum case of the current sampler design can resist >100 m depth.

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Effects of Blood Collection by the Sampler on Stress Hormones

Cortisol is the major glucocorticoid in phocid seals as in other carnivores (7, 13, 14, 28). We confirmed this using HPLC analyses in a female grey seal. However, a significant amount of cortisone was also present in the plasma of a male grey seal, a finding that has also been reported in humans (29). The reason for the difference between the two individuals is not known, but may be related to higher absolute plasma levels of glucocorticoids in the male seal.

The concentration of ACTH in the plasma was significantly lower in the blood collected using the sampler than that collected directly from the animal. Plasma cortisol concentrations were also lower when collected using the sampler, but the difference was not statistically significant. However, it is likely that animals are under less external stress using the sampler than during direct collection. Plasma cortisol concentrations have been measured in various species of pinnipeds (8, 14, 25, 43). In two subspecies of harbor seals, the concentration exhibits a circadian rhythm with higher levels in the morning (13, 28), as is found in many other mammalian species (20). In this study, blood was collected during morning hours, and the concentrations measured were similar to those reported in the previous studies in the same species (13, 28).

Cardiovascular Hormone Levels When Seals Are on Land or in Water

ANG II has been recognized as an active component of the renin-angiotensin system (RAS), but emerging evidence suggests that NH2-terminally truncated forms, ANG III and ANG IV, are also involved in the RAS (11). In this study, we found that the major circulating angiotensin in the grey seal is not ANG IV, are also involved in the RAS (11). In two subspecies of harbor seals, the concentration exhibits a circadian rhythm with higher levels in the morning (13, 28), as is found in many other mammalian species (20). In this study, blood was collected during morning hours, and the concentrations measured were similar to those reported in the previous studies in the same species (13, 28).

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Perspectives and Significance

The primary significance of this study is the development of a compact and reliable blood sampler with data-logging functions, which enables the collection of experimental and control blood samples without contamination by luminal fluids remaining in the tubing, under minimal handling stress. In the future, the sampling regime can be triggered by a variety of signals that are detectable by additional sensors on the device. Although the sampler needs further improvement before use in the wild and on smaller animals such as fish, deployment of the animal-borne blood sampler on free-ranging animals will open up new possibilities within bio-logging science and behavioral physiology.

The additional significance is the evolutionary perspective in the regulation of cardiovascular hormone secretion. Aquatic animals are almost free from the effects of gravity on blood circulation due to the buoyancy of water. Accordingly, fully aquatic cetaceans do not experience the cardiovascular regulation against gravity during their life span. Therefore, the
upregulation of ANP and downregulation of ANG II and AVP after water immersion, typically observed in humans, is absent in cetaceans. However, some regulation still exists in the semi-aquatic phocid seals, as suggested in this study. Pinnipeds regularly transit from a terrestrial to an aquatic habitat, which may have influenced the nature of their hormonal system for cardiovascular control.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
Y.T., I.S., K.S., and A.H. conceived and designed the research; Y.T., I.S., M.K.-S.W., S.M., and A.H. performed experiments; Y.T., I.S., M.K.-S.W., and A.H. analyzed data; Y.T., I.S., M.K.-S.W., K.S., and A.H. interpreted results of experiments; Y.T., I.S., and M.K.-S.W. prepared figures; Y.T. drafted manuscript; Y.T., K.S., and A.H. edited and revised manuscript; Y.T., I.S., M.K.-S.W., and A.H. approved final version of manuscript.

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