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

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Running title: Cardiovascular hormones in submerged marine mammals

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Abstract

An animal-borne blood sampler with data logging functions was developed for phocid seals which collected two blood samples for the comparison of endocrinological/biochemical parameters under two different conditions. The sampler can be triggered by preset hydrostatic pressure, acceleration (descending or ascending), temperature and time, and also manually by light. The sampling was reliable with >78% successful attempts to collect blood samples. Contamination of fluids in the tubing to the next blood sample was <1%, following the prior clearance of the tubing to a waste syringe. In captive harbor seals (*Phoca vitulina*) the automated blood sampling method was less stressful than direct blood withdrawal as plasma levels of stress hormones were lower in the former ($p<0.05$ for ACTH and $p=0.078$ for cortisol). HPLC analyses showed that both cortisol and cortisone were circulating in seal blood. Using the sampler, plasma levels of cardiovascular hormones, atrial natriuretic peptide (ANP), arginine vasopressin (AVP), and angiotensin II (AngII), were compared in grey seals (*Halichoerus grypus*), between samples collected when the animals were on land and in the water. HPLC analyses determined that [Met^{12}] ANP (1-28) and various forms of angiotensins (AngII, III and IV) were circulating in seal blood. Although water immersion profoundly changes the plasma levels of cardiovascular hormones in terrestrial mammals, there were only tendencies towards an increase in ANP ($p=0.069$) and a decrease in AVP ($p=0.074$) in the seals. These results suggest that cardiovascular regulation in phocid seals may have undergone adaptation during evolution of the carnivore to a semi-aquatic lifestyle. (249 words)

Keywords: data logger, sea mammal, stress hormone, cardiovascular hormone, gravity

Introduction

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. Due to 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 adrenocorticotropic hormones (ACTH) and cortisol, or metabolic hormones such as thyroid hormones (T3 or T4), insulin-like growth factor I (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), angiotensin II (AngII) and aldosterone have also been measured[31-33,47,48]. Thus, whilst 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 AngII and AVP are more important than vasodepressor hormones such as ANP and adrenomedullins for high blood pressure maintenance in terrestrial 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 AngII concentrations did not change after stranding[27]. The complete adaptation of cetaceans to a fully aquatic lifestyle may explain the loss of a response to gravity. It is therefore 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 after 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 high performance liquid chromatography (HPLC), were also characterized. Finally, an initial study to compare plasma concentrations of ANP, AngII 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 radioimmunoassay (RIA) developed in our laboratory, and the major molecular form of ANP and AngII 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 x 8.6 cm o.d.) 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 (i.d.: 1 mm, o.d.: 3 mm, Tigers Polymer Co. Ltd., Osaka, Japan) to the inlet of the device (Fig. 1B). The access to each syringe was regulated by a valve (PSK-1015NC, Takasago Electric, Inc. Aichi, Japan), which could be activated by pre-programmed 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 accelerometry (Fig. 2). The internal pressure of the sampler was kept negative by vacuuming the air from the case to allow smooth blood sucking 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 Inc., 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 4 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 was shown in Fig. 3. The sampler was operated by four lithium batteries (CR123A). The device was connected to a vascular catheter (Instech Solomon CBAS C70 7Fr 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, USA).

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, respectively. They were anesthetized using a combination of midazolam (Hypnovel, Roche Products Ltd, 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 extradural vein using a 18G needle into a tube containing a protein inhibitor cocktail containing 0.05 M 1,10-phenanthroline, 0.225 M potassium EDTA, and 0.1 trypsin inhibitor unit (TIU) aprotinin (30 μl/ml blood) to obtain control data for comparison with the blood collected by the sampler. While anaesthetized, the heparin coated catheter (Linton Instrumentation) was inserted using a Dispomedica (Dispomedica GmBH, Denmark) 8Fr peel away sheath introducer into the extradural vein. Two catheters of different sizes (i.d./o.d.: 3/5 mm or 4/6 mm) were tested for more efficient blood sampling. The catheter was kept from coagulating at the luer connector with heparinized saline (10 U heparin/ml 0.9% NaCl) and was connected to the blood sampler via 3-way stopcock (Fig. 1A). The blood sampler was attached to the back of the seal via a Velcro patch glued to the fur using superglue (Loctite 422, Loctite, Dusseldorf, Germany) and further fixed by cable ties. The sampler was retrieved by sedating the animal within 15 min after the second sampling. All the studies were licensed under the Animal (Scientific Procedures Act) 1986 by the UK Home Office issued to SMRU, Project License number 70/7806.

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 acetone (acetone: water: 1 M HCl = 40: 5: 1), centrifuged at 12,000 rpm for 5 min in a micro refrigerated centrifuge (Model 3700, Kubota Corp., Tokyo) and the supernatant was freeze-dried. The residue was reconstituted in 1 ml of 40% acetonitrile, and subjected to a reverse-phase ODS-120T column (4.6 x 250 mm, Tosoh Corp., 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 Inc., Oxford, MI, USA), was determined in this HPLC condition using authentic steroids as standard.

Characterization of seal ANP and angiotensins in plasma. Initially, identity of seal ANP to human [Met^{12}] 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 Ang peptides, the relative amounts of AngII and its N-terminal truncated forms (AngIII and AngIV) 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 AngII was raised against human [Ile^5] AngII, but seal AngII was [Val^5] AngII as mentioned, and the cross-reactivities to [Val^5] AngII, [Val^5] AngIII (AngII-(2-8)) and [Val^5] AngIV (AngII-(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 (ANP, AngII and AVP). For the stress hormone analyses, the two blood samples were collected 1 h apart more than 3 h after the catheterization, and the samples were collected when the seals were on land. For the cardiovascular hormone analyses, the first blood was collected more than 3 h after the seal was moving freely on the available haulout land area. The door to the pool was opened and the second blood was collected 1 h after the animal entered the water. The seal was in the pool in most of the second sample collections, which were used for comparison with the concentrations collected while the animal was on land. The plasma samples were extracted using acidic acetone, freeze dried, and subsequently measured by RIA for ANP[24], AngII[49] and AVP[18] established after iodination of each peptide. EIA kits were used for measurement of cortisol (IBL International GMBH, Hamburg, Germany) and ACTH (MD Bioproducts, St. Paul, MN, USA) according to the manufacturer's instruction. The cross-reactivity of this cortisol EIA for cortisone was low (4.2%). The antiserum used for the ACTH EIA was directed to the N-terminal 23 amino acids, which are identical in all mammals thus far examined.

Statistical analyses. All values are expressed as means ± SE of the mean. 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 one attempt out of fourteen failed using the larger catheter, while three out of eighteen 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 (i.d.: 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 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.5A, B). Thus the major glucocorticoid that responds to stress (ACTH) in phocid seals is cortisol, not corticosterone.

Angs and ANP. Elution positions of AngIII and AngIV were very close even after the best separable conditions using HPLC (Fig. 5C). [Val^5] AngII and AngIII/IV were identified in the plasma of the male grey seal, but AngIII/IV was the major form in that

of the female seal. Therefore, it is apparent that significant amounts of AngIII and AngIV were circulating in the seal blood in addition to AngII. Seal ANP was eluted at the position of [Met^{12}] ANP (1-28) but not that of [Ile^{12}] ANP after HPLC separation (data not shown). Thus, the seal has [Met^{12}] ANP as in other carnivores.

Effect of blood sampling on plasma stress hormones

In order 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. 7A, B). Plasma AngII 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). Thus the blood was collected by the sampler in this series of experiments was 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 that 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 underwater in animals in captivity[19,21]. Therefore, studies in free-ranging

315 animals under minimal stress are required in order to understand the true nature of the
316 cardiovascular response, particularly to various forms of external and environmental
317 stress in marine mammals.
318 Previous efforts have been dedicated to collecting blood automatically from free-
319 ranging animals underwater without stress. For example, Hill[16] was the first to develop
320 a microcomputer-assisted, back-pack blood sampler, which allowed blood to be
321 collected at depth in Weddell seals (300-400 kg body mass). The blood was withdrawn
322 by a pressure-resistant peristaltic pump that was reversible to flush the blood in the
323 circuit (i.e. in the tubing and dead space) in the case of multiple blood samplings. The
324 blood collection could be triggered by pressure and/or time during either the ascending
325 or descending phase of the dive through a microcomputer monitor. The size and weight
326 of the sampler were not described, but it was probably quite large judging from the
327 assembly of a flush reservoir (1 liter), pump, sample collector and microcomputer
328 monitor and thus was only applicable to large pinnipeds. Subsequently, Ponganis et
329 al.[34] produced a more compact blood sampler (1.25 kg, 24 cm x 8.5 cm o.d.) for
330 emperor penguins. The sampler collected one blood sample using two samplings; one
331 for waste and the second for the sample, and was programmed to commence sampling
332 at a specified depth or after a prefixed time interval. However, changes in plasma
333 hormone concentrations were not measured in the samples collected using these
334 devices.
335 In the present study, we have developed a downsized blood sampler which reliably
336 collects blood samples from free-diving, small seals by utilizing rapidly developing
337 electronics technology. The sampling can be triggered by preset parameters that are
338 detectable with the electronic sensors (currently using hydrostatic pressure, posture,
339 temperature and time), and additional parameters can be added if suitable sensors are
340 available (e.g., salinity). Furthermore, two test samples of 5 ml can be collected by the
341 three syringes (two samples and a waste) with <1% contamination of heparinized saline
342 or the previous blood sample, most of which are removed from the vascular catheter and
343 sampler tubing by the larger waste syringe. Thus, we expect this sampler to be
344 applicable to various species in the wild, although several improvements being
345 developed, such as the use of a pressure-resistant electromagnetic valve for deeper
346 diving animals as discussed below.
347 The success rate of blood collection by the sampler was 78% in 50 trials. As
348 sampling was carried out not only in water but also on land in this study, we set a
349 negative pressure inside the device for smooth suction from the vein within 2 min of the
350 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.

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 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] and in two subspecies of harbor seals exhibit 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

AngII has been recognized as an active component of the renin-angiotensin system (RAS), but emerging evidence suggests that N-terminally truncated forms, AngIII and AngIV, are also involved in the RAS[11]. In this study, we found that the major circulating Ang in the grey seal is not AngII but AngIII or AngVI using the HPLC analysis. In other mammalian species, significant amounts of AngIII and AngIV have been detected in plasma in addition to AngII, and their ratio increases in disease states such as atherosclerosis[30]. We also identified [Met^{12}] ANP in seal plasma using the HPLC analysis. This agrees well with the phylogenetic position of pinnipeds[2], which is

closely related to the black bear (*Ursidae*) that has [Met[12]] ANP as deduced from its genome database (data not shown).

In order to test the performance of the blood sampler, we examined the change in plasma cardiovascular hormones, ANP, AngII and AVP after they entered the pool and compared the concentrations with when they were hauled out on the land area. In humans, plasma ANP concentrations profoundly increase and plasma AngII and AVP concentrations decrease following head-out water immersion[9]. It is well known that there is a central shift in blood volume in humans during water immersion, which induces atrial distension to increase ANP secretion and reduce perfusion pressure at the afferent arterioles of the kidney glomerulus thus decreasing renin secretion. AVP secretion decreases through the inhibitory signal from the stretch (volume) receptors in the venous circulation, as well as through changes in plasma ANP and AngII levels. ANP and AngII are the potent inhibitory and stimulatory hormones, respectively, controlling AVP secretion[1].

In contrast to the profound responses of ANP, AngII, and AVP to gravitational effects in humans, we could not detect any changes in plasma ANP, BNP and AngII concentrations in the bottlenose dolphin after stranding. This result indicates that the regulation of cardiovascular hormone secretion is fully adapted to the aquatic environment where gravity effect is almost nullified in totally aquatic cetaceans[27]. In this study we could not detect significant differences in the cardiovascular hormone levels when seals were on land or in water, but there was an apparent tendency toward an increase in the ANP level and a decrease in the AVP level in the grey seal. It seems that seals may still retain some gravitational responses in cardiovascular hormone secretion as they spend a large proportion of their time on land during the breading season and regularly haul out during other times of the year[15].

Several studies have examined the plasma levels of cardiovascular hormones in pinnipeds. Hochachka et al.[17] reported a change in plasma concentrations of catecholamines (epinephrine and norepinephrine for vascular contraction) and cGMP (a marker for nitric oxide production for vascular relaxation) after voluntary diving in the cannulated Weddell seals that returned to the same ice hole for breathing. They found that plasma catecholamines increased as a function of dive duration for splenic red cell sequestration and then rapidly recovered in parallel with the increase in cGMP. Zenteno-Savin and Castellini[47] measured plasma ANP, AngII and AVP concentrations in several species of pinnipeds and found species, geographic and developmental variations. They also found an increase in plasma ANP and a decrease in plasma AngII and AVP after apnea in elephant seal pups and Weddell seal pups when they are on land[48].

Prolonged fasting in postweaned seal pups is accompanied by adipsia, which may affect water balance and change plasma ANP, AngII and AVP as these hormones are not only cardiovascular hormones but are also osmoregulatory hormones controlling water balance, urine formation and mineralocorticoid secretion[40]. In the fasting, postweaned elephant seal pups, plasma renin activity and aldosterone concentrations increased during the fasting periods but not plasma AVP[31]. Interestingly, AVP administration to the pups induced diuresis and natriuresis[33], which suggests that suppressed AVP may help maintain water and electrolyte balance during adipsia.

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 future, the sampling regime can be triggered by a variety of signals which 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 a 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 cardiovascular function when in water, as they do not need to circulate blood against gravity. This results in low arterial pressure, as has been shown in fish that have average arterial pressures of 20 mmHg. Therefore, the upregulation of ANP and downregulation of AngII and AVP after water immersion, typically observed humans, is absent in cetaceans but some regulation still exists in the semi-aquatic phocid seals. Pinnipeds regularly transition from a terrestrial to an aquatic habitat which has influenced the nature of their hormonal system for cardiovascular control.

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Author contributions

Y.T., I.S., A.H. and K.S. designed research; I.S., M.K.S.W., R.M., S.M., A.H. and Y.T. performed experiments; I.S. and Y.T. analyzed data; Y.T., I.S., M.K.S.W. and A.H. interpreted the data; A.H. and K.S. edited manuscript; Y.T. drafted manuscript; all authors approved final version of manuscript.

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Table 1. Sampling periods in seconds (Mean ± SEM) to fill each syringe with blood from two grey seals (HG1 and HG 2) and a harbor seal (PV). Only the samples that were collected with an accurate volume (5 ml) in 2 min are shown (HG1 and PV catheter o.d. = 2.3 mm, HG2 catheter o.d. = 1.2 mm).

	First sampling			Second sampling		
Animal	No	Waste	Sample	No	Waste	Sample
HG1	6	0.7±0.4	5.0±0.6	6	9.5±0.7	5.0±0.4
HG2	7	9.9±3.3	31.7±1.6	7	52.0±4.4	32.7±1.8
PV	6	0.5±0.5	1.5±0.3	5	8.4±1.5	3.4±0.4

Figure Legends

Figure 1. Schematic drawing of the blood sampler. (A) General organization of the device showing its major parts and casing. The aluminum case was 5 mm thick with corrosion-resistant treatment on the external surface, which is resistant at >100 m depth. Although the current valve was resistant to <10 m depth, the experiments with deeper diving animals become possible if a pressure-resistant electromagnetic valve is substituted. (B) Blood sampling system showing on/off valves and tube circuits after the first blood sampling. As blood was collected into the waste syringe just prior to the sample syringe and contamination of the test samples by heparinized saline was negligible. The dead space of the sample syringes was filled with an inhibitor cocktail. CP, catheter port; DS, depth (pressure) sensor; PS1, photosensor for external signal; PS2-4, photosensor to stop plunger.

Figure 2. Electric circuit for signal transduction of the blood sampler. (A) Programmable control system for triggering the sampler. After turning on the timer, the sampling was triggered by a preset timer, depth or acceleration, or light. The first sampling can also be triggered by the maximum depth if the same depth continues for 3 sec during descent, and the second sampling by the depth during ascent (e.g., when the animal reaches the surface). The maximum depth of this device is limited by the pressure resistance of the valves used. (B) Microcomputer-based control circuit of the sampler. After retrieval from the animal, the data in the memory are downloaded for analysis. UART, Universal Asynchronous Receiver Transmitter.

Figure 3. Photograph of the case and inside view of the blood sampler after the first blood sampling. Blood filled half the waste syringe. For detailed organization of the device, see Fig. 1A.

Figure 4. An example of time series data of depth, temperature, acceleration and valve status recorded in the memory of blood sampler. The recorded longitudinal-axis (broken line) was low-pass filtered to extract the static acceleration (solid line) which was used to calculate the pitch angle of the animal's body. The valve status indicates which valve was open (0, closed; 1, sample syringe 1; 2, sample syringe 2; 3, waste syringe). The valve of waste syringe opens just before the second sampling to clear blood in the tubes before the valve of sample syringe 2 opens. Each valve is turned on and off every five seconds to avoid closure of the catheter tip by the vessel wall.

Figure 5. HPLC profiles of glucocorticoids in the plasma of (A) a female and (B) a male grey seal. Arrows show elution positions of (a) cortisone, (b) cortisol, (c) corticosterone, and (d) 11-deoxycortisol. (C) HPLC profiles of angiotensins in the plasma of a female (open circle) and a male (closed circle) grey seal. Arrows show elution positions of (a) AngII, (b) AngIII and (c) AngIV. The peak height of each steroid and Ang was corrected for cross-reactivity to the antiserum used in the assay.

Figure 6. Plasma adrenocorticotropic hormone ACTH (A) and cortisol (B) concentrations in a harbor seal when blood was collected directly from the animals (Captured, n=6) or using the blood sampler (Free, n=12). The seal was hauled out on the land area when blood was collected by the sampler in both groups. Values are means $\pm$ SEM. *$p<0.05$.

Figure 7. Comparison of (A) atrial natriuretic peptide (ANP), (B) arginine vasopressin (AVP), (C) angiotensin II (AngII), (D) adrenocorticotropic hormone (ACTH), and (E) cortisol concentrations in seal plasma collected by the sampler when seals were on land (Land) or in water (Water). Plasma for ANP, AVP and AngII were collected from grey seals (n=8 for both Land and Water) and plasma for cortisol and ACTH was collected from a harbor seal (n=7 for Land and n=6 for Water). Values are means $\pm$ SEM. *$p<0.05$.

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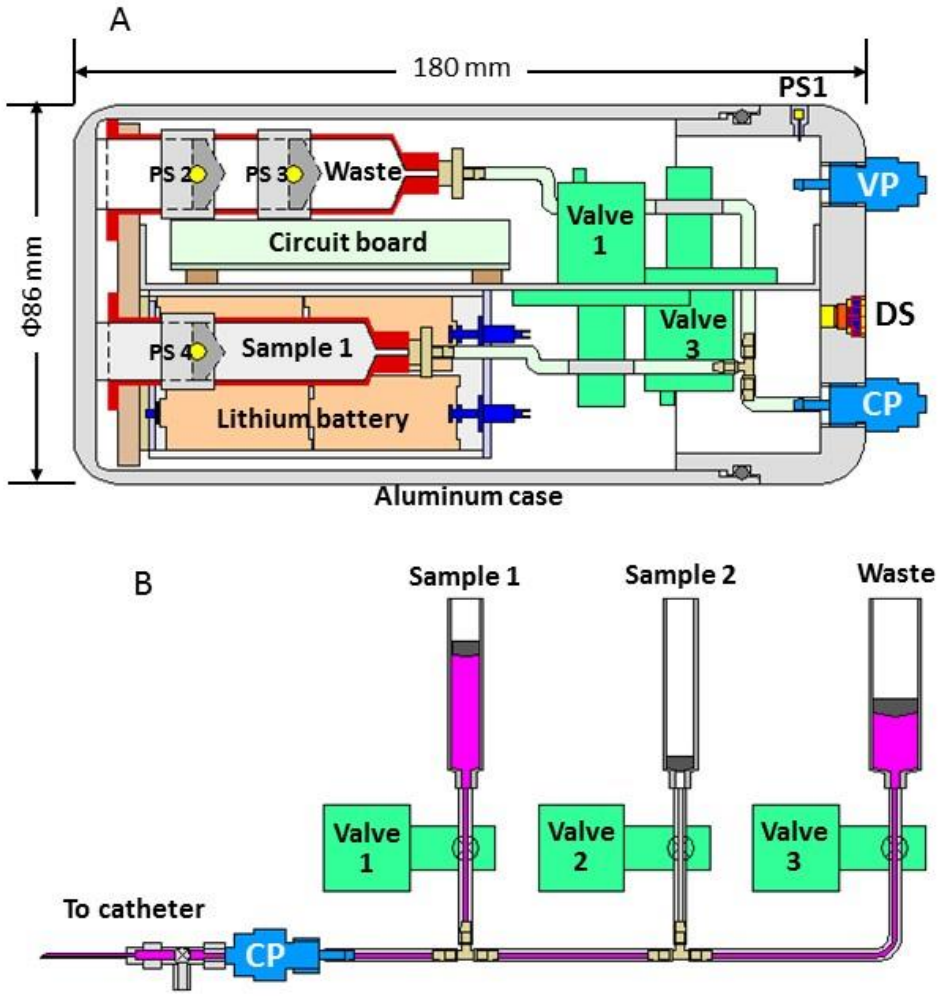

Fig. 1 Takei et al.

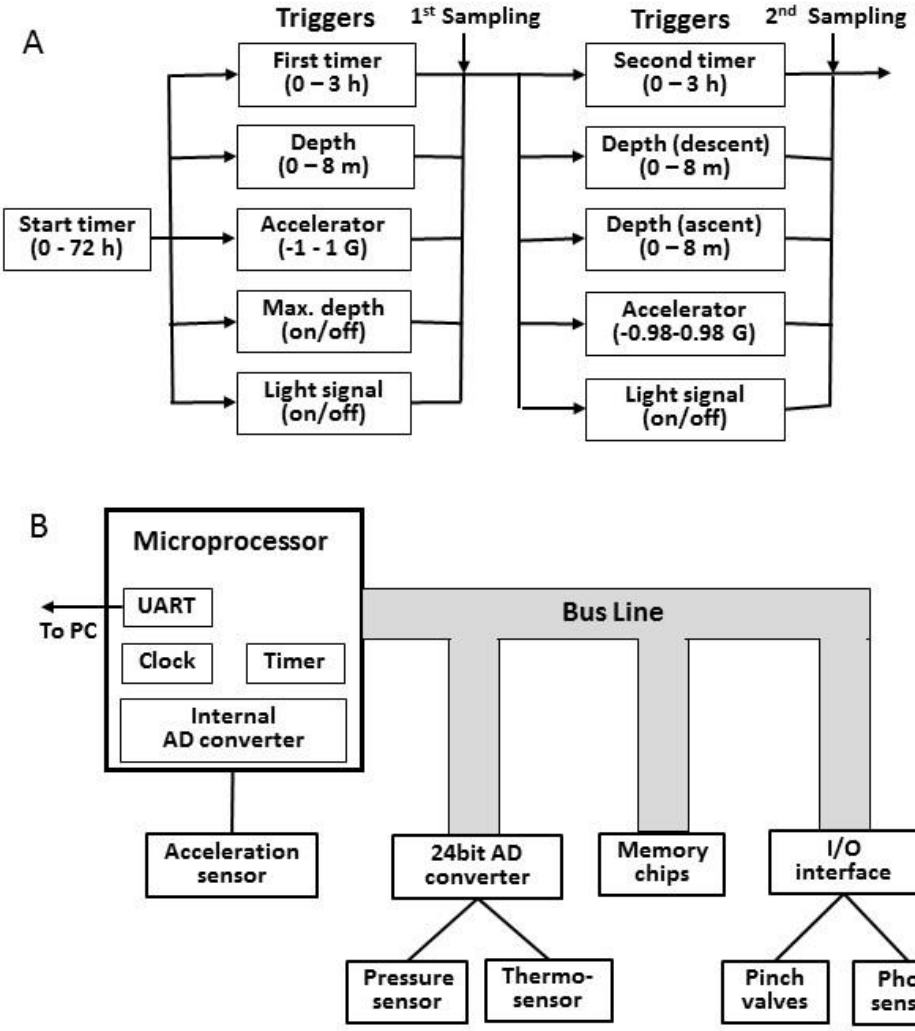

Fig. 2 Takei et al.

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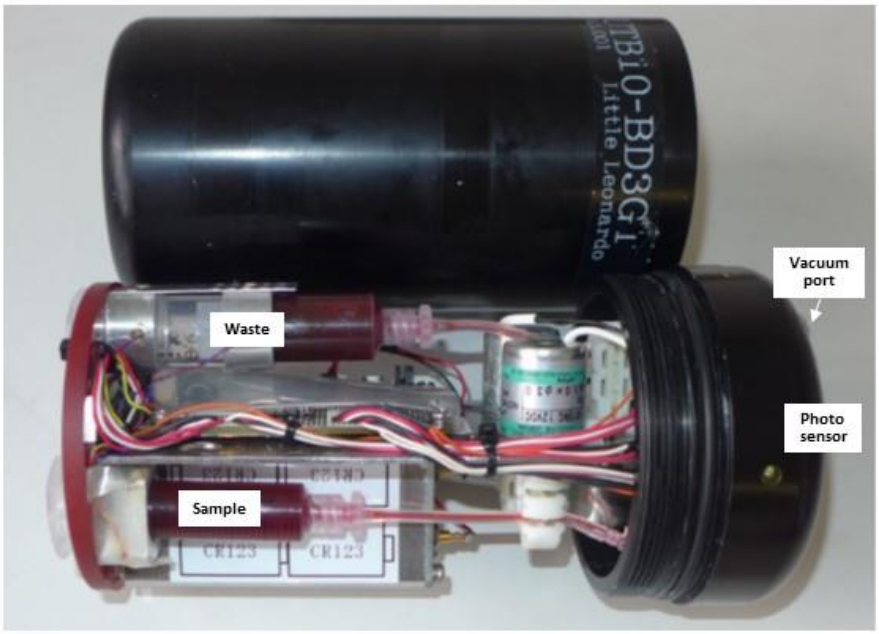

Fig. 3 Takei et al.

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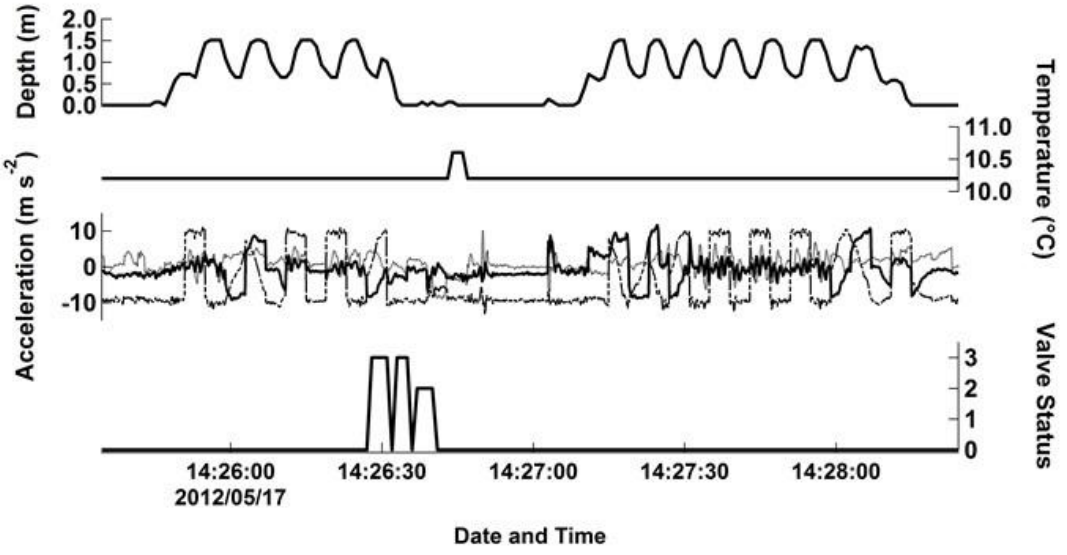

Fig. 4 Takei et al.

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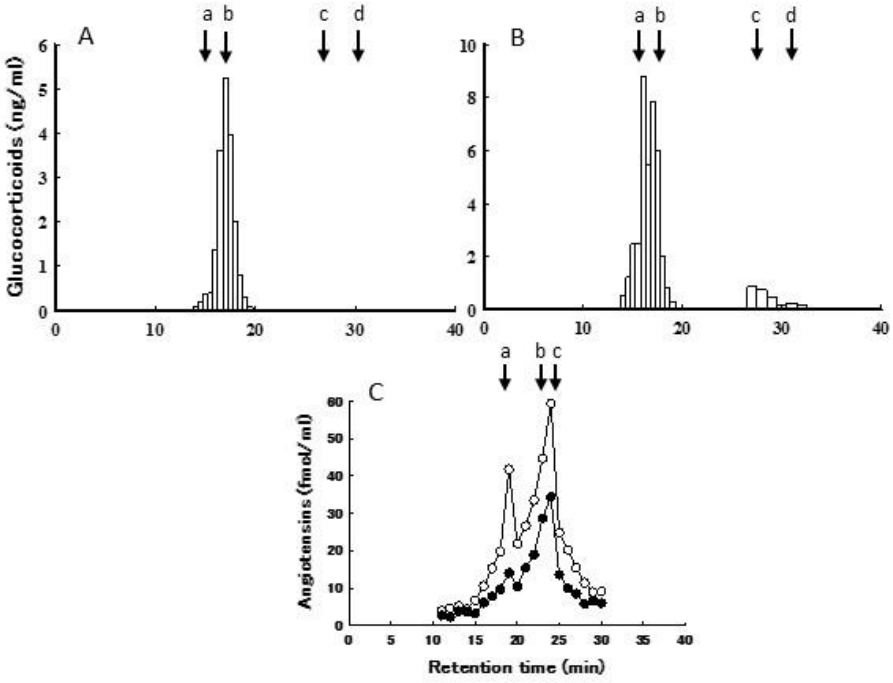

Fig. 5 Takei et al.

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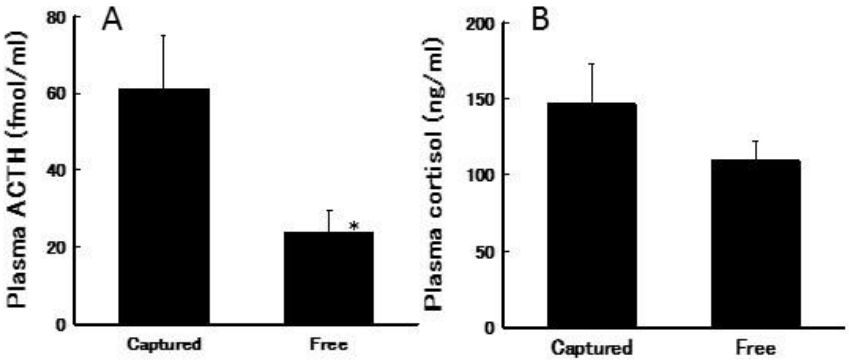

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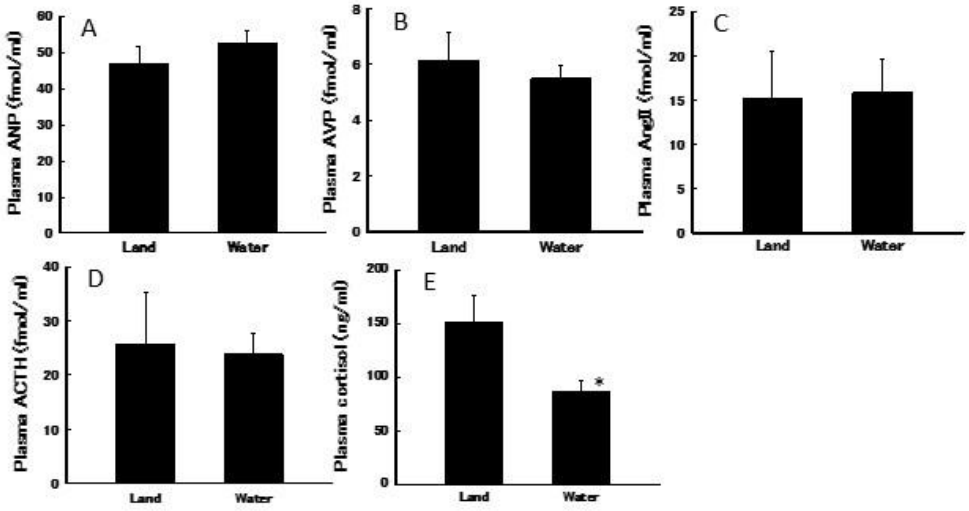

Fig. 7 Takei et al.

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