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PHYSIOLOGY OF THE ENDOCRINE, CARDIORESPIRATORY AND NERVOUS SYSTEMS IN PINNIPEDS. INTEGRATIVE APPROACH AND BIOMEDICAL CONSIDERATIONS

A

THESIS

Presented to the Faculty

of the University of Alaska Fairbanks

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for the Degree of

DOCTOR OF PHILOSOPHY

By

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Fairbanks, Alaska

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PHYSIOLOGY OF THE ENDOCRINE, CARDIORESPIRATORY AND NERVOUS SYSTEMS IN PINNIPEDS. INTEGRATIVE APPROACH AND BIOMEDICAL CONSIDERATIONS

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ABSTRACT

This thesis explored several aspects of the hormonal and cardiovascular physiology in pinnipeds (seals and sea lions). Plasma concentrations of the vasoactive hormones angiotensin II (Ang II), arginine vasopressin (AVP, the antidiuretic hormone) and atrial natriuretic peptide (ANP) were studied in six species of seals and sea lions. Resting levels of AVP, ANP and Ang II in these pinnipeds were similar to those reported for other vertebrate species, including humans. Age-related differences were found in the concentrations of these hormones in seals and sea lions. Geographic differences in hormone concentrations were found in Steller sea lions and harbor seals.

To address the endocrine and cardiovascular responses to breath-holding (apnea) in marine mammals, heart rates and plasma levels of Ang II, AVP and ANP were studied in Weddell seal (*Leptonychotes weddellii*) and northern elephant seal (*Mirounga angustirostris*) pups during periods of spontaneous breathing (eupnea) and apnea. Ang II, AVP, and ANP, as well as the autonomic nervous system, were found to contribute differently to the control of heart rate in seal pups, depending whether the respiratory system was in eupnea or apnea. Because of changes in seals of different ages, it appeared that the integration of cardiorespiratory and hormonal function is not fully mature at birth, but develops post-natally, probably simultaneously to the development of diving behavior. These studies also suggested that the factors affecting cardiorespiratory function, including hormones, may differ by species.

Plasma concentrations of AVP, ANP and Ang II were measured during food limitation and fasting in captive Steller sea lions (*Eumetopias jubatus*) and compared to levels in free-ranging conspecifics. The results suggest that Steller sea lions have a remarkable capacity to maintain hydrosmotic and endocrine balance during short-term food limitation and fasting. Hormonal studies did not provide conclusive evidence that Steller sea lions in Alaskan waters are currently affected by long-term food limitation.

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This thesis is dedicated to the memory of my grandfather,
Fidel de la Vega y Diaz, my light, my song, my spirit.

1. *INTRODUCTION*

The structure (anatomy and histology) and function of the marine mammal endocrine system appear to fit a general mammalian model (for a review see 40). However, little is known about the cellular mechanisms of action of some of the major hormones in marine mammals, including their biosynthesis rate, half-life, control of secretion, receptors, second messengers and signal transduction. Important hormonal systems, such as neurotransmitters and neuromodulators, gastrointestinal hormones and hormonal regulation of calcium homeostasis have not been studied in marine mammals. The interaction of hormones with development and metabolic regulation, and their integration with the nervous, cardiovascular and respiratory systems are areas that need to be explored, especially as they relate to the natural capacity of marine mammals for relatively prolonged periods of breath-holding and fasting.

In this project, various aspects of the role of the hormonal systems in coordinating physiological processes and maintaining cardiorespiratory homeostasis in marine mammals have been examined. I studied plasma concentrations of angiotensin II (Ang II), arginine vasopressin (AVP, the antidiuretic hormone) and atrial natriuretic peptide (ANP) in seals and sea lions. These hormones were selected for this study based on their ability to modulate resistance of blood vessels, their purported intervention in the control of cardiorespiratory function, and their participation in the maintenance of water and electrolyte balance. Of these vasoactive hormones, only AVP has been previously studied in marine mammals, and exclusively in its antidiuretic role (49, 50, 58).

A first step in studying hormonal effects and interactions with other systems is to establish baseline, or control, concentrations in the subjects at rest. I obtained plasma samples from six species of captive and free-ranging pinnipeds (seals and sea lions), to measure baseline levels of Ang II, ANP and AVP. These results are presented in Chapter 2. Differences in hormone levels among age groups, species and geographic locations

were found. Potential implications to the biology, ecology and health of these animals are discussed.

The specific role of the vasoactive hormones in cardiorespiratory function is addressed in Chapter 3. For this phase of the project, aspects of the hormonal and cardiovascular response to breath-holding (apnea) in marine mammals were studied. Blood samples to measure plasma concentrations of ANP, AVP and Ang II and heart rates were collected from Weddell seals (*Leptonychotes weddellii*) and northern elephant seals (*Mirounga angustirostris*) during periods of spontaneous breathing (eupnea) and sleep-associated apnea. Possible interactions between the vasoactive hormones, heart rate and breathing status (eupnea or apnea) are considered.

Developmental aspects of the cardiorespiratory function and hormonal contribution in phocid pups are presented in Chapter 4. During this stage of the project, age-dependent changes in heart rate and plasma levels of ANP, AVP and Ang II of Weddell seal and northern elephant seal pups during periods of spontaneous eupnea and apnea were examined.

Chapter 5 reviews the differential effects of the sympathetic and parasympathetic nervous contribution to eupneic and apneic heart rate in Weddell seal and northern elephant seal pups. Indices of the activity of each component of the autonomic nervous system were obtained from instantaneous heart rate measurements using a computer program (Coarse Grain Spectral Analysis, University of Toronto, Canada) designed and validated for humans.

Chapter 6 summarizes results from studies of hormone levels in Steller sea lions held in captivity and fed controlled diets. Changes in plasma levels of ANP, AVP and Ang II in response to food limitation and fasting are reported and compared to hormone concentrations in free-ranging Steller sea lions. Possible use of these vasoactive hormones as indicators of health and nutritional status of pinniped populations is addressed.

A general conclusion, prospects for future research, and how these results may be applied in biomedical research are presented in Chapter 7. The specific topics discussed in the individual chapters will be introduced by reviewing relevant aspects of the cardiovascular physiology of marine mammals and the characteristics of the hormonal systems (ANP, AVP and Ang II) that comprise the core of this thesis.

1.1. *Cardiovascular Adjustments to Apnea in Marine Mammals*

Seals have a high tolerance for long duration apnea associated to diving (up to 2 hours) (42) and sleep (25 minutes) (18), which, in contrast to terrestrial mammals, is not linked to pathologic disturbances. Furthermore, the changes in heart rate and blood flow distribution observed in seals during sleep-associated apnea are quite similar to those changes recorded during natural dives (18). The ability to withstand long apneic periods may be an adaptive process in seals, in which the congenital neonatal resistance to apnea is not lost during early development, as in most mammals (7, 20), but is maintained and even enhanced through adulthood.

Marine mammals reduce heart rate (bradycardia) and cardiac output, increase hematocrit and redirect blood flow in order to manage oxygen stores during apnea (17, 34, 53, 66, 67), but maintain a relatively constant blood pressure (27, 36). Selective vasoconstriction ensures that, by decreasing blood supply to kidneys, liver, and skeletal muscle (15, 28, 84), oxygen-dependent tissues (i.e., central nervous system and the brain) are maintained under optimum working conditions (53).

In marine mammals, the central control of the cardiovascular system during diving appears to be exerted primarily through the hypothalamus (8). During diving apnea, while an increase in parasympathetic activity may initiate bradycardia (14, 15), peripheral vasoconstriction may be mediated by sympathetic efferents via α-adrenoreceptors (23). In those birds and mammals which hold their breath upon expiration, removal of the excitatory influences from central inspiratory neurons and pulmonary afferent feedback

contribute to the initial bradycardia (13). At the end of the dive, cardiovascular recovery does not result merely from the removal of inhibitory and commencement of excitatory sensory stimuli (13). Sympathetic α-receptors could be involved in increasing cardiac stroke volume (30) and/or in the vasodilation occurring upon surfacing (15), perhaps including an element of sympathetic cholinergic activity (51).

1.2. *Endocrine Control of Heart Rate*

The cardiovascular system is a natural oscillator that receives input from respiration (61), blood pressure waves (12), and vasomotor activity (38). The cardiac response to these factors is mostly due to nervous transmission, primarily autonomic drive to the heart (37, 63). Only a few studies have focused on identifying contributions from vasoactive hormones in the overall pattern of heart rate control (1, 11, 26, 33). However, some of the factors involved in the endocrine control of heart rate and cardiovascular function are markedly influenced by experimental conditions, such as anesthesia, drugs and surgery, which are likely to distort our understanding of the integrated function of the circulation.

Existing data suggest an endocrine role in the control of mammalian heart rate. In terrestrial mammals, the tone of veins and arteries is, to an extent, determined by the circulating concentrations of the vasoactive hormones Ang II, AVP and ANP. While Ang II and AVP produce constriction of the vascular smooth muscle (11, 19, 64), ANP has a vasorelaxing effect (4). Vasoconstriction increases the resistance against which the heart must pump, causing a rise in blood pressure (10, 60); vasorelaxation has the opposite effects (6, 41). These changes in blood pressure, via the baroreceptor reflex and/or interactions with the nervous system, may translate into changes in heart rate and cardiac output (74). Both a direct and an indirect action of AVP to decrease heart rate have been observed (26, 45). By resetting the baroreceptor reflex to a lower pressure, AVP can elicit a large decrease in heart rate for a given increase in blood pressure (45). The action of

ANP appears to reset the baroreceptor control of the heart towards cardioinhibition in humans (78). It has been suggested that Ang II resets the baroreflex control of heart rate to a higher blood pressure, such that a given increase in blood pressure is accompanied by a mild bradycardia (82). It has also been postulated that this hormone may stimulate ventilation in dogs (55). Thus, through these effects, the vasoactive hormones can affect blood pressure, blood flow, cardiac output and heart rate both directly and indirectly.

1.2.1. *The Renin-Angiotensin System*

Renin is an internal secretion of the kidney. It is different from most hormones in that it does not act on a tissue, but on a protein in the blood, angiotensinogen, effectively acting as an enzyme (59, 62). Angiotensinogen is synthesized in the liver as a prohormone with molecular weight of approximately 58,000 daltons (79). From it renin splits off the inactive decapeptide angiotensin I (Ang I) (56, 81). Further cleavage of the dipeptide histidyl-leucine from the C terminal end of Ang I results in the formation of Ang II, a polypeptide containing eight amino acid residues (73). Synthesis of Ang II is catalyzed by angiotensin converting enzyme (ACE), and occurs primarily in the pulmonary vascular endothelium (57).

Decreased blood flow to the kidney, changes in posture, or blockade of one or both renal arteries may lead to increased production of renin by the kidney (5, 80), and therefore increased levels of Ang II (Figure 1.1). The circulating Ang II is rapidly metabolized in part by proteolytic enzymes in the blood, but also to a large degree by a poorly characterized mechanism in the peripheral vascular bed that appears to remove the peptides from the circulation. Its half-life in humans is less than one minute; renin has a somewhat longer half-life, about 30 to 60 minutes, and is catabolized mainly in the liver (54, 71).

Some of the physiological properties ascribed to Ang II are the regulation of renin secretion, renal excretory function, adrenal steroidogenesis, smooth muscle contraction

and nervous activity. All of these effects are pertinent to regulating blood volume and blood pressure (22, 57, 72, 75, 77, 81) (Figure 1.1). By a direct action on the adrenal cortex, Ang II stimulates the secretion of aldosterone and cortisol, influencing metabolism of electrolytes and water (2, 48). It also causes the release of catecholamines from the adrenal medulla and stimulates renal reabsorption of sodium, which in turn favors water retention. Angiotensin II acts on the vascular smooth muscle to produce constriction of small arteries and arterioles. This increases the resistance against which the heart must pump, causing a rise in both systolic and diastolic blood pressure (60). In addition, Ang II affects baroreceptor control of heart rate (11) and baroreflex control of lumbar sympathetic activity (65). However, there is evidence that Ang II does not exert a direct action on either the carotid sinus or aortic arch baroreceptors (33). Receptor sites for Ang II have been found on the plasma membrane of target cells in blood vessels, kidney, brain, adrenal glands, and other tissues (16, 76).

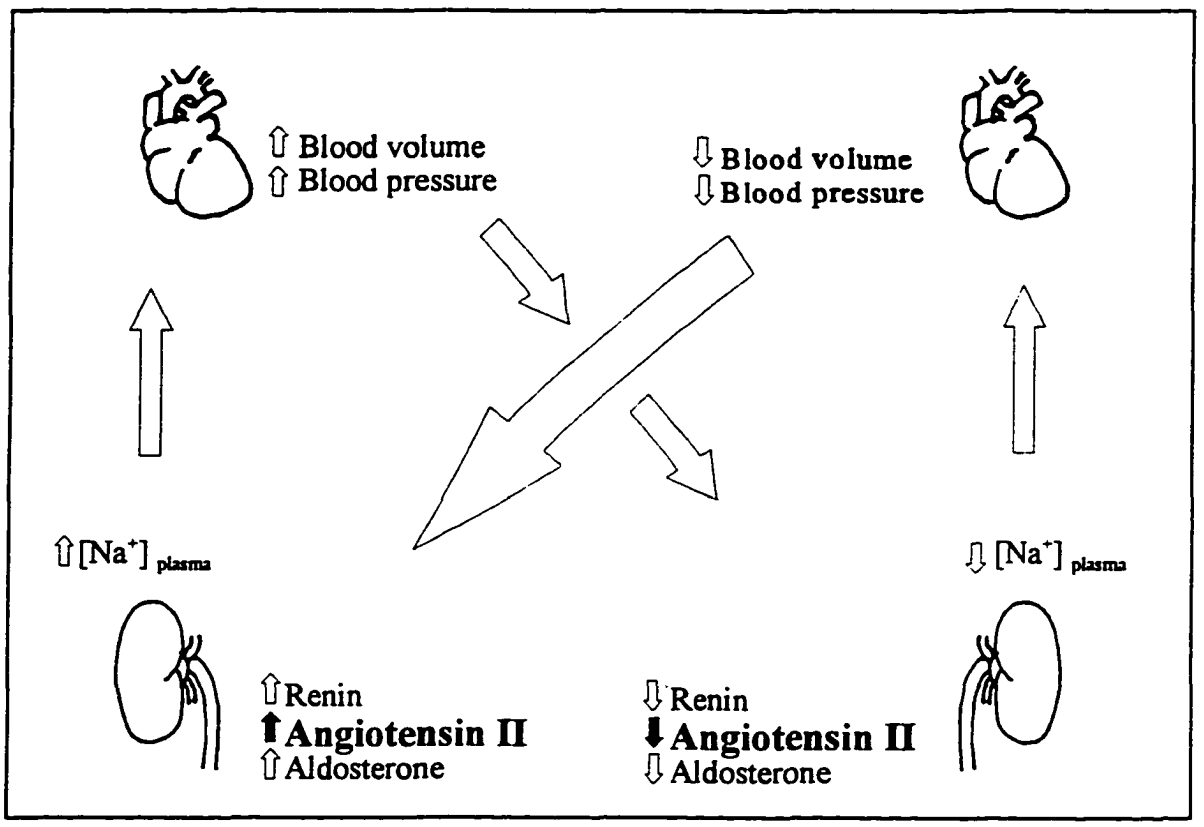

FIGURE 1.1. The role of Angiotensin II in the regulation of cardiovascular function in mammals.

1.2.2. *Arginine Vasopressin*

Arginine vasopressin, also known as the antidiuretic hormone (ADH), is a peptide with molecular weight of 1,087 daltons and half-life of 7.8 minutes in humans (25). Synthesized in the hypothalamus by proteolytic cleavage from its precursor molecule, AVP is stored in neurosecretory granules until it is released to the circulation. Secretion of AVP is stimulated by reduction in blood and extracellular fluid volume, decreased mean arterial pressure, and by increased blood osmolality (68) (Figure 1.2). Secretion of AVP may also be stimulated by insulin and hypoglycemia (86). Circulating levels of AVP are mainly controlled by extracellular fluid tonicity (24, 68). The sensitivity and osmotic threshold for AVP secretion appear to be in part genetically determined (85).

Vasopressin has two major physiological actions, contraction of vascular smooth muscle and movement of water and sodium across epithelial tissue in the distal tubule of the mammalian kidneys. This hormone has been shown to exert a variety of effects on the cardiovascular system, including increases in arterial and atrial pressures (19), decreases in heart rate and cardiac output, and changes in baroreflex function (64). These effects are mediated via V_1 receptors (44).

While AVP may not be required for a normal reflex decrease in heart rate (10), it appears to increase the sensitivity or gain of the baroreceptor reflex (65), such that it elicits a larger decrease in heart rate for any given increase in pressure (45). However, AVP does not appear to potentiate the baroreflex in rats as it does in dogs (70).

Bonjour and Malvin (9) presented evidence that AVP secretion is stimulated by increases in plasma Ang II levels. Subsequent reports demonstrated that intracerebroventricular (icv) injection of Ang II also increases AVP release (39). However, in experiments performed in anesthetized animals, intravenous (iv) infusion of a wide range of doses of Ang II consistently failed to stimulate AVP secretion (69).

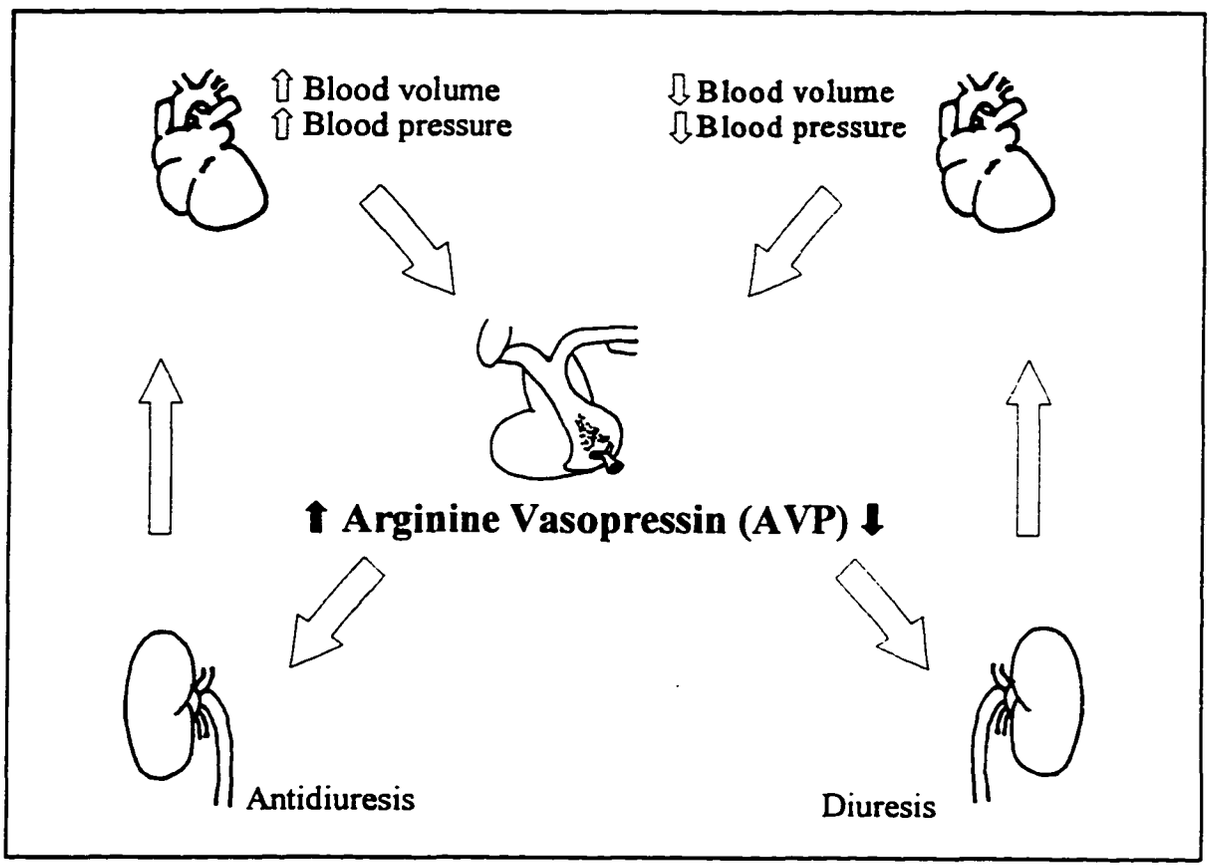

FIGURE 1.2. The role of Arginine Vasopressin in the regulation of cardiovascular function in mammals.

1.2.3. *Atrial Natriuretic Peptide*

The heart can act as an endocrine organ secreting ANP (21). ANP is a peptide composed of 28 amino acids with molecular weight of 3,062 daltons and a half-life of 3.1 minutes in humans (83). The principal source of ANP is atrial cardiocytes, where the hormone is synthesized and stored.

Secretion of ANP is stimulated by atrial distension, via mechanoreceptors sensitive to volume changes, increased central blood volume, and increased blood pressure (3, 47) (Figure 1.3). The release of ANP may also be stimulated by chronic sodium loading, changes in posture, AVP, and water immersion (29, 52).

The main physiological action of ANP is to induce diuresis and natriuresis. This is achieved via a modification of the intrarenal distribution of blood flow which increases medullary flow (31, 47). Administration of ANP evoked a marked and sustained increase in glomerular filtration rate in rats (35). Interactions of ANP with other hormonal systems include inhibition of aldosterone production, inhibition of renin release, inhibition of AVP secretion, and counteracting vasoconstrictor effects of Ang II (3, 43).

Cardiovascular actions of ANP include shifting fluid from the intravascular to interstitial compartments, thus increasing hematocrit (46), lowering cardiac output and cardiac filling pressure (41), and dose-dependent reductions in arterial blood pressure (32, 46). However, the blood pressure-lowering effect of ANP is not consistently associated with the expected reflex tachycardia (1). Therefore, it appears that the actions of ANP reset the baroreceptor control in the heart towards cardioinhibition. Some of the cardiovascular effects of ANP are accomplished by interactions with autonomic control mechanisms (78).

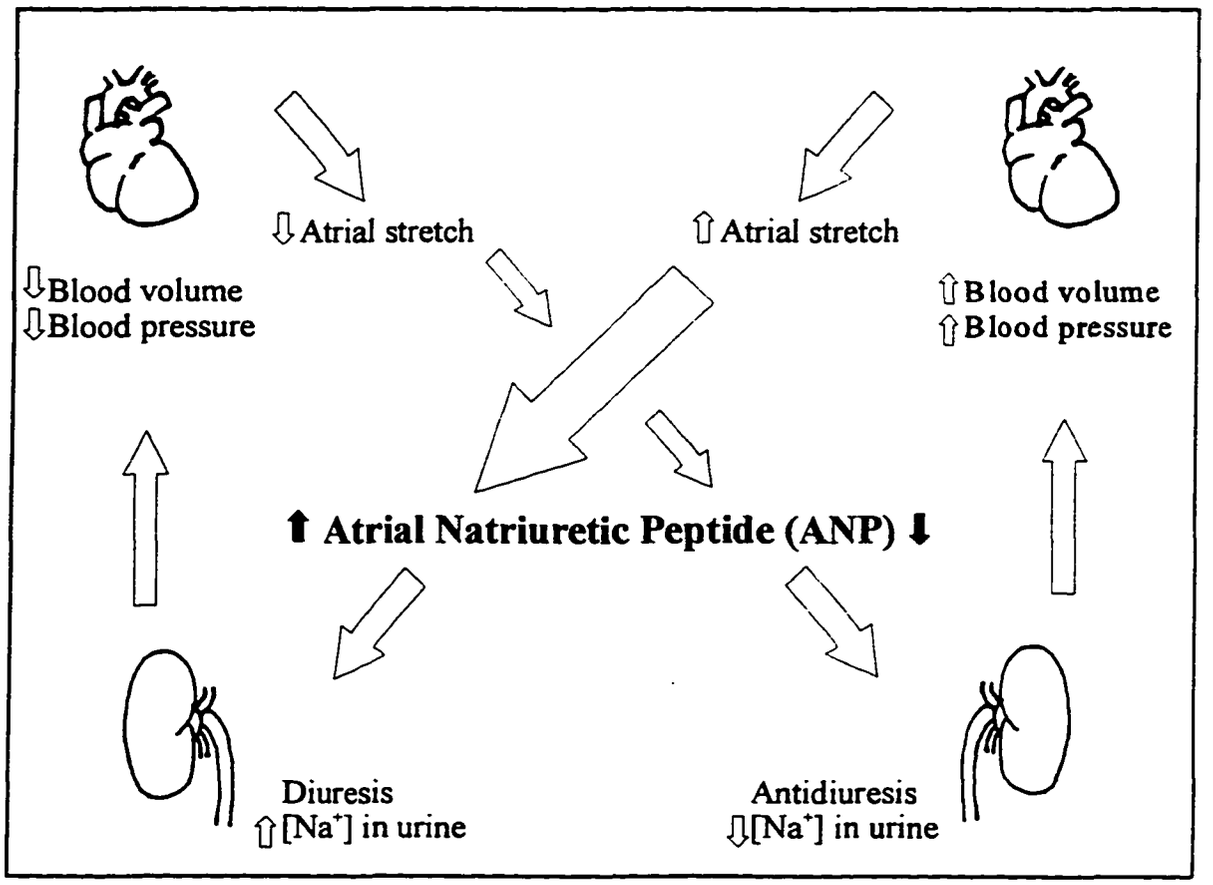

FIGURE 1.3. The role of Atrial Natriuretic Peptide in the regulation of cardiovascular function in mammals.

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2. *PLASMA ANGIOTENSIN II, ARGININE VASOPRESSIN AND ATRIAL NATRIURETIC PEPTIDE IN FREE RANGING AND CAPTIVE SEALS AND SEA LIONS.*[1]

2.1. *Abstract*

We used radioimmunoassay methods to quantify arginine vasopressin (AVP), atrial natriuretic peptide (ANP) and angiotensin II (Ang II) in plasma samples from harbor seals (*Phoca vitulina richardsii*), Weddell seals (*Leptonychotes weddellii*), northern elephant seals (*Mirounga angustirostris*), ringed seals (*Phoca hispida*), California sea lions (*Zalophus californianus*) and Steller sea lions (*Eumetopias jubatus*). Plasma concentrations of AVP, ANP and Ang II in these pinniped species were within the ranges reported for other vertebrates under resting conditions. However, there were species, geographic and developmental variations in these hormones: Levels of AVP in plasma samples from adult Steller sea lions and harbor seals were higher than in pups of the same species; higher levels of plasma ANP were found in wild captured Alaskan Steller sea lions and in hunted ringed seals; differences in plasma levels of all three hormones were found throughout the geographic distribution of harbor seals and Steller sea lions in Alaska. This is the first report on circulating concentrations of vasoactive hormones in pinnipeds, and demonstrates that further studies are needed to ascertain the natural variability in these levels with the impact of molting, fasting, diving and environmental factors.

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[1]Plasma angiotensin II, arginine vasopressin and atrial natriuretic peptide in free ranging and captive seals and sea lions. Zenteno-Savin, T. and M. A. Castellini. Comparative Biochemistry and Physiology, *in press*.

2.2. *Introduction*

As vasoactive hormones, the role of both angiotensin II (Ang II) and arginine vasopressin (AVP) is to produce constriction of vascular smooth muscles (16, 51), while that of atrial natriuretic peptide (ANP) is to antagonize such constrictor effect (7). Deviations from baseline plasma concentrations of ANP, AVP, and Ang II during exercise and water-immersion have been reported for a variety of vertebrate species. In humans and other mammals, plasma concentrations of ANP, AVP and aldosterone, and plasma renin activity increase in response to exercise in an intensity-related fashion (26, 34). During head-out immersion the plasma level of ANP increases in humans (22) and dogs (47), while AVP (14, 30, 39) and the renin-angiotensin-aldosterone systems (RAAS) (14) are inhibited. Freshwater turtles responded to water immersion and diving with changes in circulating vasoactive hormones (6) similar to those observed in humans and dogs. It is possible that Ang II, AVP and ANP participate in the control of the changes in heart rate, blood pressure and redistribution of blood flow observed in marine mammals during natural diving- and sleep-associated apnea (10, 11, 21). However, in order to explore this hypothesis it is first necessary to obtain information on the circulating levels of these hormones in marine mammals at rest. Of these hormones, only AVP has been studied in marine mammals, and mostly in its antidiuretic role (43, 44, 50). The purposes of the present study were to determine plasma concentrations of ANP, AVP, and Ang II in a variety of diving mammals and to establish potential species and geographic differences.

2.3. *Materials and Methods*

Blood samples were collected from Steller sea lions (*Eumetopias jubatus*) and harbor seals (*Phoca vitulina*) at haul-out sites and rookeries at various locations in the Aleutian Islands and the Gulf of Alaska. Weddell seals (*Leptonychotes weddellii*) were studied in McMurdo Sound, Antarctica, while northern elephant seals (*Mirounga angustirostris*) were studied at Año Nuevo State Reserve, California. Additional samples

were obtained from Steller sea lions that had been captured as pups in the wild and raised at the Vancouver Aquarium, Vancouver, British Columbia, Canada, and from harbor seals maintained at SeaWorld-Hubbs Research Institute, San Diego, CA. Samples were also obtained from California sea lions (*Zalophus californianus*) from SeaWorld-Hubbs Research Institute, San Diego, CA, and from ringed seals (*Phoca hispida*) from native hunters in Barrow, AK. We analyzed plasma samples from 181 Steller sea lions, 161 harbor seals, 6 California sea lions, 33 Weddell seals, 10 elephant seals and 5 ringed seals. Age was estimated based on morphometric data as follows: newly-born to 16 week old animals were classified as pups, yearlings were animals from the weaning period to about 1 year old, subadults were estimated to be 2 to 3 years old, and adults were estimated to be at least 4 years old. During sampling, pups and yearlings were manually restrained. Steller sea lion adults and subadults, as well as some pups, were darted with Telazol® and, if necessary, further anesthetized with either Halothane® or Telazol® (33); some Steller sea lion pups were anesthetized with Halothane®, and adult harbor seals (Prince William Sound) were anesthetized with ketamine/diazepam intramuscularly (i.m.) at standard doses (28).

Blood samples (5 ml) were taken by venipuncture from either a hind flipper vein, the extradural vein (phocid seals), or the dorsal pelvic vein (sea lions), immediately transferred into a chilled test tube containing 0.125 M ethylenediaminetetraacetic acid (EDTA) (Vacutainer 6450, Becton-Dickinson Ltd., Rutherford, NJ) and centrifuged at 4000 x g for 10 min. An angiotensin-converting enzyme (ACE) inhibitor, o-phenanthroline (0.025 M, 100 μl/ml plasma, P-9375, Sigma Chemicals, St Louis, MO) (19) was added to the recovered plasma. Samples were placed in a liquid nitrogen-cooled CryoPac shipper (-196°C) and transported to the University of Alaska Fairbanks, where they were archived at -70°C until extraction for radioimmunoassay (RIA). Plasma osmolality (Osm_{pl}) was not measured; osmometers were not available at the remote field

locations. In our hands, freezing/thawing of plasma samples significantly compromises the accuracy of the Osm_{pl} determinations.

Immunoreactive (ir) material was extracted from plasma by using prepacked octadecasilyl-silica cartridges (Sep-Col C_{18}, Phoenix Pharmaceuticals, Mountain View, CA) according to a method adapted from Hartter (31). Each cartridge was used for only one sample. The cartridge was sequentially washed with 100% methanol (glass distilled, HPLC grade, Sigma Chemicals), 90% methanol in 0.5% trifluoroacetic acid (TFA, HPLC/Spectrograde, Sigma Chemicals), and distilled water. Thawed plasma (1 to 2 ml) was slowly passed through the cartridge, which was then washed with 8 ml distilled water, and the immunoreactive material was eluted with a 90% methanol-0.5% TFA solution. The eluate was dried in a vacuum sample evaporator and concentrator (Labconco, Kansas City, MO) and stored at -20°C for RIA on the next day. Prior to analysis, samples were reconstituted to their original volume with RIA buffer (Phoenix Pharmaceuticals). The percent recovery from the extraction procedure was determined by adding known amounts of synthetic peptide (5 to 100 pg/ml, Phoenix Pharmaceuticals) to pooled plasma (quality control). Measurements of extracted plasma samples were not corrected for extraction efficiency, which ranged from 90 to 110%. Biochemical identity of the ir material obtained from pinniped plasma samples has not yet been assessed; thus, in this paper will be referred to as ANP-, AVP-, and Ang II-like ir material, accordingly.

The concentrations of AVP-, ANP-, and Ang II-like ir material in plasma samples were analyzed using commercially available RIA kits, which include antibodies raised in rabbits against the human peptides (Phoenix Pharmaceuticals). Using a second antibody, goat anti-rabbit immunoglobulin G serum, the antibody-bound material was removed. After centrifugation at 3000 x g for 30 min at 4°C, the supernatant was aspirated, and the radioactivity in the bound fraction was counted using an automatic gamma counter (Micromedic 200+, Micromedic Systems Inc., Horsham, PA). During all RIA procedures reagents and samples were kept on ice. All samples were run in duplicate, and replicates

were run within the same assay. Each assay included a standard curve generated with serial dilutions of the synthetic peptide provided by the manufacturer, and the quality control for the species for which unknown samples were being analyzed in that assay. Only values that fell within 20 to 80% of the maximum of the dose-response curve (the linear portion of the curve) were considered. All dilutions were made using RIA buffer. Cross-reactivity between antibodies and the material extracted from pinniped plasma samples was determined by assaying pooled plasma serially diluted in RIA buffer and then comparing the resulting curve with that given by the standard peptide dilutions. The amount of the pooled plasma which yielded 50% inhibition of binding of the labeled hormone to the antibody was compared with the amount of standard peptide giving the same inhibition, and expressed as percentage of that of the standard (12). This test was performed in all pinniped species for each hormone analyzed.

Data were analyzed using ANOVA followed by multiple comparison Student-Neuman-Keuls tests, and non-paired t-tests with Bonferroni adjustment for multiple comparisons (66), running the statistical software, SYSTAT® (SPSS, Chicago, IL). Significance was assumed when $P < 0.05$. Final results are presented as mean ± standard error of the mean. Information on gender and age was not available for all samples; results were analyzed and are presented for the subset where this information was procured.

2.4. *Results*

Concentrations of AVP-, ANP-, and Ang II-like ir material extracted from pinniped plasma samples are presented in Table 2.1. Results are expressed as picograms of ir material per milliliter (pg/ml) of extracted plasma.

TABLE 2.1. Plasma concentrations of arginine vasopressin (AVP), atrial natriuretic peptide (ANP), and angiotensin II (ANG II) in several pinniped species. Data are expressed as pg/ml extracted plasma and presented as mean ± SEM. N = Number of samples assayed.

SPECIES	N	AVP	ANP	ANG II
STELLER SEA LIONS				
ADULTS	20	14.2 ± 1.5	139.3 ± 7.8 [b]	55.8 ± 11.9
SUBADULTS	1	6.5	6.5	20.5
YEARLINGS	5	6.2 ± 1.7 [d]	32.0 ± 13.6 [d]	24.6 ± 4.0
PUPS	155	7.2 ± 0.4 [d]	88.3 ± 6.4 [d]	46.9 ± 3.3
TOTAL/AVERAGE	181	7.9 ± 0.4 [b]	92.0 ± 5.7	47.0 ± 3.1
CALIFORNIA SEA LIONS				
ADULTS	1	10.2	26.9	8.4
PUPS	5	4.7 ± 1.0	31.7 ± 5.4	7.6 ± 0.8
TOTAL/AVERAGE	6	5.6 ± 1.2	30.9 ± 4.5	7.7 ± 0.6
HARBOR SEALS				
ADULTS	68	15.9 ± 2.5	30.4 ± 4.4 [a,c]	29.5 ± 3.7
SUBADULTS	41	11.4 ±1.3	23.7 ± 4.5	34.2 ± 6.6
YEARLINGS	15	16.2 ± 4.0	46.8 ± 13.9	29.0 ± 9.6
PUPS	17	8.4 ± 2.0 [d]	25.3 ± 9.1 [a]	24.0 ± 5.5
UNKNOWN	20	10.2 ± 1.3	20.3 ± 2.8	20.8 ± 2.9
TOTAL/AVERAGE	161	13.3 ± 1.2 [a]	28.5 ± 2.7 [a,c]	29.0 ± 2.6 [a]

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SPECIES	N	AVP	ANP	ANG II
WEDDELL SEALS				
ADULTS	2	12.0 ± 0.1	20.3 ± 0.2	9.0 ± 0.2
YEARLINGS	10	7.5 ± 2.2	52.8 ± 3.8	13.7 ± 2.6
PUPS	21	2.6 ± 0.5	18.2 ± 2.3 [a]	17.0 ± 2.5 [a]
TOTAL/AVERAGE	33	4.4 ± 0.8 [b]	24.5 ± 3.1 [a,c]	15.6 ± 1.8 [a]
ELEPHANT SEALS				
PUPS	10	7.9 ± 4.9	23.5 ± 2.8	33.2 ± 3.4
TOTAL/AVERAGE	10	7.9 ± 4.9	23.5 ± 2.8 [a,c]	33.2 ± 3.4
RINGED SEALS				
ADULTS	5	9.3 ± 1.8	126.8 ± 38.4 [b]	14.0 ± 6.0
TOTAL/AVERAGE	5	9.3 ± 1.8	126.8 ± 38.4	14.0 ± 6.0

[a] = $P < 0.05$ compared to Steller sea lions. [b] = $P < 0.05$ compared to harbor seals. [c] = $P < 0.05$ compared to ringed seals. [d] = $P < 0.05$ compared to adults of the same species.

The sensitivity of the RIA systems (50% depression of tracer binding) for each hormone was as follows: AVP, 1.8 ± 0.2 pg/tube; ANP, 6.4 ± 0.6 pg/tube; Ang II, 4.1 ± 0.3 pg/tube (n=15). Intra-assay errors (coefficient of variation percent) were: AVP, 3.7 ± 0.1 %; ANP, 3.9 ± 0.3 %; Ang II, 3.1 ± 0.2 % (n=10). Inter-assay coefficients of variation were: AVP, 5.3 ± 0.2 %; ANP, 4.2 ± 0.4 %; Ang II, 5.6 ± 0.3 % (n=15). For all three hormones, the least detectable concentration was 0.1 pg/ml. Dilutions of all seal and sea lion species showed parallelism with the synthetic peptides provided by the manufacturer, yielding 75 to 85% cross-reactivity (Figure 2.1).

No diurnal, seasonal or annual cyclic patterns in hormone concentrations were detected because insufficient information precluded testing for effects of time of day or year. There was no statistically significant effect of gender or anesthesia on AVP-, ANP- or Ang II-like ir material concentration in any of the pinniped species analyzed. Thus, data was combined for further statistical analyses and the results presented include data for both males and females, as well as anesthetized and manually restrained animals.

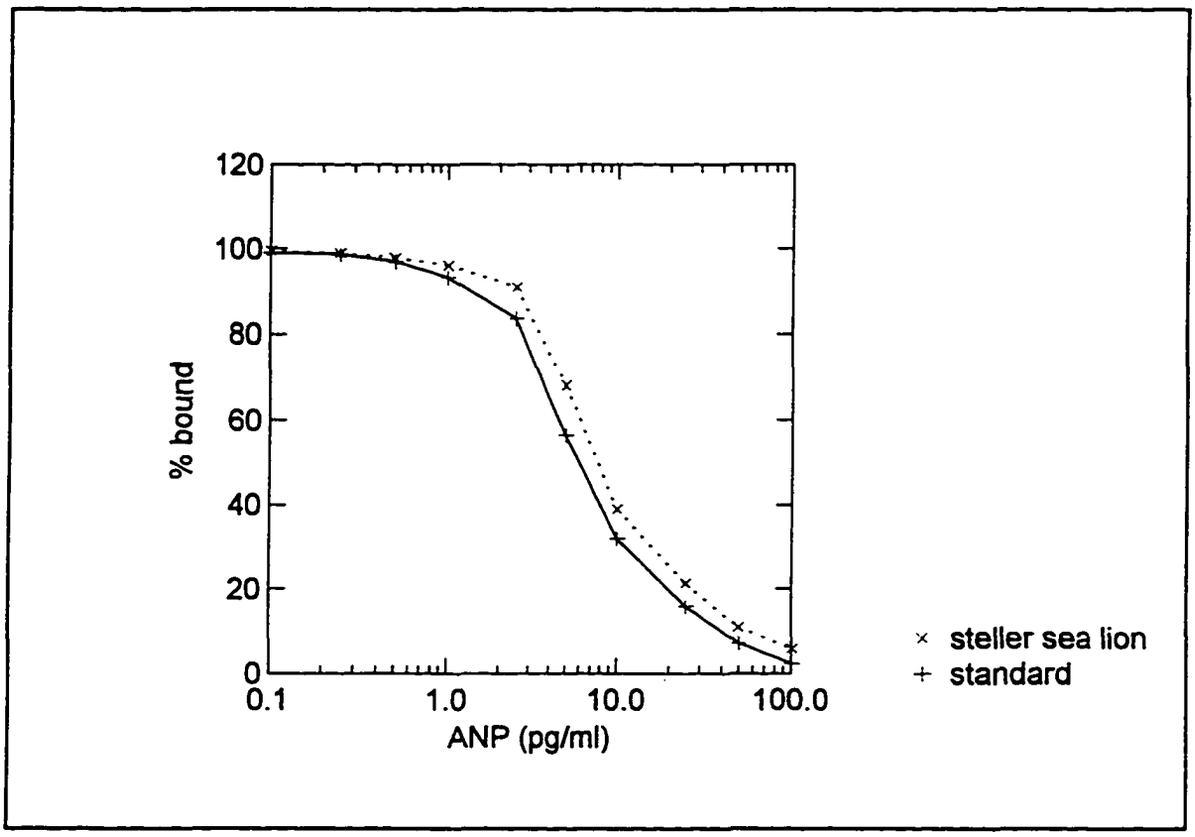

FIGURE 2.1. Representative estimation of the percentage cross-reaction from the amount of material required to produce 50% inhibition in the radioimmunoassay. Results shown are for estimating cross-reactivity between the atrial natriuretic peptide (ANP) standard provided by the manufacturer (Phoenix Pharmaceuticals) and Steller sea lion plasma. Solid line, standard; dashed line, Steller sea lion pooled plasma.

2.4.1. *Vasopressin*

The use of antibodies raised against human AVP yielded 80% cross-reactivity with pinniped plasma samples. The concentration of AVP-like ir material in plasma samples from all seal and sea lion species analyzed ranged from 1 pg/ml to over 16 pg/ml and demonstrated significant developmental and regional differences. Steller sea lion pups and yearlings and harbor seal pups had lower levels of AVP-like ir material compared to adults of the same species (Table 2.1). Additional analyses revealed that AVP-like ir material was significantly lower ($P < 0.05$) in Steller sea lion pups from Southeast Alaska (4.7 ± 0.4 pg/ml, n=41) (where the population is stable) than in pups from Aleutian Islands (7.8 ± 0.6 pg/ml, n=46) and Gulf of Alaska (8.8 ± 0.9 pg/ml, n=53), both locations at which the populations are declining. Harbor seal pups sampled in Southeast Alaska (stable population) appeared to have higher concentrations of AVP-like ir material (26.1 ± 9.1 pg/ml, n=2) than did captive harbor seals in California (6.2 ± 1.4 pg/ml, n=11) and wild harbor seals in Prince William Sound (5.6 ± 0.5 pg/ml, n=4) (population declining). However, this pattern changed in yearlings, such that harbor seal yearlings from Kodiak (population increasing) appeared to have lower levels of AVP-like ir material (8.4 ± 2.6 pg/ml, n=2) than yearlings from Prince William Sound (20.3 ± 7.2 pg/ml, n=8) (population decreasing).

2.4.2. *Atrial Natriuretic Peptide*

Anti-human ANP serum displayed a 75% cross-reactivity with pinniped plasma samples and also showed patterns by region, age and species. The average levels of ANP-like ir material in plasma samples from Steller sea lions and ringed seals were significantly higher than in samples from harbor seals, Weddell seals, and elephant seals ($P < 0.05$) (Table 2.1). Adult Steller sea lions had significantly higher ($P < 0.05$) levels of ANP-like ir material than did younger conspecifics. Among the Steller sea lion pups, those sampled in the Aleutian Islands (115.2 ± 13.1 pg/ml, n=46) and the Gulf of Alaska (118.9 ± 10.0

pg/ml, n=55) (declining populations) had significantly higher ($P < 0.05$) levels of circulating ANP-like ir material than those from Southeast Alaska (31.6 ± 3.5 pg/ml, n=41) (population stable) and those kept at the Vancouver Aquarium (21.7 ± 0.6 pg/ml, n=9). Levels of ANP-like ir material in hunted ringed seals (126.8 ± 38.4 pg/ml, n=5) were as high as those in Steller sea lions (all age classes) from Aleutian Islands (113.4 ± 12.9 pg/ml, n=47) and Gulf of Alaska (124.3 ± 7.7 pg/ml, n=75) (declining populations). This pattern was age-dependent and subadult harbor seals in Southeast Alaska had higher levels of ANP-like ir material (59.1 ± 20.2 pg/ml, n=3) than subadult harbor seals in Kodiak (16.2 ± 5.1 pg/ml, n=6) and Prince William Sound (21.6 ± 5.0 pg/ml, n=28) ($P<0.05$).

2.4.3. *Angiotensin II*

Plasma samples from pinnipeds exhibited an average 85% cross-reactivity with the human Ang II antiserum. Samples from Steller sea lions had a higher average concentration of Ang II-like ir material than harbor seals, and Weddell seals, ($P < 0.05$). However, there was no statistically significant effect of age on circulating levels of Ang II-like ir material in any of the seal and sea lion species analyzed. The concentration of Ang II-like ir material in plasma samples of Steller sea lion pups from the Gulf of Alaska (69.2 ± 6.4 pg/ml, n=49) was significantly higher ($P < 0.05$) than that in samples of pups from the Aleutian Islands (32.4 ± 4.8 pg/ml, n=43), Southeast Alaska (40.7 ± 5.4 pg/ml, n=41) and the Vancouver Aquarium (23.8 ± 2.0 pg/ml, n=9). Plasma samples of harbor seals of all ages from Prince William Sound had significantly ($P < 0.05$) higher Ang II-like ir material (36.2 ± 4.7 pg/ml, n=79) than did samples of harbor seals from Southeast Alaska (20.3 ± 1.7 pg/ml, n=45).

2.5. *Discussion*

Sufficient cross-reactivity was obtained between each of the antibodies against the human peptides and plasma samples from pinnipeds to justify the use of commercial kits. Successful determination of AVP-, ANP- and Ang II-like ir material in plasma samples from Weddell seals, harbor seals, northern elephant seals, ringed seals, California sea lions and Steller sea lions was achieved by the use of commercially available antibodies. This suggests not only that these hormones are present in the plasma of pinnipeds, but also that the amino acid sequences of the pinniped peptides are similar to those of humans. This is consistent with the highly conserved sequences reported for these peptide hormones among vertebrates (1, 36, 64). However, detailed biochemical analyses are needed in order to verify the biochemical identity of the ir material found in seal and sea lion plasma samples.

2.5.1. *Vasopressin*

The levels of AVP-like ir material in pinnipeds (Table 2.1) were higher than those reported for humans, in which the average circulating AVP concentration is 4 pg/ml (52). However, plasma AVP concentrations in rabbits (9.4 ± 3.2 pg/ml, 37) and rats (10.4 ± 2.5 pg/ml, 65) are similar to values found in the pinnipeds sampled in this study. Similarly, the levels of plasma AVP-like ir material found in all pinniped species were comparable to those found in a previous study on fasting, postweaned northern elephant seal pups (34.8 ± 18.2 pg/ml (early fasting) to 4.8 ± 1.3 pg/ml (late fasting), 50). The northern elephant seal pups in the present study were in the mid to late fasting stage. In general, pups had lower levels of AVP-like ir material than adults, but this was statistically significant ($P<0.05$) only for Steller sea lions and harbor seals. Johnson *et al.* (35) reported higher plasma AVP concentration in healthy elderly than in younger humans; however, Clark *et al.* (13) did not find differences between healthy young and old subjects. In addition, some of the yearling, subadult and adult harbor seals were molting at the time of sampling.

Evidence suggests that hypophysial peptides may regulate molting in vertebrates and invertebrates (20, 46, 53).

2.5.2. *Atrial Natriuretic Peptide*

The ANP-like ir material detected in plasma samples from California sea lions, Weddell seals, harbor seals and northern elephant seals were similar to values reported for humans (54.0 ± 5.0 pg/ml, 25) and fresh water turtles (47.0 ± 3.5 pg/ml, 6). However, the concentrations of ANP-like ir material were relatively high in samples from adult Steller sea lions and ringed seals (Table 2.1).

2.5.3. *Angiotensin II*

The concentration of Ang II-like ir material in samples from northern elephant seals, ringed seals, harbor seals, Weddell seals, and California sea lions (Table 2.1) were similar to values reported for humans (12.0 ± 2.1 pg/ml, 59) and other terrestrial mammals (rabbit, 18.5 ± 4.0 pg/ml, 58; rat, 29.3 ± 3.5 pg/ml, 65; calf 57.2 ± 1.0 pg/ml, 8).

Unlike samples collected from all other species, samples from ringed seals were taken from animals that had been shot, which may explain the elevated ANP-like ir material concentrations in this species. However, their levels of AVP- and Ang II-like ir material were not distinct from other species. All other seals and sea lions were captured when they were on land, and presumably had been on land for some time. It is unlikely that the high levels of ANP-like ir material found in Steller sea lions were brought about by differences in sample manipulation or the diving history of the animals just prior to blood sampling. Molting may not by itself be responsible for these higher values since, unlike the harbor seals, Steller sea lions were not molting at the time of sampling.

Plasma ANP levels as high as those found in Steller sea lion and ringed seal plasma samples are typically found in humans with congestive lung disease and pulmonary artery hypertension (3), and in experimental situations, in humans after 60 minutes water

immersion (39), in fresh water turtles during diving (6) and in pigs breathing a mixture of 89% N_2 and 11% O_2 for 15 minutes (2). Plasma Ang II and ANP levels similar to those found in Steller sea lion plasma samples were observed in humans with impaired left ventricular systolic function (62).

Most interestingly, the circulating levels of ANP- and Ang II-like ir material in Steller sea lion samples collected in the Aleutian Islands and the Gulf of Alaska were higher than those from Steller sea lions in Southeast Alaska and the Vancouver Aquarium. Similarly, the concentration of AVP- and Ang II-like ir material in harbor seal samples from Prince William Sound was higher than that in harbor seal samples from Kodiak, Southeast Alaska and California. Furthermore, concentrations of the vasoactive peptides in samples of Steller sea lions and harbor seals from Southeast Alaska, the Vancouver Aquarium and California were closer to those in California sea lions, northern elephant seals and Weddell seals, as well as to those in terrestrial mammals.

The populations of Steller sea lions and harbor seals at the Aleutian Islands, Gulf of Alaska and Prince William Sound have been declining for the past 20 years (41, 54) to the point that Steller sea lions have recently been proposed as "Endangered" (23). The cause of the decline is not obvious, but we are addressing the possibility that the hormonal differences we found may reflect nutritional, physiological and/or genetic distinctions among the populations.

Malnutrition and eating disorders are accompanied by disturbances in the metabolism of vasoactive hormone systems. Abnormal concentrations of ANP, AVP and Ang II, and impaired responses to these hormones have been reported in patients with bulimia and anorexia nervosa (18, 45, 49), acute and chronic starvation (40), and also in rats with dietary obesity (15). Dietary copper deficiency has been shown to increase plasma concentrations of ANP (9), to increase the angiotensin-converting enzyme (ACE) activity in renal microvilli (56), to decrease both plasma ACE activity and blood pressure in weanling rats (24) and to increase blood pressure in mature rats (42). Zenteno-Savin *et*

al. (67) reported that plasma levels of the acute phase protein haptoglobin (Hp) in samples of harbor seals and Steller sea lions in the Gulf of Alaska, the Aleutian Islands and Prince William Sound are elevated as compared to those in samples of animals from Southeast Alaska. Besides increases in Hp and other plasma proteins, the acute phase response involves vasodilation (38). This is consistent with our findings of relatively elevated levels of circulating ANP-like ir material in samples of Steller sea lions and harbor seals from the Gulf of Alaska, the Aleutian Islands and Prince William Sound. In addition, certain Hp phenotypes appear to be associated with the risk of developing hypertension in humans (17, 32). Hypertension is normally accompanied by a rise in circulating ANP and activation of the RAAS (61, 63). Conversely, Hp levels were significantly lower in human transplant recipients who had been given angiotensin-converting enzyme inhibitors (29). Other pathologic states in which Hp, ANP and the RAAS may be increased include some types of anemia (5, 48, 60, 62), heart failure (27, 57), and pericarditis (4, 55).

In summary, the plasma concentrations of AVP-, ANP- and Ang II-like ir material in samples from northern elephant seals, harbor seals, Weddell seals and California sea lions are similar to those reported for many terrestrial mammals, but showed interesting natural variation. Studies in our laboratory are being conducted to address the possibility that the differences in vasoactive hormone concentrations found in Alaskan pinnipeds may be inherent to genetic stocks or may be due to physiological and/or pathological states. Clearly, further research is needed to evaluate interactions among vasoactive hormones and physiological events pertinent to the natural histories of seals and sea lions, such as molting, fasting and diving.

2.6. *Acknowledgments*

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3. *CHANGES IN THE PLASMA LEVELS OF VASOACTIVE HORMONES DURING APNEA IN SEALS.*[2]

3.1. *Abstract*

Prolonged and repetitive breath-hold periods (apnea) during diving and sleep are a routine component in the ecological physiology of marine mammals. Seals are among the few mammals in which control of heart rate (HR) can be studied independent of respiration, without pharmacochemical manipulation. We hypothesized that the vasoactive hormones angiotensin II (Ang II), arginine vasopressin (AVP) and atrial natriuretic peptide (ANP) were involved in the control of cardiovascular function in seals, and that the relationship was dependent upon input from the respiratory system. Venous plasma samples were collected and electrocardiograms were recorded from northern elephant seal (*Mirounga angustirostris*) and Weddell seal (*Leptonychotes weddellii*) pups during both spontaneous breathing (eupnea) and apnea. Instantaneous HR and simultaneous plasma levels of ANP, AVP and Ang II from periods of eupnea and apnea were compared. In these seal pups, apnea was associated with bradycardia, increased ANP and decreased AVP and Ang II. The results support the hypothesis of a complex involvement between the vasoactive hormones and the control of cardiovascular function, and provide evidence for differential levels of control during periods of eupnea and apnea.

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[2]Changes in the plasma levels of vasoactive hormones during apnea in seals. Zenteno-Savin, T. and M. A. Castellini. Comparative Biochemistry and Physiology, *in press*.

3.2. *Introduction*

Of the cyclical factors (respiration, blood pressure (BP), vasomotor activity, neural regulation and thermoregulation) that drive heart rate (HR) and HR variability, breathing has the strongest influence in determining the prevailing HR (33). The study of intrinsic HR patterns and the factors that affect them in mammals, independent of respiratory influences, requires a unique model. Prolonged and repetitive breath-hold periods (apnea), associated with diving (reviewed in 34) and sleep (10, 12), are a routine component in the respiratory patterns of seals. Apnea in seals is characterized by decreases in HR and cardiac output, peripheral vasoconstriction and redistribution of blood flow (61), as well as increases in hematocrit (9, 12). However, systolic, mean and diastolic arterial BP remain relatively unchanged (24, 32).

Several studies using terrestrial mammals suggest that the vasoactive hormones may be important factors in the control of cardiorespiratory function. The vasoactive hormones arginine vasopressin (AVP, the antidiuretic hormone), angiotensin II (Ang II) and atrial natriuretic peptide (ANP) affect vascular resistance, thus modifying, directly or indirectly, blood flow, cardiac output and HR (36, 59, 60). In addition, AVP has a direct cardiodepressant action in rabbits (23), Ang II stimulates ventilation in dogs (44), and ANP resets the baroreceptor control of the heart towards cardioinhibition in humans (59).

This project studied the changes in HR and plasma levels of ANP, AVP and Ang II in northern elephant seal (*Mirounga angustirostris*) and Weddell seal (*Leptonychotes weddellii*) pups during periods of spontaneous eupnea and apnea. We hypothesized that in seals, as in other mammals, HR and HR variability are strongly driven by respiration, and that in the absence of ventilation, the integrity of cardiovascular function is maintained, at least in part, in response to the interaction of the vasoactive hormones ANP, AVP and Ang II. Furthermore, we hypothesized that the bradycardia and decreased cardiac output driven by apnea at a constant arterial pressure would increase intra-cardiac pressure, thus increasing plasma levels of ANP and decreasing the concentrations of AVP and Ang II.

We speculated that differences between seal species in the intensity or extent of the interaction of the vasoactive hormones and the cardiorespiratory system may reflect differential diving capacities.

3.3. *Materials and Methods*

The endocrine control of cardiorespiratory function was estimated in 10 northern elephant seal pups from Año Nuevo State Reserve, CA, and 5 Weddell seal pups from McMurdo Sound, Antarctica. Because most of these animals were tagged when born as part of long-term population studies, their age was known. When birth-dates were not known, age was calculated from morphometric data. Pup age ranged between 4 and 16 weeks. The mean age for Weddell seal pups was 6 weeks. Northern elephant seal pups were divided by age into two groups, mean ages 8 and 14 weeks. Handling and sampling techniques followed routine procedures for the study of sleep-associated apnea in northern elephant seals (10, 12). Briefly, seals were captured, transported to laboratory facilities (Long Marine Laboratory, University of California Santa Cruz (elephant seals), or an adapted fish-hut anchored on the sea ice at McMurdo Sound (Weddell seals)), and weighed. Body mass was, for elephant seal pups 74.2 to 95.2 kg; for Weddell seal pups 106 to 130 kg. Under light anesthesia (3.0 mg/kg ketamine, Ketaset®, Aveco Co., New York, NY, and 1.25 μg/kg Diazepam®, Abbott Laboratories, North Chicago, IL) and sterile conditions, a percutaneous catheter (14 gauge, 5 ¼ inch, Becton Dickinson, Sandy, UT) was implanted in the extradural intravertebral vein and needle electrodes (21 gauge, 1.5 inch stainless needles) were anchored subdermally across the thoracic area to monitor HR. An antibiotic was administered intravenously (iv) (0.5 g Keflin®, Lilly Co., New York, NY). Animals were allowed to recuperate from this minimal anesthesia for at least 3 hours. The northern elephant seal pups were studied while they slept or rested in a large, quiet room. The Weddell seal pups were examined while they slept or rested between diving bouts; they had free access to water through a hole in the sea ice under the

laboratory hut (reference for general method: 11). The animals were not handled or restrained during the sampling period. After the experiment was completed, the antibiotic dosage was repeated, the electrodes and catheter were removed, and the animals were kept for an additional period (up to 12 hours) for observation. After this time, the seals were returned to the colonies.

Heart rate was recorded continuously by directing the electrocardiogram signal through a cardiotachometer (BIOTACH, ufi, Morro Bay, CA). Heart rate and respiratory chest movement were simultaneously collected directly into a multichannel physiological recorder (Microscribe, Houston Instruments, The Recording Co., San Marcos, TX) for later analysis. Heart rate polygraphic data was digitized (DrawingBoard II, CalComp Digitizer Products Group, Scottsdale, AZ) to obtain mean and instantaneous HR for each eupneic and apneic period.

Venous blood samples were collected for hormone analysis during at least 5 independent periods of spontaneous eupnea and apnea. Samples were collected 1 to 2 min into apnea and 2 min after the first breath. If an apneic period lasted more than 5 min, additional samples were taken at 1 to 2 min intervals. Blood samples (5 ml) were collected into chilled test tubes containing 0.125 M EDTA (Vacutainer 6450, Becton-Dickinson Ltd., Rutherford, NJ). Plasma was separated by centrifugation at 4000 x g at 4°C for 10 min. To the recovered plasma an angiotensin-converting enzyme inhibitor, o-phenanthroline (0.025 M, 100 μl/ml plasma, Sigma Chemicals, St Louis, MO) was added (21). All samples were stored at -70°C, transported to University of Alaska Fairbanks (UAF) and kept frozen until analyzed.

Prior to analysis, samples were thawed and immunoreactive (ir) material was extracted by using prepacked octadecasilyl-silica cartridges (SepCol, Phoenix Pharmaceuticals, Mountain View, CA), according to a method adapted from Hartter (29, 63). The percent recovery from the extraction procedure was determined by adding known amounts of synthetic peptide (5 to 100 pg/ml, Phoenix Pharmaceuticals) to pooled

plasma (quality control). Measurements of extracted plasma were not corrected for extraction efficiency, which ranged from 89 to 112%.

The vasoactive hormone concentrations were analyzed in plasma samples using radioimmunoassay (RIA) kits for AVP, ANP and Ang II (Phoenix Pharmaceuticals), which include antibodies raised in rabbits against the human peptides. We have found sufficient cross-reactivity between antisera raised in rabbits against the human peptide hormones and ir material in plasma samples of several species of seals and sea lions using these kits (63). All plasma samples were run in duplicate, and replicates were run in a single assay, along with a quality control sample for the species for which unknown samples were being analyzed in that assay. Only values that fell within the linear portion of the dose-response curve (20 to 80%) were considered. All dilutions were made with RIA buffer. The sensitivity of the RIA systems (50% depression of radiolabeled hormone binding to antibody) (17) for each hormone was as follows: AVP, 1.8 ± 0.2 pg/tube; ANP, 6.4 ± 0.6 pg/tube; Ang II, 4.1 ± 0.3 pg/tube (n=15). Intra-assay errors (coefficient of variation percent) were: AVP, 3.7 ± 0.1%; ANP, 3.9 ± 0.3%; Ang II, 3.1 ± 0.2% (n=10). Inter-assay coefficients of variation were: AVP, 5.3 ± 0.2%; ANP, 4.2 ± 0.4%; Ang II, 5.6 ± 0.3% (n=15). The least detectable concentration for all three hormones was 0.1 pg/ml. Samples from both seal species showed parallelism with the synthetic peptides provided by the manufacturer, yielding 75 to 85% cross-reactivity. Biochemical identity of the ir material obtained from elephant seal and Weddell seal plasma samples has not yet been assessed; thus, in this paper will be referred to as ANP-, AVP- and Ang II-like ir material, accordingly.

Statistical analyses were carried out as follows: Differences in HR and hormone concentrations between periods of eupnea and apnea were identified using paired t-tests and non-paired t-tests with Bonferroni adjustment for multiple comparisons where applicable (62) (SYSTAT®, SPSS Inc., Chicago, IL). Interactions between HR, breathing status (eupnea or apnea, scored as 1 or 0, respectively) and hormone

concentrations were detected with analysis of covariance (ANCOVA) (62) (SYSTAT®, SPSS Inc.). Predictors of breathing status based on HR and hormone concentrations were obtained using discriminant analysis (DA) (62) (SYSTAT®, SPSS Inc.). Significance was assumed when $P < 0.05$. Data are presented as mean ± standard error of the mean (SE). Heart rate units are beats per minute (bpm), and hormone concentrations are expressed as picograms of ir material per milliliter (pg/ml) of extracted plasma.

3.4. *Results*

In all cases, average HR values during periods of apnea were lower ($P < 0.05$) than those during eupnea in northern elephant seal and Weddell seal pups (Table 3.1). The HR recorded for seal pups during eupnea was not significantly different between species or among age groups. The HR obtained during periods of sleep-associated apnea was lower ($P < 0.05$) in the 14 week old than in the 8 week old elephant seal pups. Apneic HR in Weddell seal pups (6 week old) was lower ($P < 0.05$) than that in elephant seal pups of either age group.

Plasma concentrations of ANP-, AVP- and Ang II-like ir material in northern elephant seal and Weddell seal pups during periods of eupnea and apnea are also presented in Table 3.1. Circulating levels of ANP-like ir material in Weddell seal pups was significantly ($P < 0.05$) higher during apnea than during eupnea. Mean concentrations of Ang II- and AVP-like ir material were significantly ($P < 0.05$) lower during periods of apnea than eupnea in Weddell seal pups, as well as in the older (14 weeks old) elephant seal pups.

TABLE 3.1. Mean heart rate and average concentrations of vasoactive hormones in extracted plasma samples from northern elephant seal and Weddell seal pups during periods of eupnea and apnea. Data are presented as mean ± standard error of the mean. HR= heart rate; ANP= atrial natriuretic peptide; Ang II= angiotensin II; AVP= arginine vasopressin. Sample size is indicated in parentheses.

		HR (bpm)	ANP (pg/ml)	Ang II (pg/ml)	AVP (pg/ml)
Northern Elephant Seal Pups					
age 8 weeks					
Eupnea	(40)	93.7 ± 1.6	22.7 ± 3.6	24.3 ± 4.6	1.7 ± 0.3
Apnea	(52)	63.9 ± 1.4*	24.1 ± 3.3	17.7 ± 3.0	1.8 ± 0.1
age 14 weeks					
Eupnea	(29)	93.9 ± 2.0	20.9 ± 2.2	30.9 ± 1.4	3.9 ± 0.5
Apnea	(53)	53.2 ± 1.1*	26.3 ± 1.8	16.5 ± 0.9*	2.5 ± 0.2*
Weddell Seal Pups					
age 6 weeks					
Eupnea	(32)	94.0 ± 1.4	12.5 ± 0.5	39.6 ± 1.7	7.2 ± 0.4
Apnea	(35)	46.2 ± 0.6*	30.6 ± 1.2*	12.2 ± 0.4*	3.2 ± 0.1*

*$P < 0.05$ compared to values during eupnea.

Graphical representations of the relationships between HR and hormone concentrations for northern elephant seal and Weddell seal pups are displayed as scattergrams in Figures 3.1, 3.2, and 3.3; opened and closed circles distinguish periods of eupnea and apnea, respectively. These scatter-plots, also known as casement plots, show separate graphs for each possible pair of variables (HR, ANP-, Ang II-, and AVP-like ir material) in a single matrix-style display (16). The density plots on top of each column show the relative concentration of data points for each variable during periods of apnea (solid line) and eupnea (dashed line), in which case the plotted variable is displayed in the X-axis and number of cases in the Y-axis.

In the younger elephant seal pups (average age 8 weeks) only HR was significantly different between periods of eupnea and apnea (Figure 3.1, Table 3.1). In this group of younger elephant seal pups, Ang II- and AVP-like ir material and breathing status (eupnea or apnea) were significant in explaining HR (ANCOVA, $P < 0.05$), and AVP-like ir material and HR significantly contributed to predicting breathing status (ANCOVA, R^2=0.719, $P < 0.05$). Classification functions for eupnea and apnea were determined as follows (DA, Wilk's Lambda = 0.281, $P < 0.05$): *Eupnea* = -48.723 + 1.057 *Heart Rate* + 0.051 *ANP* - 0.091 *Ang II* - 0.111 *AVP*, and *Apnea* = -22.900 + 0.710 *Heart Rate* + 0.044 *ANP* - 0.063 *Ang II* - 0.050 *AVP*.

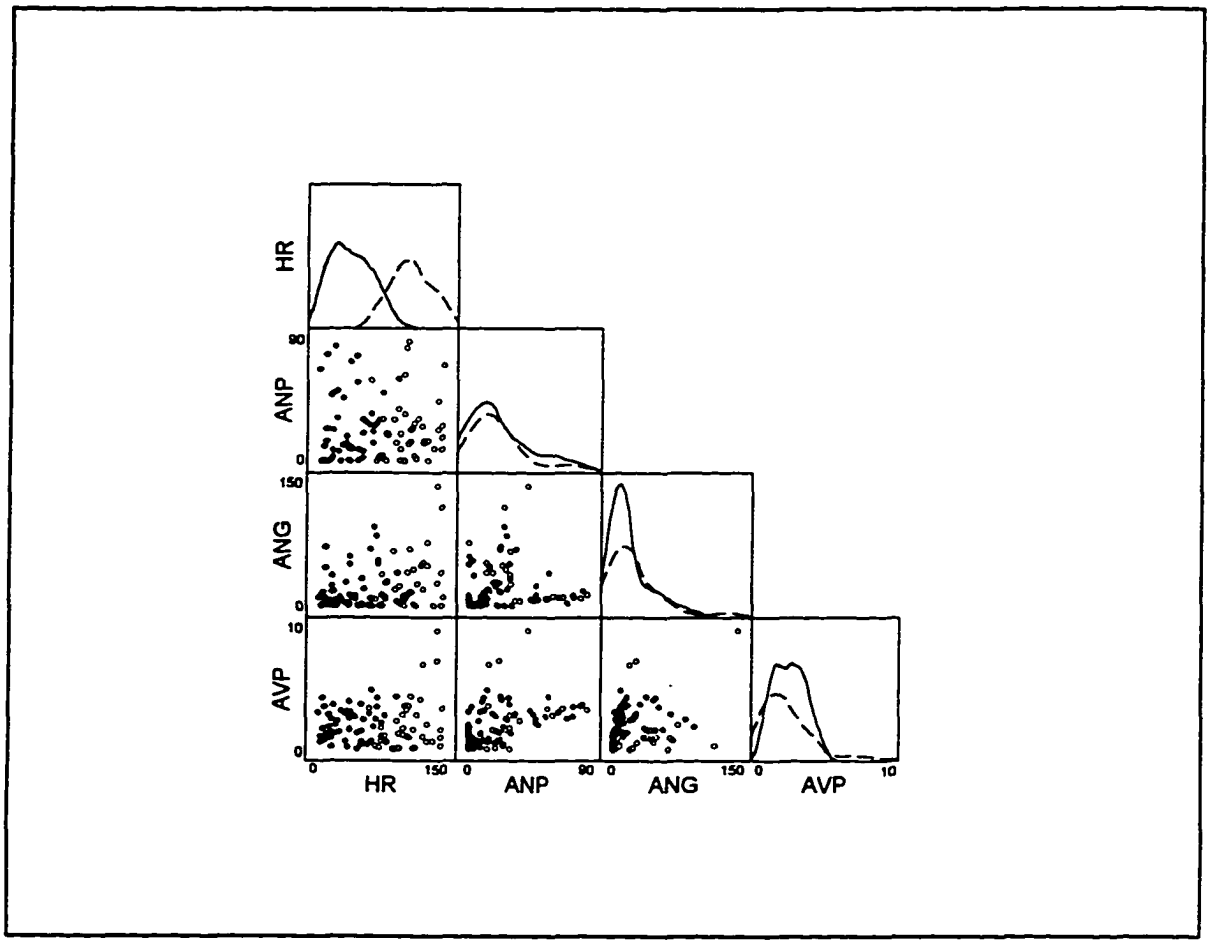

FIGURE 3.1. Scattergram showing separate plots for each possible pair of variables, heart rate (HR, bpm) and hormone concentrations (ANP= atrial natriuretic peptide, ANG= angiotensin II, AVP= arginine vasopressin, pg/ml), during eupnea (open circles) and apnea (closed circles) in 5 northern elephant seal pups, average age 8 weeks. The density plots on top of each column show the relative concentration of data points for each variable during eupnea (dashed line) and apnea (solid line).

The relationships between HR and the vasoactive hormone concentrations in the older elephant seal pups (Figure 3.2) differ in slope and dispersion compared to those of the younger elephant seal pups (Figure 3.1). In this group of older elephant seals, circulating levels of Ang II- and AVP-like ir material increased with HR (Figure 3.2). Regression and descriptive functions between variables were different for periods of eupnea and apnea; for example, Ang II-like ir material tended to decrease with ANP-like ir material during eupnea but not during apnea (Figure 3.2). In this group of elephant seal pups, ANP-like ir material and breathing status (eupnea or apnea) significantly contributed to describing HR (ANCOVA, $P < 0.05$), and breathing status could be predicted from HR and Ang II- and AVP-like ir material (ANCOVA, $R^2=0.850$, $P < 0.05$). Classification functions for eupneic and apneic episodes were determined as follows (DA, Wilk's Lambda = 0.150, $P < 0.05$): *Eupnea* = -73.311 + 1.232 *Heart Rate* + 0.347 *ANP* + 0.466 *Ang II* + 2.070 *AVP*, and *Apnea* = -27.742 + 0.735 *Heart Rate* + 0.289 *ANP* + 0.254 *Ang II* + 1.234 *AVP*.

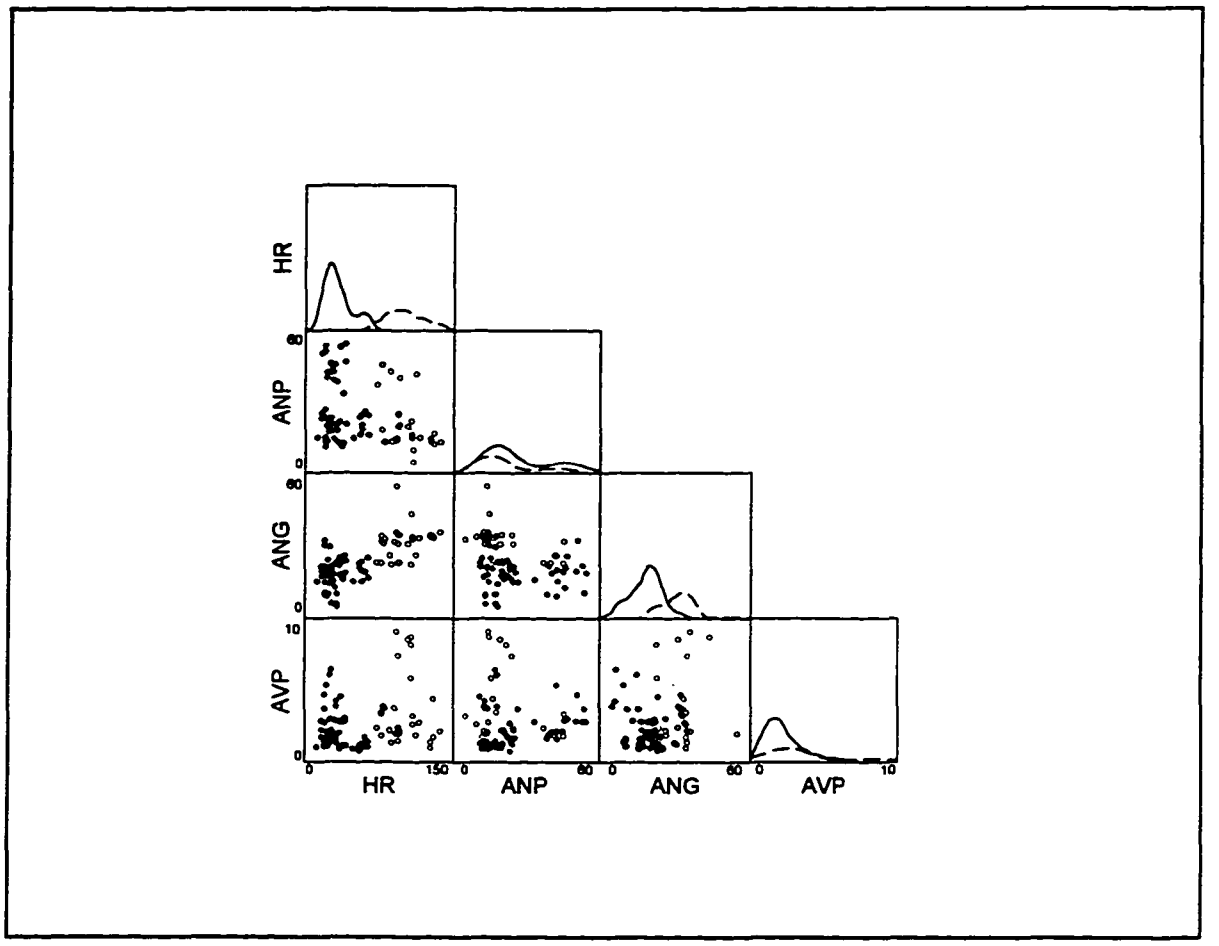

FIGURE 3.2. Scattergram showing separate plots for each possible pair of variables, heart rate (HR, bpm) and hormone concentrations (ANP= atrial natriuretic peptide, ANG= angiotensin II, AVP= arginine vasopressin, pg/ml), during eupnea (open circles) and apnea (closed circles) in 5 northern elephant seal pups, average age 14 weeks. The density plots on top of each column show the relative concentration of data points for each variable during eupnea (dashed line) and apnea (solid line).

Periods of eupnea and apnea are completely separated and easily identifiable in Weddell seal pups, average age 6 weeks (Figure 3.3). Circulating levels of Ang II- and AVP-like ir material increased, while concentrations of ANP-like ir material decreased with HR. The concentration of AVP-like ir material increased with Ang II-like ir material during periods of eupnea, and increased with ANP-like ir material during apnea. Ang II- and AVP-like ir material and breathing status (eupnea or apnea) contributed significantly to explaining HR (ANCOVA, $P < 0.05$), while ANP- and AVP-like ir material and HR had the highest significance in predicting breathing status (ANCOVA, $R^2=0.953$, $P < 0.05$). Classification functions for eupnea and apnea were determined as follows (DA, Wilk's Lambda = 0.047, $P < 0.05$): *Eupnea* = -143.194 + 2.696 *Heart Rate* + 0.628 *ANP* - 0.132 *Ang* II - 4.006 *AVP*, and *Apnea* = -52.399 + 1.398 *Heart Rate* + 1.130 *ANP* - 0.015 *Ang II* + 1.385 *AVP*.

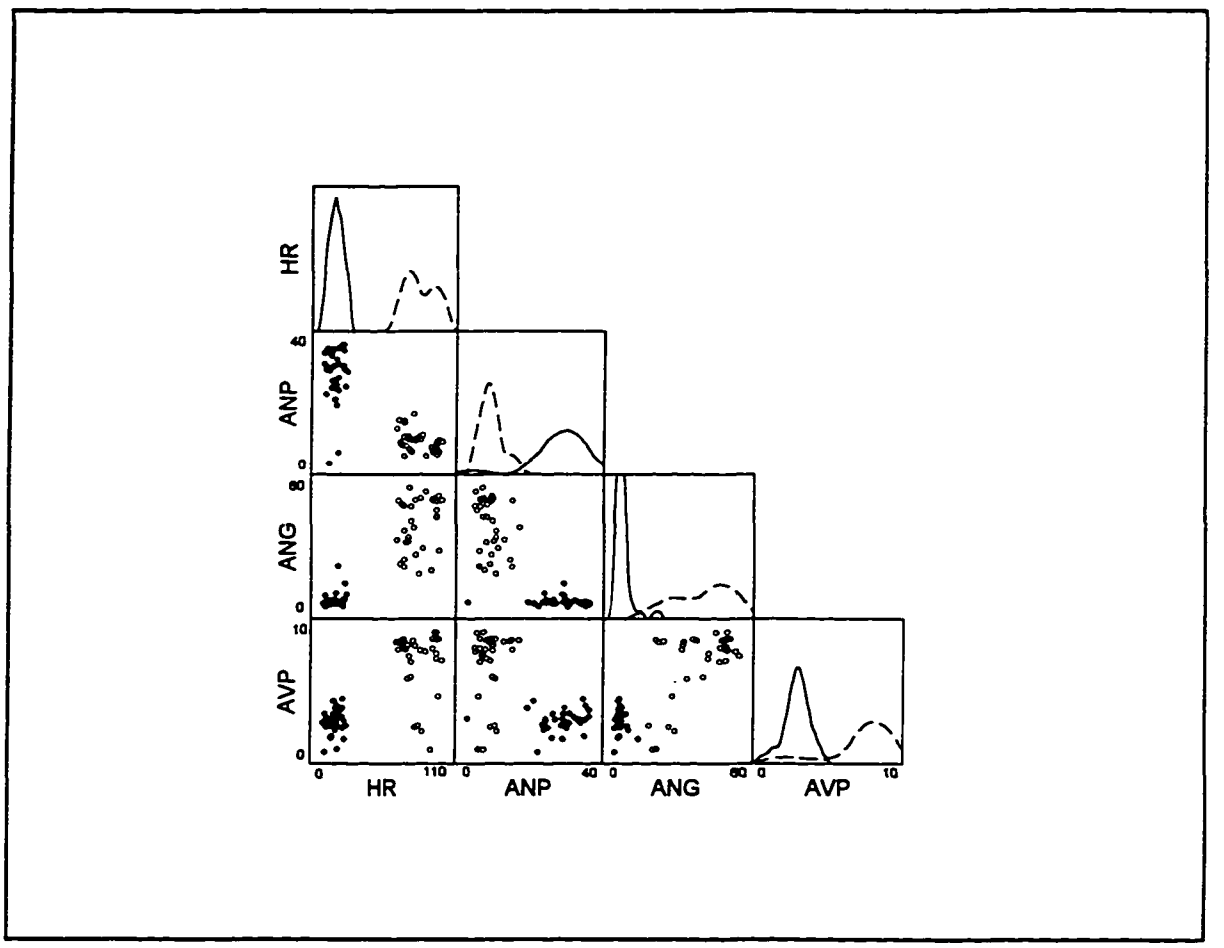

FIGURE 3.3. Scattergram showing separate plots for each possible pair of variables, heart rate (HR, bpm) and hormone concentrations (ANP= atrial natriuretic peptide, ANG= angiotensin II, AVP= arginine vasopressin, pg/ml), during eupnea (open circles) and apnea (closed circles) in 5 Weddell seal pups, average age 6 weeks. The density plots on top of each column show the relative concentration of data points for each variable during eupnea (dashed line) and apnea (solid line).

3.5. *Discussion*

In both northern elephant seals and Weddell seals, apneic HR was significantly lower than eupneic HR, consistent with previous findings (reviewed in 14). Respiratory sinus arrhythmia (RSA), acceleration of the heart during inspiration and slowing of the heart during the expiratory phase of respiration, was observed in the three groups of seal pups (data not shown). In all cases, apnea was initiated after exhalation and the apneic HR was maintained at the average rate recorded during expiration in the course of spontaneous breathing. The differences between eupneic and apneic HR were smaller in the younger elephant seals and largest in the Weddell seals, suggesting development of RSA and control of the cardiorespiratory function to a higher degree in the 14 week old than in the 8 week old elephant seal pups, as was reported by Castellini *et al.* (13), and higher in Weddell seals than in the northern elephant seal pups. Differences between species may be associated with the development of a strong RSA at an earlier postnatal age in Weddell seal pups than in elephant seal pups.

Circulating levels of ANP-, Ang II- and AVP-like ir material in elephant seal and Weddell seal pups were within the ranges reported for a variety of terrestrial and marine mammals (27, 46, 48, 54, 63). In both elephant seal and Weddell seal pups, concentrations of the three hormones were found to change rapidly under resting conditions, in some cases within 5 minutes. This suggests that in seal pups there is a fast turnover rate for these vasoactive hormones, which is consistent with the short half-life reported for these hormones in terrestrial mammals (Ang II, 2 min (4); ANP, 2-3 min (15); AVP, 7.8 min (22)).

In parallel with HR data, the differences between apneic and eupneic hormone concentrations were larger in Weddell seal pups than in elephant seal pups and among the latter, in the 14 week old than in the 8 week old pups. These differences between periods of eupnea and apnea suggest both a developmental and a species-specific component to the integration of cardiorespiratory function in seal pups.

Circulating levels of ANP-like ir material were elevated, while concentrations of Ang II- and AVP-like ir material (except in the youngest elephant seal pups) were lower during periods of apnea in elephant seals and Weddell seals. It may be expected, following the terrestrial mammal model, that as a consequence of decreased HR during breath-holding, CO would decrease and thus, arterial BP would decrease while cardiac filling would increase. The latter is a known stimulus for increased ANP and decreased AVP concentrations. In order to maintain a relatively constant BP, as has been reported to occur in seals during periods of apnea, the activity of the baroreceptors will result in vasoconstriction and increased Ang II levels. However, Ang II-like ir material was lower during apnea than during eupnea in both Weddell seal and elephant seal pups (Table 3.1). Zapol *et al.* (61) reported that during breath-hold diving in the Weddell seal, blood flow to the kidneys virtually ceased. It may be that, during apneic periods, although the reduced blood flow to the kidneys is a stimulus for renin secretion and synthesis of Ang II (45), the former would not have access to the general circulation, delaying the increase in Ang II levels until blood flood were re-established. However, we do not have direct measurements of renal blood flow in these seals to corroborate this idea.

In agreement with increased hematocrit (data not shown) and lower HR during breath-holding in seals, ANP induces shifting fluid from the intravascular to the interstitial compartments, and increases vagal afferent sensitization (31, 57). Also, ANP lowers cardiac output in sheep and rats (5, 39), and inhibits renin and AVP secretion (52, 57). One of the main effects of ANP is to reduce BP (20). The ANP-induced decrease in BP appears to be related to the degree of renin stimulation (38). The lower Ang II-like ir material in elephant seal and Weddell seal pups suggests that renin stimulation was minimal during periods of apnea, thus the ANP-induced lowering of BP would not be observed.

The vast majority of biologically active Ang II is produced as blood passes through the lungs (47). There is scarce information on pulmonary circulation in seals. Nevertheless,

in breath-hold diving the cardiac output, and thus pulmonary blood flow, is reduced. This response is accompanied by a decrease in pulmonary arterial diastolic pressure to the same level as the right atrial pressure (55, Elsner personal communication). It is possible that the same hemodynamic changes occur during sleep apnea when marked cardio-inhibitory effects are observed. In any event, the combined restoration of blood flow to the kidneys and lungs when eupnea is resumed would release renal renin to the general circulation, starting the series of reactions which will ultimately lead to an increase in Ang II levels.

Compatible with our finding relatively higher levels of Ang II-like ir material in seal plasma samples during breathing, the effects of Ang II include, besides a strong vasoconstriction and increases in BP, HR and sympathetic activity, stimulation of respiration (8, 28, 44). The increases in HR may result from the ability of Ang II to inhibit vagal tone to the heart (49, 51), or its direct chronotropic effect (43). Angiotensin II also stimulates AVP release from the posterior pituitary (7, 53).

In general, AVP is thought to decrease HR via V_1 receptors and to increase HR via V_2 receptors. In dogs and rats, AVP was shown to increase HR by acting at V_1 and V_2 receptors (6, 35, 58). Small increases in plasma AVP increased temperature, pulse and respiration (18, 42). Studies in humans demonstrated a biphasic effect of AVP, such that higher doses of the peptide cause vasodilation, an effect which appeared to be mediated by nitric oxide (56). Microinjection of AVP into the nucleus tractus solitarius produced tachycardia in rats (40). If circulating AVP-like ir material is able to cross the blood-brain barrier, the higher levels of this peptide may contribute to the increased HR during eupnea in elephant seal and Weddell seal pups.

One of the most striking features of breath-holding in seals is that arterial BP remains relatively constant in the face of decreasing HR, apparently because of simultaneous increases in vascular resistance (reviewed in 24, 25). Angell-James *et al.* (1) demonstrated that during breath-hold diving the baroreceptor control of HR in seals is reset towards bradycardia at a given level of mean arterial BP. All three hormones, ANP,

AVP and Ang II, affect the baroreceptor reflex control of HR by resetting its set point and/or altering vagal activity (3, 30, 35, 39). The actions of ANP appear to induce resetting of the baroreceptor control of HR towards cardioinhibition (59), while those of AVP amplify the increase in HR produced by hypotension (6) and those of Ang II reset the baroreflex control of HR without changing its sensitivity (50, 51). Furthermore, Wong *et al.* (60) suggested that basal levels of endogenous Ang II exert a tonic action on the cardiac baroreflex to increase the set-point around which the baroreflex regulates HR. The observed increases in circulating ANP-like ir material during sleep apnea and the higher levels of AVP- and Ang II-like ir material during eupnea in elephant seal and Weddell seal pups could contribute to the mechanisms by which the baroreceptor reflex is modified by breath-holding. However, we do not have direct evidence that baroreflex was reset in these animals during periods of spontaneous apnea.

Several reviews of the mechanisms of cardiovascular adjustments to breath-holding in marine mammals, with major emphasis on autonomic nervous system interactions, have been published (19, 24, 26). From these, it appears that seals are primarily defending BP during apneic episodes. It is possible that, since northern elephant seal and Weddell seal pups exhale at the onset of apnea, the combined influence from glossopharyngeal and trigeminal baroreceptors, chemoreceptors and pulmonary stretch receptors, as demonstrated by Angell-James *et al.* (2) in harbor seals (*Phoca vitulina richardsii*), initiates the activation of cardiac vagal motorneurons and thus leads to a decrease of HR. Under these circumstances, following the conventional responses to baroreceptor activation (41), BP would tend to increase. The observed increase in ANP-like ir material will ensure that normal BP is maintained. Conversely, HR is restored to pre-apneic levels with the first breath; this sudden increase in HR, via the baroreceptor reflex, would tend to lower BP, a known stimulus for secretion of both AVP and Ang II. The combined action of these peptide hormones would explain the maintenance of a relatively constant BP during the eupnea/apnea cycles in seals.

These results provide support for the hypothesis of a complex involvement among the vasoactive hormones and the control of cardiovascular function and suggest differential levels of control during periods of eupnea and apnea in seal pups. The data from Weddell seal and northern elephant seal pups are in agreement with the hypothesized increase in plasma levels of ANP and decrease in levels of AVP and Ang II in response to apnea-driven bradycardia and lowered cardiac output. We assume that these changes in HR and hormone concentrations are driven by apnea rather than sleep since a few samples obtained from awake animals during breath-holding periods yielded identical results (data not shown).

Differences in HR and vasoactive hormone concentrations between elephant seal pups at 8 and 14 weeks of age suggest that in these seals the factors affecting cardiorespiratory function, including vasoactive hormones, are modified during development. Differences in HR and circulating levels of ANP-, AVP- and Ang II-like ir material among species may reflect the more precocious development of diving behavior in Weddell seal pups compared with northern elephant seal pups. We propose that the intervention of the vasoactive hormones ANP, AVP and Ang II maintains the functional integrity of the cardiovascular system in seals during repetitive cycles of eupnea and apnea.

To resolve the question of whether differences in cardiorespiratory and endocrine function among species are indeed due to differential rates of diving development, further studies using pups of the same age and at the same developmental stage, as well as seal species which start diving at a younger age, are needed. Expanding these studies to include measurements of BP and other determinants of cardiovascular function and variablility would provide a better understanding of the factors that control cardiorespiratory function in seals. Whether the observed changes in vasoactive hormone concentrations during sleep-associated apnea also occur during breath-hold diving remains unknown.

3.6. *Acknowledgments*

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4. *POSTNATAL DEVELOPMENT OF CARDIORESPIRATORY AND ENDOCRINE FUNCTIONS IN SEALS.*[3]

4.1. *Abstract*

We have previously reported that in seals the vasoactive hormones angiotensin II (Ang II), arginine vasopressin (AVP) and atrial natriuretic peptide (ANP) are involved in the control of cardiovascular function, and provided evidence for differential levels of control during periods of eupnea and apnea. We have expanded this study to test the hypothesis that the control of cardiorespiratory function and the involvement of these vasoactive hormones is under postnatal development in seal pups. Heart rate and plasma levels of ANP, Ang II and AVP were measured in northern elephant seal (*Mirounga angustirostris*) and Weddell seal (*Leptonychotes weddellii*) pups during spontaneous breathing (eupnea) and breath-holding (apnea). The observed changes with age in both eupneic and apneic heart rate and vasoactive hormone levels suggest a developmental, as well as a species-specific, component to the integration of cardiorespiratory function, which may be related to diving behavior.

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[3]Postnatal development of cardiorespiratory and endocrine functions in seals. Zenteno-Savin, T. and M. A. Castellini.

4.2. *Introduction*

In mammals, lung function starts at birth when asphyxia, due to blockade of the umbilical vessels, plus cooling of the body activate the respiratory center. When the lungs first fill with air, pulmonary resistance increases inducing a large blood flow through the lungs to the left atrium and increased left atrial pressure. Progressive anatomical and physiological changes in the pulmonary and cardiovascular systems occur over a period of weeks after birth. Concomitant to the entrainment and fine-tuning of the cardiopulmonary system, changes in circulating and tissue concentrations of vasoactive hormones have been observed in newborn mammals (19, 32, 34). At the same time, seal pups develop high tolerance for long-duration apnea and the ability to dive deeper and longer within a few weeks after birth.

The ability to withstand prolonged and repetitive breath-hold periods (apnea) may be an adaptive process in seals, in which the congenital neonatal resistance to apnea is not lost early in development, as occurs in most mammals (4, 14), but is maintained and even enhanced through adulthood. Long-duration apneas, associated with diving (reviewed in 20) and sleep (7, 9), are a routine component in the respiratory patterns of seals. Apnea in seals is characterized by decreases in HR and cardiac output, peripheral vasoconstriction and re-distribution of blood flow (36), as well as increases in hematocrit (6, 9). One of the most striking features of breath-holding in seals is that arterial BP remains relatively constant in the face of decreasing HR because of simultaneous increases in vascular resistance (reviewed in 16, 17).

This project studied postnatal age-dependent changes in HR and plasma levels of ANP-, AVP- and Ang II-like immunoreactive (ir) material of northern elephant seal (*Mirounga angustirostris*) and Weddell seal (*Leptonychotes weddellii*) pups during periods of spontaneous eupnea and apnea. We hypothesized that in these seal pups the integrity of cardiovascular, respiratory and endocrine functions are under post-natal developmental control.

4.3. *Materials and Methods*

High quality electrocardiograms (EKG), respiration data and plasma samples from 5 northern elephant seal pups (*M. angustirostris*) and 5 Weddell seal pups (*L. weddellii*) were collected during periods of eupnea and apnea. Weddell seal pups were sampled in the austral summer of 1994 near McMurdo Station, Antarctica, and northern elephant seal pups were studied in spring of 1995 in Año Nuevo State Reserve, CA. Because these animals were tagged when born as part of long-term population studies, their age was known. Pup age ranged between 37 and 107 days. The mean age for Weddell seal pups was 42 days and for northern elephant seal pups 98 days.

Handling and sampling techniques followed routine procedures for the study of sleep-associated apnea in northern elephant seals (7, 9). Briefly, seals were captured, transported to laboratory facilities (Long Marine Laboratory, University of California Santa Cruz (elephant seals), or an adapted fish-hut anchored on the sea ice at McMurdo Sound (Weddell seals)), and weighed. Under light anesthesia (3.0 mg/kg ketamine, Ketaset®, Aveco Co., New York, NY, and 1.25 μg/kg Diazepam®, Abbott Laboratories, North Chicago, IL) and sterile conditions, a percutaneous catheter (14 gauge, 5 ¼ inch, Becton Dickinson, Sandy, UT) was implanted in the extradural intravertebral vein and needle electrodes (21 gauge, 1.5 inch stainless needles) were anchored subdermally across the thoracic area. An antibiotic was administered intravenously (iv) (0.5 g Keflin®, Lilly Co., New York, NY). Animals were allowed to recuperate from this minimal anesthesia for at least 3 hours. The northern elephant seal pups were studied while they slept or rested in a large, quiet room. The Weddell seal pups were examined while they slept or rested between diving bouts; they had free access to water through a hole in the sea ice under the laboratory hut (reference for general method: 8). The animals were not handled or restrained during the sampling period. After the experiment was completed, the antibiotic dosage was repeated, the electrodes and catheter were removed, and the animals

were kept for an additional period (up to 12 hours) for observation. After this time, the seals were returned to the colonies.

The EKG signal was directed through a cardiotachometer (BIOTACH, ufi, Morro Bay, CA), and heart rate and respiratory chest movement were simultaneously collected into an analog multichannel physiological recorder (Microscribe, Houston Instruments, The Recording Co., San Marcos, TX) for later analysis. Heart rate polygraphic data was digitized (DrawingBoard II, CalComp Digitizer Products Group, Scottsdale, AZ) to obtain mean and instantaneous HR for each eupneic and apneic period.

Blood samples were collected for hormone analysis during at least 5 independent periods of spontaneous eupnea and apnea. Samples were collected 1 to 2 minutes into apnea and 2 minutes after the first breath. Blood samples (5 ml) were collected into chilled test tubes containing 0.125 M EDTA (Vacutainer 6450, Becton-Dickinson Ltd., Rutherford, NJ). Plasma was separated by centrifugation at 4000 x g at 4°C for 10 minutes. To the recovered plasma an angiotensin-converting enzyme inhibitor, o-phenanthroline (0.025 M, 100 μl/ml plasma, Sigma Chemicals, St Louis, MO) was added (15). All samples were stored at -70°C, transported to University of Alaska Fairbanks (UAF) and kept frozen until analyzed. The vasoactive hormone concentrations were analyzed in plasma samples using radioimmunoassay (RIA) kits for AVP, ANP and Ang II (Phoenix Pharmaceuticals, Mountain View, CA), which include antibodies raised in rabbits against the human peptides. We have found sufficient cross-reactivity between antisera raised in rabbits against the human peptide hormones and ir material in plasma samples of several species of seals and sea lions using these kits (38). For all three hormones, recovery during the extraction procedure was typically about 90%; the mean intra-assay coefficient of variance was 6%. All plasma samples were run in duplicate in a single assay. Biochemical identity of the ir material obtained from elephant seal and Weddell seal plasma samples has not yet been assessed; thus, in this paper will be referred to as ANP-, AVP- and Ang II-like ir material, accordingly.

Statistical analyses were carried out as follows: To assess age-dependent changes in HR and hormone concentrations, separate linear regressions for each variable were performed. Interactions between HR, breathing status (eupnea or apnea, scored as 1 or 0, respectively), hormone concentrations and age were detected with analysis of covariance (ANCOVA) (37) (SYSTAT®, SPSS Inc.). Predictors of HR based on breathing status, hormone concentrations and age were obtained using discriminant analysis (37). Significance was assumed when $P < 0.05$. Data are presented as mean ± standard error of the mean (SE). Heart rate units are beats per minute (bpm), and hormone concentrations are expressed as picograms of ir material per milliliter (pg/ml) of extracted plasma.

4.4. *Results*

The HR recorded for seal pups during eupnea was not significantly different between species (Table 4.1). Apneic HR in Weddell seal pups was lower ($P < 0.05$) than that in elephant seal pups. During eupnea, the concentration of ANP-like ir material was lower ($P < 0.05$), and the concentrations of Ang II- and AVP-like ir material were higher ($P < 0.05$) in Weddell seal pups than in elephant seal pups. During apnea, the concentration of Ang II-like ir material was lower ($P < 0.05$) and the concentration of AVP-like ir material was higher ($P < 0.05$) in Weddell seal pups than in elephant seal pups.

TABLE 4.1. Mean heart rate and average concentrations of vasoactive hormones in extracted plasma samples from northern elephant seal and Weddell seal pups during periods of eupnea and apnea. Data are presented as mean ± standard error of the mean. HR= heart rate; ANP= atrial natriuretic peptide; Ang II= angiotensin II; AVP= arginine vasopressin. Sample size is indicated in parentheses.

		HR (bpm)	ANP (pg/ml)	Ang II (pg/ml)	AVP (pg/ml)
Northern Elephant Seal Pups					
mean age 98 days					
Eupnea	(29)	93.9 ± 2.0	20.9 ± 2.2	30.9 ± 1.4	3.9 ± 0.5
Apnea	(53)	53.2 ± 1.1*	26.3 ± 1.8	16.5 ± 0.9*	2.5 ± 0.2*
Weddell Seal Pups					
mean age 42 days					
Eupnea	(32)	94.0 ± 1.4	12.5 ± 0.5‡	39.6 ± 1.7‡	7.2 ± 0.4‡
Apnea	(35)	46.2 ± 0.6*‡	30.6 ± 1.2*	12.2 ± 0.4*‡	3.2 ± 0.1*‡

*P < 0.05 compared to values during eupnea. ‡P<0.05 compared to elephant seal pups.

Among the northern elephant seals (age 95 to 107 days) average eupneic and apneic HR were higher ($P < 0.05$) in the youngest animals (Figure 4.1 A). Average ANP-like ir material during eupnea and apnea was lower ($P < 0.05$) in the younger pups (age 95 and 96 days) (Figure 4.1 B). Apneic Ang II-like ir material did not vary with age (Figure 4.1 C), but apneic AVP-like ir material was higher ($P < 0.05$) in the older pups (age 100 and 107 days) (Figure 4.1 D). In these elephant seal pups, age accounted for 8.4% ($P=0.035$) of apneic HR variability and 5.3 % ($P=0.231$) of eupneic HR variability. In elephant seal pups, age, ANP-like ir material and breathing status (eupnea or apnea) were the most significant variables describing HR (ANCOVA, $P < 0.05$): *Heart Rate = 91.898 - 0.369 Age + 38.546 Breathing - 0.131 ANP + 0.134 Ang II - 0.346 AVP* ($R^2=0.841$).

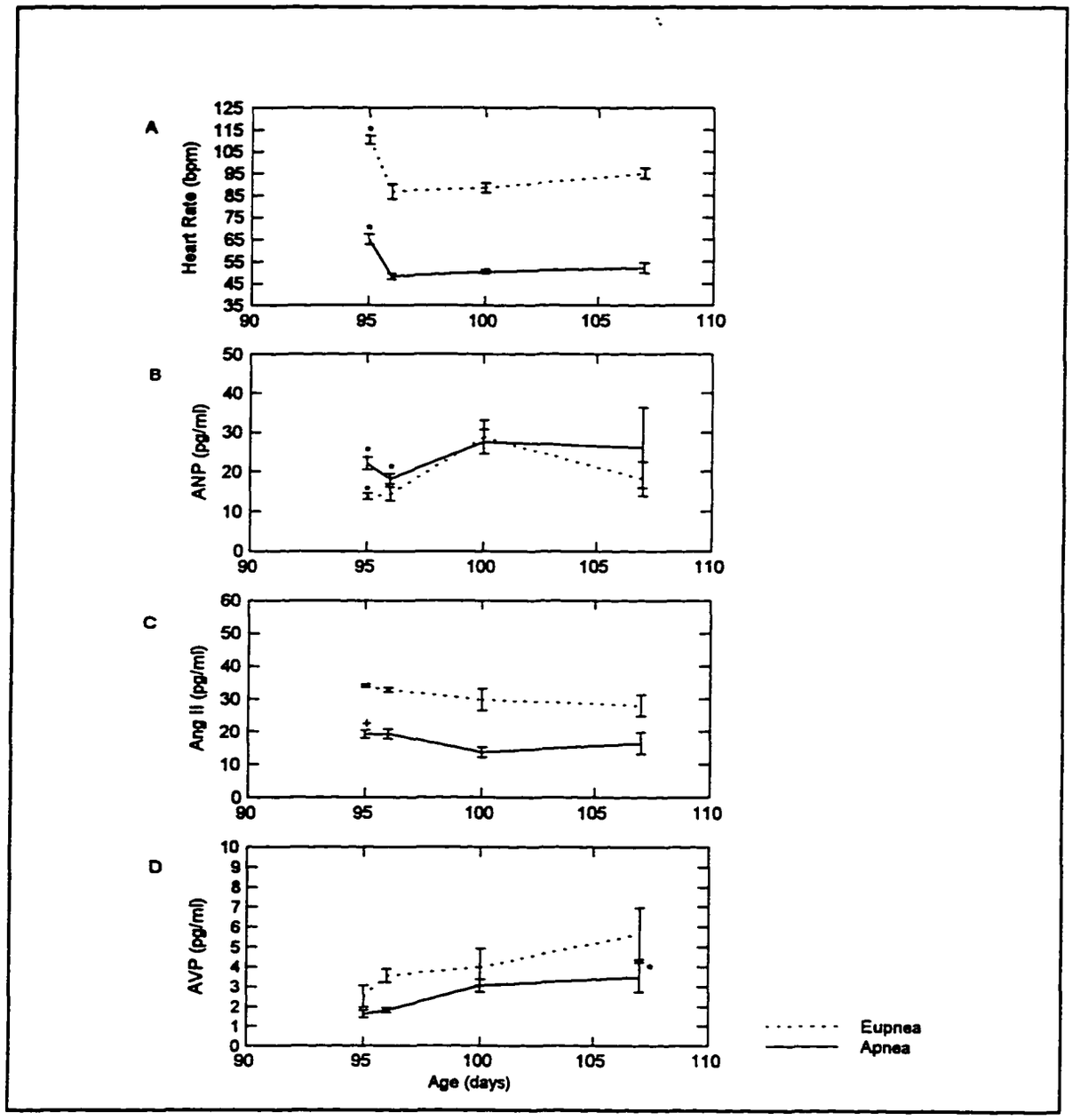

FIGURE 4.1. Heart rate (A: HR, bpm) and hormone concentrations (B: ANP= atrial natriuretic peptide, C: ANG= angiotensin II, D: AVP= arginine vasopressin, pg/ml) against age (days) during eupnea (broken line) and apnea (continuous line) in 5 northern elephant seal pups. *P<0.05. +P<0.1.

Among Weddell seals (Figure 4.2 A-D), the average eupneic HR was higher ($P < 0.05$) and ANP-like ir material was lower ($P < 0.05$) in the youngest pup (age 37 days). However, eupneic Ang II- and AVP-like ir material did not show variation with age ($P < 0.1$). Except for HR, which was lower ($P < 0.05$) in the oldest (50 days old) pup, there was no change in the measured parameters with age during apnea in Weddell seal pups. Age accounted for 19.3% (P=0.008) and 34.9% (P=0.001) of apneic and eupneic HR variability, respectively, in Weddell seal pups. Levels of Ang II- and AVP-like ir material, age and breathing status (eupnea or apnea) were the most significant variables explaining HR (ANCOVA, $P < 0.05$): *Heart Rate = 74.998 - 0.783 Age + 48.270 Breathing - 0.218 ANP + 0.458 Ang II - 2.228 AVP* (R^2=0.971).

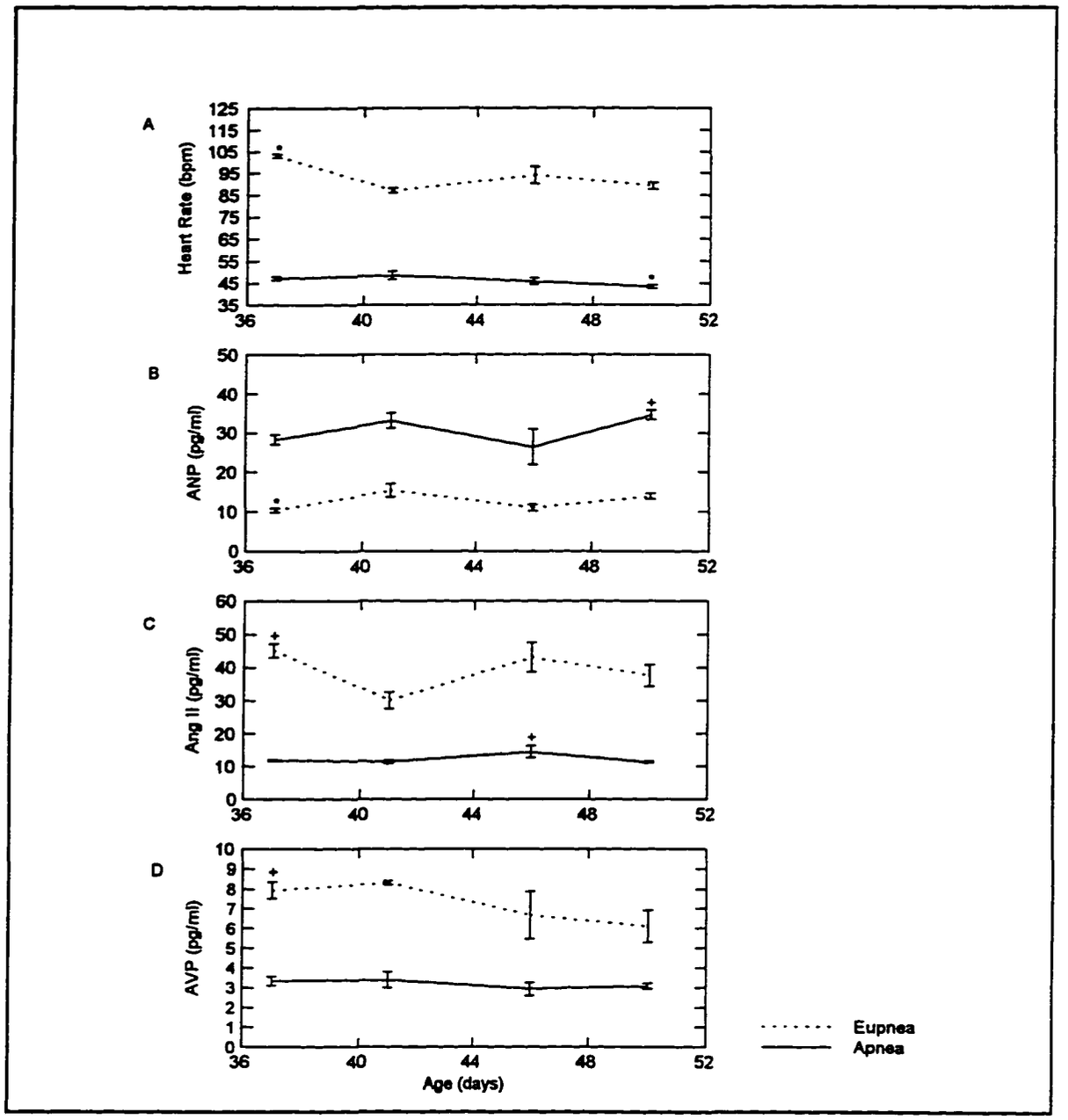

FIGURE 4.2. Heart rate (A: HR, bpm) and hormone concentrations (B: ANP= atrial natriuretic peptide, C: ANG= angiotensin II, D: AVP= arginine vasopressin, pg/ml) against age (days) during eupnea (broken line) and apnea (continuous line) in 4 Weddell seal pups. *P<0.05. +P<0.1.

4.5. *Discussion*

The differences between eupneic and apneic HR were smaller in the younger seal pups. This suggests development of respiratory sinus arrhythmia (RSA) to a higher degree in the older seal pups, as was reported for northern elephant seals by Castellini *et al.* (10). Differences between species may be associated with the development of a strong RSA at an earlier postnatal age in Weddell seal pups than in northern elephant seal pups. In parallel with HR data, the differences between apneic and eupneic hormone concentrations were larger in Weddell seal pups than in elephant seal pups, and within each species in the older than in the young pups. These differences suggest both a developmental and a species-specific component to the integration and control of the cardiorespiratory function in seal pups.

Circulating levels of ANP-, Ang II- and AVP-like ir material changed with age in both elephant seal and Weddell seal pups. ANP-like ir material tended to increase and Ang II-like ir material tended to decrease with age in both species, while AVP-like ir material appeared to increase in elephant seals and decrease in Weddell seals. Plasma concentrations of ANP decreased with age in newborn humans (3). ANP levels may be a consequence of the pulmonary haemodynamics during the perinatal period (after 33). In rats and humans, plasma angiotensin-converting enzyme (and presumably Ang II) levels display a biphasic pattern, rising a few days after birth and decreasing toward adult values within the first month of life (12, 31). Changes in plasma levels of AVP during the early postnatal period are correlated to development of the hypothalamo-hypophyseal-adrenocortical system in mammals (13). Thus, it is possible that ANP, Ang II and AVP contribute to the adaptation of the neonate mammal to extrauterine life, as related to blood volume homeostasis and development of cardiovascular, pulmonary and/or renal structures and functions (1, 29, 32).

During the first days and months after birth, the circulation of the newborn is in a transitional state, constantly changing. In the pig the mean pulmonary arterial pressure at

birth falls by about 50% in 12 hours, and by 6 months of age the pulmonary arterial pressure and resistance are down to adult levels (14, 28). Development of the sympathetic nervous system is incomplete at birth in the rat, chick, mouse, and sheep (11, 21, 22), and, because there is no baroreflex feedback control of the sympathetic outflow, the cardiovascular control is still not fully mature (25). Simultaneously, the vascular smooth muscle contractile mechanisms that mediate noradrenergic vasoconstriction are maturing rapidly during this period (26). Resting blood pressure in the early postnatal period is maintained near the physiologic level by circulating noradrenergic vasoactive substances, including AVP and Ang II, in rats (27). Similarly, the actions of ANP induce resetting of the baroreceptor control of HR towards cardioinhibition (35), while those of AVP amplify the increase in HR produced by hypotension (5) and those of Ang II reset the baroreflex control of HR without changing its sensitivity (30). Thus, ANP, AVP and Ang II affect control of HR by resetting the baroreceptor reflex set point and/or altering its vagal activity (2, 18, 23, 24). The observed changes in circulating ANP-, AVP- and Ang II-like ir material with age in elephant seal and Weddell seal pups could contribute to the maintenance of blood pressure during the early postnatal stages.

These results provide support for the hypothesis that in Weddell seals and northern elephant seals the factors affecting cardiorespiratory function, including vasoactive hormones, are modified during postnatal development, and provide a suggestion for differential levels of control during periods of eupnea and apnea in seal pups. We propose that the intervention of the vasoactive hormones ANP, AVP and Ang II maintains the functional integrity of the cardiovascular system in developing seals during repetitive cycles of eupnea and apnea.

4.6. *Acknowledgments*

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5. *DIFFERENTIAL CONTRIBUTION FROM THE AUTONOMIC NERVOUS SYSTEM TO THE CONTROL OF CARDIORESPIRATORY FUNCTION IN SEALS.*[4]

5.1. *Abstract*

Spectral analysis of continuous electrocardiogram records allows identification of contributing factors to heart rate (HR) and its variability. However, some of the properties of HR variability inherent to the cardiovascular system, as well as interactions with other systems, may be concealed by movements due to respiration. Seals are the only mammals able to hold their breath for a sufficiently long period at normal body temperatures to allow studies of HR control and variability independently of the respiratory input. We used northern elephant seal (*Mirounga angustirostris*) and Weddell seal (*Leptonychotes weddellii*) pups as models of differential nervous contribution to the control of cardiovascular function, by comparing spectral peaks during spontaneous breathing (eupnea) and long-duration breath-holding (apnea). We found differential contribution of the autonomic nervous system to the control of HR in the these seal pups dependent on the input from the respiratory system. The results from this study provide evidence for distinct levels of cardiovascular control during periods of apnea and eupnea in both Weddell seal and elephant seal pups, and suggest that the factors affecting cardiorespiratory function are under developmental control and may differ by species.

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[4]Differential contribution from the autonomic nervous system to the control of cardiorespiratory function in seals. Zenteno-Savin, T. and M. A. Castellini.

5.2. *Introduction*

The cardiovascular system is a natural oscillator that receives input from respiration (17), blood pressure (BP) waves (2), and vasomotor activity (13). Spectral analysis of beat-to-beat heart rate (HR) variability has been an important tool in identifying these cyclical factors and is widely used to assess cardiovascular function (13, 14). It is well established that the cardiac response to these factors is mostly due to nervous transmission, primarily autonomic drive to the heart (12, 18). Additional influences (for example, from the endocrine system) may contribute to integrate incoming signals from the lungs and blood vessels.

Spectral analysis provides a means for separating sources of variance to a time-based phenomenon, such as HR, if these sources can be distinguished in the frequency domain. While spectral analysis of HR is a very powerful tool, it remains difficult to differentiate cardiorespiratory pathologies with this technique because intrinsic patterns in HR variability may be masked by mechanically induced changes in breathing pattern (11, 20). For example, to gain a clearer view of the hormonal control of HR essentially requires a study where the subject is not breathing. Consequently, only a few studies have focused on identifying contributions from the endocrine system in the overall pattern of HR control (25). Furthermore, some of the factors involved in the control of HR and cardiovascular function are markedly influenced by experimental conditions, such as anesthesia, drugs and surgery, which are likely to distort our understanding of the integrated function of the circulation. Inevitably, the relative dominance or priority when cardiovascular responses and reflexes interact can be studied only in an intact preparation.

Studying HR patterns in the absence of respiratory influences calls for a different kind of model. In this sense, seals offer a unique opportunity. Prolonged and repetitive breath-hold periods (apnea), associated to dive (up to 2 hours) and sleep (25 min) (4, 6, 16), are a routine component in the ecological physiology of pinnipeds (seals and sea lions). The ability to withstand long apneic periods may be an adaptive process in seals, in

which the congenital neonatal tolerance of apnea is not lost early in development, as occurs in most mammals (1, 10), but is maintained and even enhanced through adulthood. Changes in HR, cardiac output, distribution of blood flow and hematocrit are characteristic of dive- and sleep-apnea in seals (3, 23).

This project studied medical aspects of how marine mammals can withstand both long duration and repetitive apnea during sleep. We explored the differential contribution of the nervous system to the control of HR by comparing spectral signatures of HR from northern elephant seal (*Mirounga angustirostris*) and Weddell seal (*Leptonychotes weddellii*) pups during normal ventilation (eupnea) and breath-holding. The working hypotheses were that during eupneic periods in seals, HR variability was strongly driven by respiration; that in the absence of ventilation, with the withdrawal of mechanical influences from chest movements, intrinsic characteristics of HR and HR variability would prevail; and that, under these circumstances, internal variation of HR occurred as a response to differential input from the nervous system.

5.3. *Materials and Methods*

High quality electrocardiograms (EKG) and respiration data from northern elephant seal and Weddell seal pups during periods of apnea and eupnea (scored as 0 and 1, respectively) were collected as part of a larger study on developmental changes in sleep apnea in seals (6, 7). Cardiorespiratory variability in these data was quantified and correlated to endocrine patterns in the plasma samples; these results have been reported elsewhere (25).

Northern elephant seal pups were sampled in spring of 1994 and 1995 in California, and Weddell seal pups were sampled in the austral summer of 1994 near McMurdo Station, Antarctica. Because most of these animals were tagged when born as part of long-term population studies, their age was known. When birth-dates were not known, age was calculated from mor phometric data. Pup age ranged between 4 and 16

weeks. The mean age for Weddell seal pups was 6 weeks. Northern elephant seal pups were divided by age into two groups, mean ages 8 and 14 weeks. Handling and sampling techniques followed routine procedures for the study of sleep-associated apnea in northern elephant seals (4, 6). Briefly, seals were captured, transported to laboratory facilities (Long Marine Laboratory, University of California Santa Cruz (elephant seals) or an adapted fish-hut anchored on the sea ice at McMurdo Sound (Weddell seals)), and weighed. Under light anesthesia (3.0 mg/kg ketamine, Ketaset®, Aveco Co., New York, NY, and 1.25 μg/kg Diazepam®, Abbott Laboratories, North Chicago, IL), needle electrodes were anchored subdermally across the thoracic area. Animals were allowed to recuperate from this minimal anesthesia for at least 3 hours. The northern elephant seal pups were studied while they slept or rested in a large, quiet room. The Weddell seal pups were examined while they slept or rested between diving bouts; they had free access to water through a hole in the sea ice under the laboratory hut (for general method see: 5). The animals were not handled or restrained during the sampling period. After the experiment was completed, the electrodes were removed, and the animals kept for an additional period (up to 12 hours) for observation. After this time, the seals were returned to the colonies.

Data on time, EKG, HR and chest breathing movements were simultaneously collected. Sample sets were obtained for at least 5 independent eupneic and apneic periods from each animal. Cardiorespiratory data were obtained from a total of 5 Weddell seal pups and 10 northern elephant seal pups. Heart rate was recorded continuously by directing the electrocardiogram signal through a cardiotachometer (BIOTACH, ufi, Morro Bay, CA). Heart rate and respiratory chest movement were simultaneously collected directly into an analog multichannel physiological recorder (Microscribe, Houston Instruments, The Recording Co., San Marcos, TX) for later analysis.

Analog electrocardiograms were digitized (Drawing Board II, CalComp Digitizer Products Group, Scottsdale, AZ), instantaneous HR calculated and data transformed to

interbeat (RR) intervals in milliseconds. Discrete Fourier transformations and power spectral analyses were applied to HR data to identify spectral peaks. To assess the relative contribution of the sympathetic (SNS) and parasympathetic (PNS) innervation to the spontaneous HR and HR variability during eupneic and apneic periods, coarse graining spectral analysis was applied using computer software (CGSA, Toronto, Ontario, Canada). Briefly, the total power of HR variability was broken down into harmonic and nonharmonic (fractal) components, and the contribution of the fractal component to total HR variability power was calculated (22). The high-frequency (0.15-0.5 Hz) component may be used as a marker of PNS modulation of HR, while the ratio of low- (0.04-0.15 Hz) to high-frequency indicates activity of the cardiac SNS component.

Paired t-tests with Bonferroni adjustment were applied to instantaneous HR and indices of PNS and SNS activity to determine differences between species (24) (SYSTAT®,SPSS Inc., Chicago, IL). Stepwise discriminant analysis and analysis of covariance (ANCOVA) were applied to the full data set. This introduced the variables (HR, breathing status -eupnea or apnea, scored as 1 or 0, respectively-, and indicators of PNS and SNS activity) into the analysis in the order of their ability to discriminate between the two groups (eupnea and apnea) and at the same time brought out the correlation structure of the variables. Predictors of breathing status based on HR and PNS and SNS indices were obtained using logistic regression (15) (SYSTAT®, SPSS Inc.). Significance was assumed when $P < 0.05$. Data are presented as mean ± standard error of the mean (SE). Heart rate units are beats per minute (bpm), indices of SNS and PNS activity are dimension-less.

5.4. *Results*

Results obtained for instantaneous HR, RR interval, and indices of SNS and PNS activity for northern elephant seal and Weddell seal pups are summarized in Table 5.1.

TABLE 5.1. Instantaneous heart rate (HR, beats per minute), interbeat interval (RR Interval, milliseconds), and indicators of the parasympathetic (PNS) and sympathetic (SNS) activity estimated for periods of eupnea and apnea from digitized electrocardiogram records of northern elephant seal and Weddell seal pups. Data are presented as mean ± standard error. Sample size is indicated in parenthesis.

	HR	RR Interval	PNS	SNS
Northern Elephant Seal Pups				
mean age 8 weeks				
Eupnea (34)	96.1 ± 3.0	643.7 ±19.1	0.715 ± 0.010	0.321 ± 0.019
Apnea (39)	66.4 ± 1.8[a]	928.9 ± 24.6[a]	0.814 ± 0.007[a]	0.211 ± 0.008[a]
Northern Elephant Seal Pups				
mean age 14 weeks				
Eupnea (28)	95.4 ± 1.6	629.1 ± 10.9	0.628 ± 0.015	0.479 ± 0.031
Apnea (55)	53.3 ± 0.5[a]	1125.9 ± 10.5[a]	0.714 ± 0.011[a]	0.205 ± 0.012[a]
Weddell Seal Pups				
mean age 6 weeks				
Eupnea (22)	90.8 ± 3.0	682.6 ± 32.7	0.672 ± 0.029	0.543 ± 0.078
Apnea (29)	47.3 ± 3.9[a]	1249.9 ± 54.5[a]	0.751 ± 0.022[b]	0.390 ± 0.045

[a]= $P<0.05$, [b]= $P<0.01$ as compared to eupnea for the same species.

As expected, RR interval was lower, and conversely HR was higher, during spontaneous breathing as compared to apneic periods in both seal species. Respiratory sinus arrhythmia (RSA), acceleration of the heart during inspiration and slowing of the heart during the expiratory phase of respiration, was observed in all seal pups (data not shown). In all cases, apnea was initiated after exhalation and the apneic RR interval was maintained at the average interval recorded during expiration in the course of spontaneous breathing. The differences between eupneic and apneic HR and RR interval were smaller in the younger elephant seal pups and largest in the Weddell seal pups.

Representative power spectra of the instantaneous HR from a northern elephant seal pup (Figure 5.1) and from a Weddell seal pup (Figure 5.2) show that during apnea the high frequency peaks corresponding to respiration (0.4 Hz) are absent from the power spectra, and that during this period HR is characterized by peaks in the low frequencies (frequency < 0.1 Hz). The indicator of PNS activity was higher during apnea than during eupnea in both elephant seals and Weddell seals (Table 5.1). The indicator of SNS activity was higher during eupnea than during apnea in elephant seal pups (Table 5.1).

In the younger group of elephant seal pups (average age 8 weeks) breathing status (eupnea or apnea) and SNS index were the most significant variables explaining HR (ANCOVA, $P < 0.05$): *Heart Rate = 63.7 + 32.7 Breathing - 26.8 SNS + 7.9 PNS* (R^2=0.702). Logistic regression produced the following model to distinguish between periods of eupnea and apnea: *Breathing = exp (0.210 + 0.338 HR + 0.006 SNS + 0.045 PNS) / (1 + exp (0.210 + 0.338 HR + 0.006 SNS + 0.045 PNS)*), where *exp* represents an exponential function.

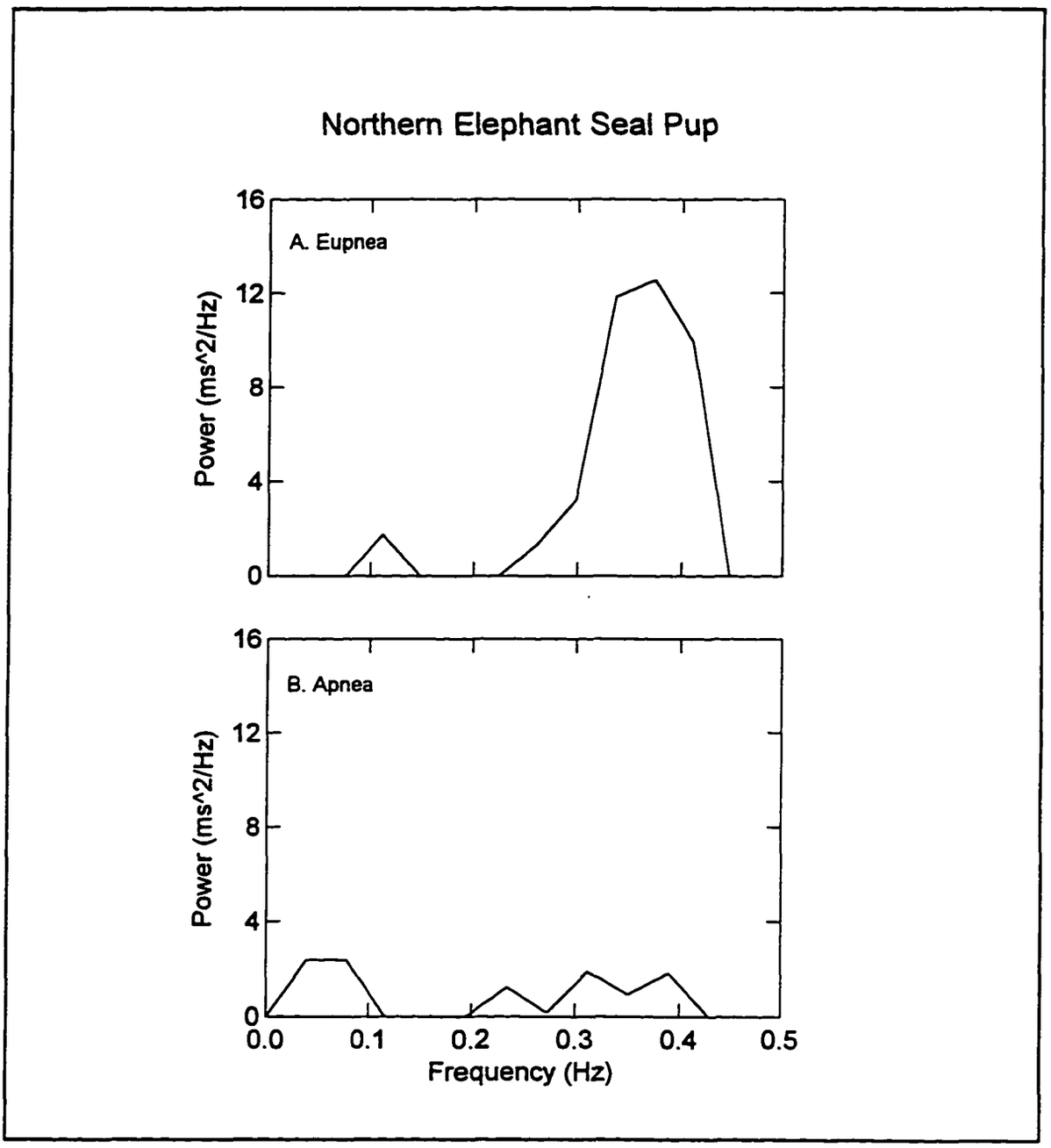

FIGURE 5.1. Representative power spectra of the instantaneous heart rate from one episode of sleep eupnea (A) and one of apnea (B) in a northern elephant seal pup (age 7 weeks).

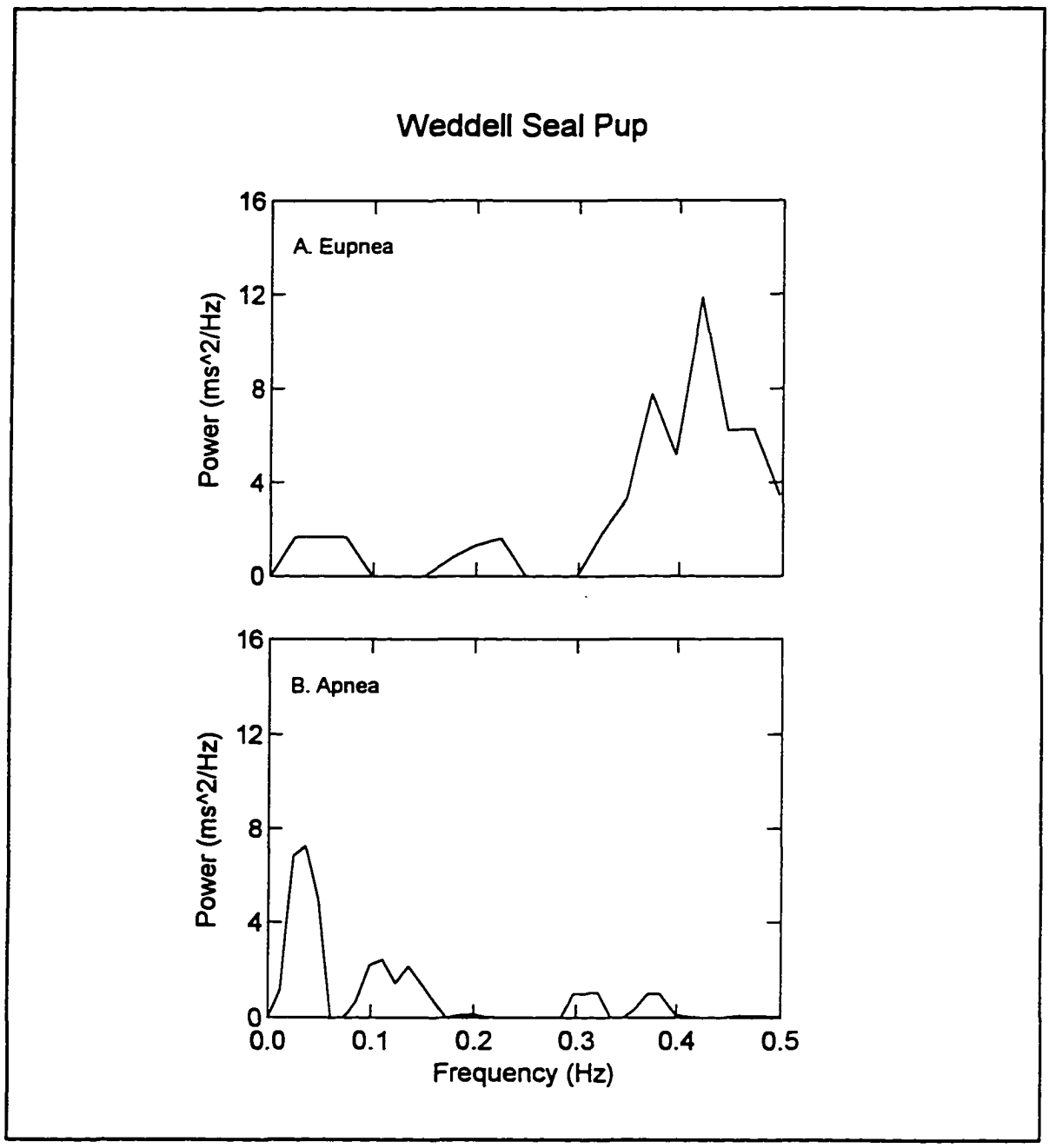

FIGURE 5.2. Representative power spectra of the instantaneous heart rate from one episode of sleep eupnea (A) and one of apnea (B) in a Weddell seal pup (age 5 weeks).

In the older group of elephant seal pups (average age 14 weeks) breathing status (eupnea or apnea), PNS and SNS indices were all significant variables describing HR (ANCOVA, $P < 0.01$): *Heart Rate = 28.7 + 39.8 Breathing + 14.0 SNS + 31.1 PNS* (R^2=0.803). Logistic regression produced the following model to distinguish between periods of eupnea and apnea: *Breathing = exp (0.100 + 0.647 HR - 0.198 SNS + 0.054 PNS) / (1 + exp (0.100 + 0.647 HR - 0.198 SNS + 0.054 PNS)*), where *exp* represents an exponential function.

In the Weddell seal pups (average age 6 weeks) breathing status (eupnea or apnea) and SNS index were the most significant variables explaining HR (ANCOVA, $P < 0.01$): *Heart Rate = 51.8 + 49.1 Breathing - 9.4 SNS - 2.7 PNS* (R^2=0.949). Logistic regression produced the following model to distinguish between periods of eupnea and apnea: *Breathing = exp (0.144 + 0.348 HR + 0.043 SNS + 0.047 PNS) / (1 + exp (0.144 + 0.348 HR + 0.043 SNS + 0.047 PNS)*), where *exp* represents an exponential function.

5.5. *Discussion*

Instantaneous HR and RR interval recorded during spontaneous breathing were significantly different to those recorded during sleep-associated apnea in both elephant seal and Weddell seal pups (Table 5.1), consistent with previous findings (reviewed in 8). Simultaneously, differences between apneic and eupneic HR and RR interval were not quantitatively equivalent among the groups, indicating development of RSA, as has been previously suggested (7, 25), and control of cardiorespiratory function to a higher degree in the 14 week old than in the 8 week old elephant seal pups, and in Weddell seal pups (average age 6 weeks) than in the northern elephant seal pups. Differences between species may be associated with the development of a strong RSA at an earlier postnatal age in Weddell seal pups than in elephant seal pups.

Spectral analyses of the RR interval from northern elephant seal and Weddell seal pups suggest that during sleep apnea, HR was characterized by peaks in the lower

frequencies and absence of peaks in the higher frequencies (the region of the respiratory frequency) in northern elephant seal and Weddell seal pups (Figures 5.1, 5.2). These results indicate differential contributions to the control of HR during periods of eupnea and apnea. Because Weddell seals were sleeping on the surface of the ocean (water temperature -1 °C) during sampling, additional peaks in the spectra may reflect activity of the nervous system and/or thermoregulatory mechanisms.

It is well established that in mammals, increases in HR are the product of cardiac SNS activation and parasympathetic withdrawal. Differential contribution of the autonomic innervation to cardiorespiratory function was indicated by the higher index of PNS activity during apnea in both species and the lower index of SNS activity during apnea in elephant seal pups. These results indicate PNS withdrawal and increased activity of the SNS component of HR during eupnea, and increased PNS activity during apnea in these seal pups. At the same time these data suggest predominance of the vagal inflow, which correlates with HR being lower during breath-holding. These results are consistent with Daly *et al.* (9) who concluded that in harbor seals (*Phoca vitulina*) bradycardia due to diving apnea is vagal in origin. In a study using human subjects, Sakakibara and Hayano (21) suggested that voluntarily slowed respiration increases the cardiac PNS activity in humans.

Although eupneic HR was lower in the older elephant seal pups and lowest in Weddell seal pups, PNS indicators did not increase as would be expected. The index of SNS activity was higher in the older elephant seal pups and highest in Weddell seal pups, which may suggest finer control of peripheral vascular muscle tone in the older elephant seal pups and Weddell seal pups. These data suggest changes in the autonomic balance with age and between species which may contribute to the different eupneic (taken in this context as basal) HR, may be related to post-natal development of cardiorespiratory function, and may correlate to the development of diving behavior in seal pups. At the time of birth in the rat (and at other times in different species) there is a period of potential

autonomic imbalance when the PNS innervation to the heart is established but the SNS innervation is not yet well developed (in 19).

These results cannot be taken as conclusive due to the fact that indicators of SNS and PNS activity in elephant seal and Weddell seal pups were obtained from computer programs designed and validated for use mostly with HR data from humans. The significance of the indicators of nervous activity as obtained from elephant seal and Weddell seal HR data needs to be evaluated. Nevertheless, these preliminary results suggest differential nervous input to the control of cardiorespiratory function in seal pups.

5.6. *Acknowledgments*

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6. *EFFECTS OF FOOD LIMITATION AND FASTING ON PLASMA VASOACTIVE HORMONE LEVELS IN JUVENILE STELLER SEA LIONS.*[5]

6.1. *Abstract*

Significantly elevated levels of plasma atrial natriuretic peptide (ANP) in the declining populations of Steller sea lions from the Aleutian Islands and the Gulf of Alaska have been revealed. Because food limitation can alter levels of osmoregulatory hormones under certain conditions in mammals, this project measured plasma concentrations of ANP, arginine vasopressin (AVP), and angiotensin II (Ang II) at weekly intervals during a 4-week restricted-intake diet and a 14-day total fast in juvenile Steller sea lions (*Eumetopias jubatus*). Neither food limitation nor fasting affected ANP or AVP levels, but at the end of both trials, plasma Ang II levels were significantly higher than initial values, although within physiological levels for mammals. These results suggest that Steller sea lions have a remarkable capacity to maintain hydrosmotic and endocrine balance during short-term food limitation and fasting. However, significant differences in plasma hormone concentrations between age-matched free-ranging Steller sea lions throughout their geographic distribution in Alaskan waters, compared with healthy conspecifics in captivity may provide preliminary evidence that Steller sea lion populations in Alaskan waters are affected by longer-term food limitation. The results of this study are important to understanding how sea lions are affected by limited food resources and crucial for interpreting data collected on free-ranging Steller sea lions.

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[5]Effects of food limitation and fasting on plasma hormone levels in juvenile Steller sea lions. Preliminary results from an on-going project. Zenteno-Savin, T., L. D. Rea and M. A. Castellini.

6.2. *Introduction*

Some populations of Steller sea lions (*Eumetopias jubatus*) in Alaskan marine waters have been declining in numbers over the past two decades (20, 23, 29). With the evidence available to date, the decreases in these populations cannot be directly attributed to either environmental or anthropogenic causes. One of the hypotheses for the decline in pinniped populations is inadequate food supply, quantity and/or quality, affecting juveniles and subadults (44).

Food restriction in humans and terrestrial mammals is often associated with dehydration and electrolyte imbalance (32, 35). Maintenance of sodium and water homeostasis depends on a balance between intake (thirst and sodium appetite) and output (mostly via the kidneys). Osmoregulatory hormones have an important role in maintaining this balance: Renal water excretion is largely dependent on the action of arginine vasopressin (AVP, the antidiuretic hormone), and sodium excretion is controlled by the renin-angiotensin-aldosterone system (RAAS) and atrial natriuretic peptide (ANP). Thirst and sodium appetite are controlled by the same hormones acting at specific sites within the brain (27, 28, 37, 38).

The plasma concentrations of these osmoregulatory hormones may be useful as indirect indicators of nutritional status. Recent research in other mammals suggests the involvement of the endocrine system in the observed responses to fasting, weight-cycling and malnutrition (7, 9, 18, 21). Starvation was accompanied by impaired secretion of AVP, ANP and Angiotensin II (Ang II) (7, 12, 22, 25). In humans, acute and chronic starvation produced a significant reduction in serum angiotensin converting enzyme (ACE) (16, 22), presumably reducing the levels of circulating Ang II. However, fasting did not alter the vascular responsiveness to Ang II in rats (7, 8). The mean plasma level of ANP in patients with anorexia nervosa was significantly higher than that in age-matched healthy subjects, and was related to an elevated cardiac atrial pressure (25). In addition, isotonic volume expansion did not increase plasma ANP concentration in anorexics, as occurs in

healthy humans (25). Patients with anorexia nervosa had lower plasma AVP levels and, although the average concentration of the peptide in the cerebrospinal fluid (CSF) did not differ from normal concentrations, the CSF-to-plasma ratio of AVP was often reversed (12). Following weight gain, ACE activity, ANP and AVP levels were restored to normal (12, 22, 25). These combined results suggest that Ang II, AVP and ANP secretion may be impaired during food limitation and fasting in mammals.

Unlike from most mammals, seals and sea lions are adapted to withstand long duration fasting as part of their reproductive and molting cycles. During these periods of food deprivation, phocid seals remain relatively active while progressively decreasing their dependence on lean tissue for energy. As the fast proceeds, total body metabolism decreases, and utilization of non-esterified fatty acids and ketone bodies replaces protein degradation as a source of energy (5) . Therefore, metabolic needs are met without compromising the requirements for thermoregulation and maintenance of muscle mass. Fast-induced changes in metabolism and biochemistry in otariids are not well known and have only recently been studied (3, 31). It would be expected that seals and sea lions retain endocrine balance during their routine fasting cycles. However, under states of starvation or prolonged malnutrition the hormonal indicators of osmoregulation and hemodynamics are expected to react in a similar fashion to that observed in other mammals. Therefore, undernourished Steller sea lions would be expected to have lower concentrations of Ang II and AVP, and higher concentration of ANP, compared to healthy animals.

6.3. *Materials and Methods*

Effects of food limitation and fasting were studied in captive Steller sea lions that had been captured as pups in the wild and raised at the Vancouver Aquarium, Vancouver, British Columbia, Canada. Before, and immediately after the experimental regimes each animal was fed 8 kg herring per day.

6.3.1. *Food Limitation*

Four juvenile (4 years old) Steller sea lions (3 males and 1 female) were maintained on a restricted herring diet for 4 weeks, such that the animals lost mass at a consistent rate (approximately 0.5 kg per day) over the experimental period. Thus, during this time, food supply was limited to 4 kg herring per day. Blood samples were collected from each animal at the beginning of the study as controls (Day 0), and on Day 7, 14, 21 and 28 of reduced food intake.

6.3.2. *Fasting*

Two juvenile male Steller sea lions underwent a complete fast for 14 days. No food was given during the experimental period, but ice cubes were given at training sessions and fresh water was available at all times. Blood samples were collected after 0, 3, 7 and 14 days of fasting.

6.3.3. *Free-ranging Steller Sea Lions*

Blood samples were collected from Steller sea lions at haul-out sites and rookeries at various locations in the Aleutian Islands, the Gulf of Alaska and Southeast Alaska. Only samples from free-ranging Steller sea lion pups and yearlings were considered in this study, in an attempt to match the ages of the sea lions in captivity. In the wild, same age class (i.e., 3-4 year old) Steller sea lions are seldom found at the haul-out sites.

6.3.4. *Blood Sample Analyses*

Captive animals were held in a restraining cage and free-ranging sea lions were manually restrained for blood sampling. In all cases, blood samples (5 ml) were taken by venipuncture from the dorsal pelvic vein. Each sample was transferred to a blood collection tube containing 0.125 M ethylenediaminetetraacetic acid (EDTA) (Vacutainer 6450, Becton-Dickinson Ltd., Rutherford, NJ), and immediately chilled. Plasma was

separated from the red blood cells by centrifugation at 4000 x g for 10 minutes. For hormone analyses, an angiotensin-converting enzyme inhibitor, o-phenanthroline (0.025 M, 100 μl/ml plasma, Sigma Chemicals, St Louis, MO) was added to the recovered plasma (10). All samples were stored frozen at -80°C for later analysis at the University of Alaska Fairbanks. Whole blood and plasma samples were submitted to the local veterinary laboratory for hematology and clinical chemistry panels, including spun hematocrit and mean corpuscular hemoglobin content (MCHC). These results are presented in detail elsewhere (31, Rea *et al.* unpublished).

6.3.5. *Hormone Analyses*

Prior to hormone analysis, samples were thawed and immunoreactive material (ir) was extracted by using prepacked octadecasilyl-silica cartridges (SepCol, Phoenix Pharmaceuticals, Mountain View, CA), according to a method adapted from Hartter (15, 42). The percent recovery from the extraction procedure was determined by adding known amounts of synthetic peptide (5 to 100 pg/ml, Phoenix Pharmaceuticals) to pooled plasma (quality control). Measurements of extracted plasma were not corrected for extraction efficiency, which ranged from 92 to 112%.

Hormone concentrations were analyzed in plasma samples using radioimmunoassay (RIA) kits for AVP, ANP and Ang II (Phoenix Pharmaceuticals), which include antibodies raised in rabbits against the human peptides. We have found sufficient cross-reactivity between antisera raised in rabbits against the human peptide hormones and ir material in plasma samples of several pinniped species using these kits (42). All plasma samples were run in duplicate, and replicates were run in a single assay, along with a quality control (pooled plasma) sample. Biochemical identity of the ir material obtained from Steller sea lion plasma samples has not yet been assessed; thus, in this paper will be referred to as ANP-, AVP- and Ang II-like ir material, accordingly.

Statistical analyses were carried out as follows: Repeated measures analysis of variance (ANOVA) was used to identify significant differences in hormone concentrations over the experimental period (41). Pearson correlation coefficients (PCC) were determined to evaluate interactions between hormone concentrations and clinical chemistry parameters (41). To assess differences between food limitation and fasting, t-tests were applied on hormone concentrations at Day 0, 7 and 14 (41). Significance was assumed when $P < 0.1$. Data are presented as mean ± standard error of the mean (SE). Hormone concentrations are expressed as picograms of ir material per milliliter (pg/ml) of extracted plasma.

6.4. *Results*

6.4.1. *Food Limitation*

Initial (Day 0) hormone concentrations in plasma samples from the captive Steller sea lions were as follows: AVP-like ir material, 7.4 ± 3.1 pg/ml; ANP-like ir material, 20.8 ± 2.8 pg/ml; Ang II-like ir material, 5.7 ± 1.3 pg/ml. By the end (Day 28) of the experimental regime, plasma concentration of Ang II-like ir material was significantly elevated over initial values ($P < 0.1$) (Figure 6.1). Under these circumstances, plasma levels of ANP-like ir material were correlated to plasma levels of Ang II-like ir material (PCC = 0.630), and both hormones were correlated to MCHC (data not shown) (PCC = -0.690 and -0.369, respectively).

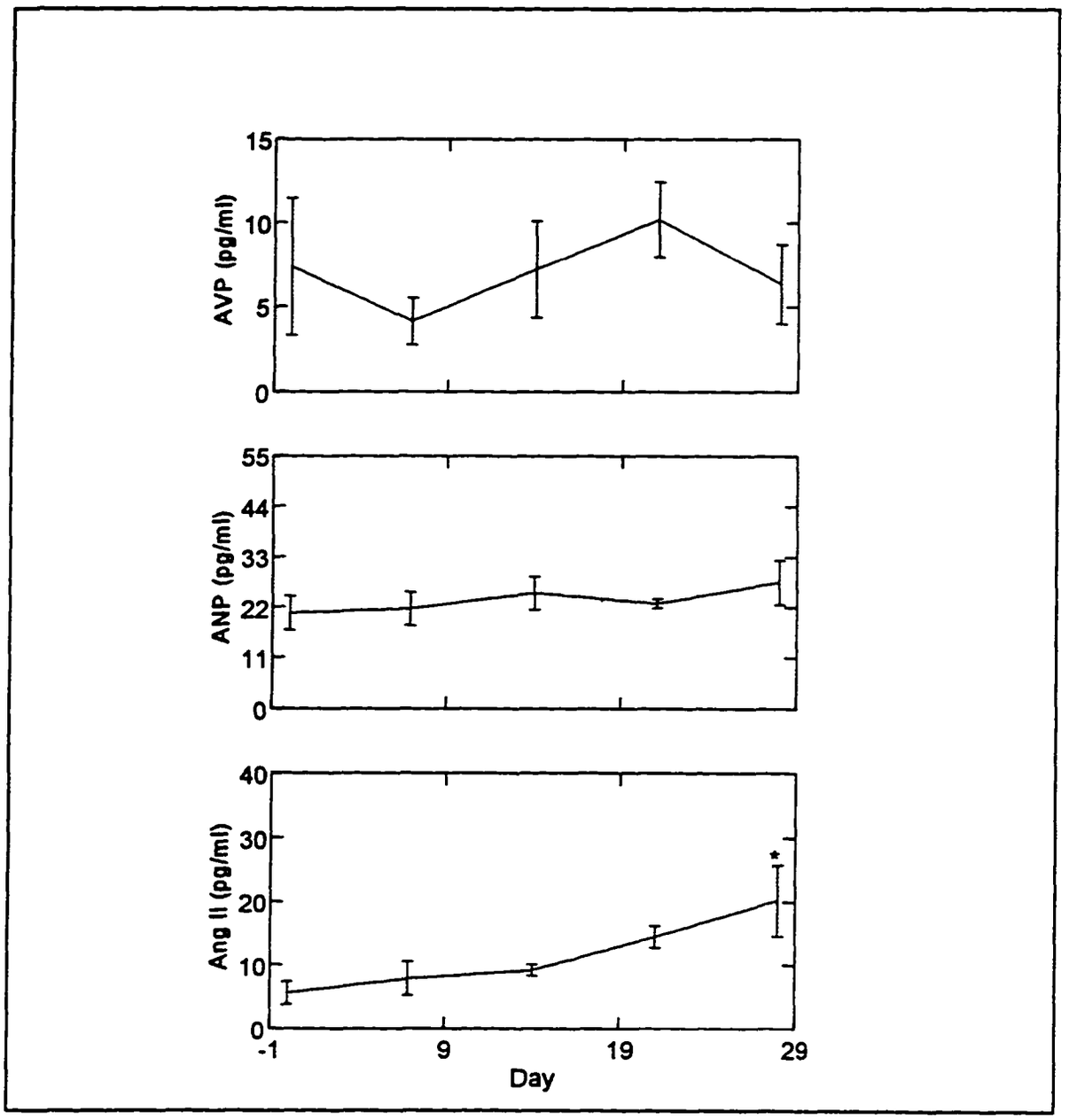

FIGURE 6.1. Plasma concentration of arginine vasopressin (AVP), atrial natriuretic peptide (ANP) and angiotensin II (Ang II) in four Steller sea lions under a food limitation regime. * $P < 0.1$ compared to initial values. Values shown are mean ± standard error.

6.4.2. *Fasting*

Plasma hormone concentrations at Day 0 of the fasting regime were: AVP-like ir material, 4.1 ± 0.6 pg/ml; ANP-like ir material, 32.1 ± 10.6 pg/ml; Ang II-like ir material, 6.5 ± 1.0 pg/ml. At the end of the 14-day long fast, plasma levels of Ang II-like ir material were significantly higher than initial values ($P < 0.1$) (Figure 6.2). During the fasting regime, plasma concentrations of ANP-like ir material and Ang II-like ir material were negatively correlated (PCC = -0.487). Plasma concentrations of ANP-like ir material were also negatively correlated to plasma water (PCC = -0.523), and plasma levels of Ang II-like ir material were positively correlated to serum water and MCHC, but negatively correlated to plasma specific gravity (data not shown) (PCC = 0.578, 0.574 and -0.759, respectively).

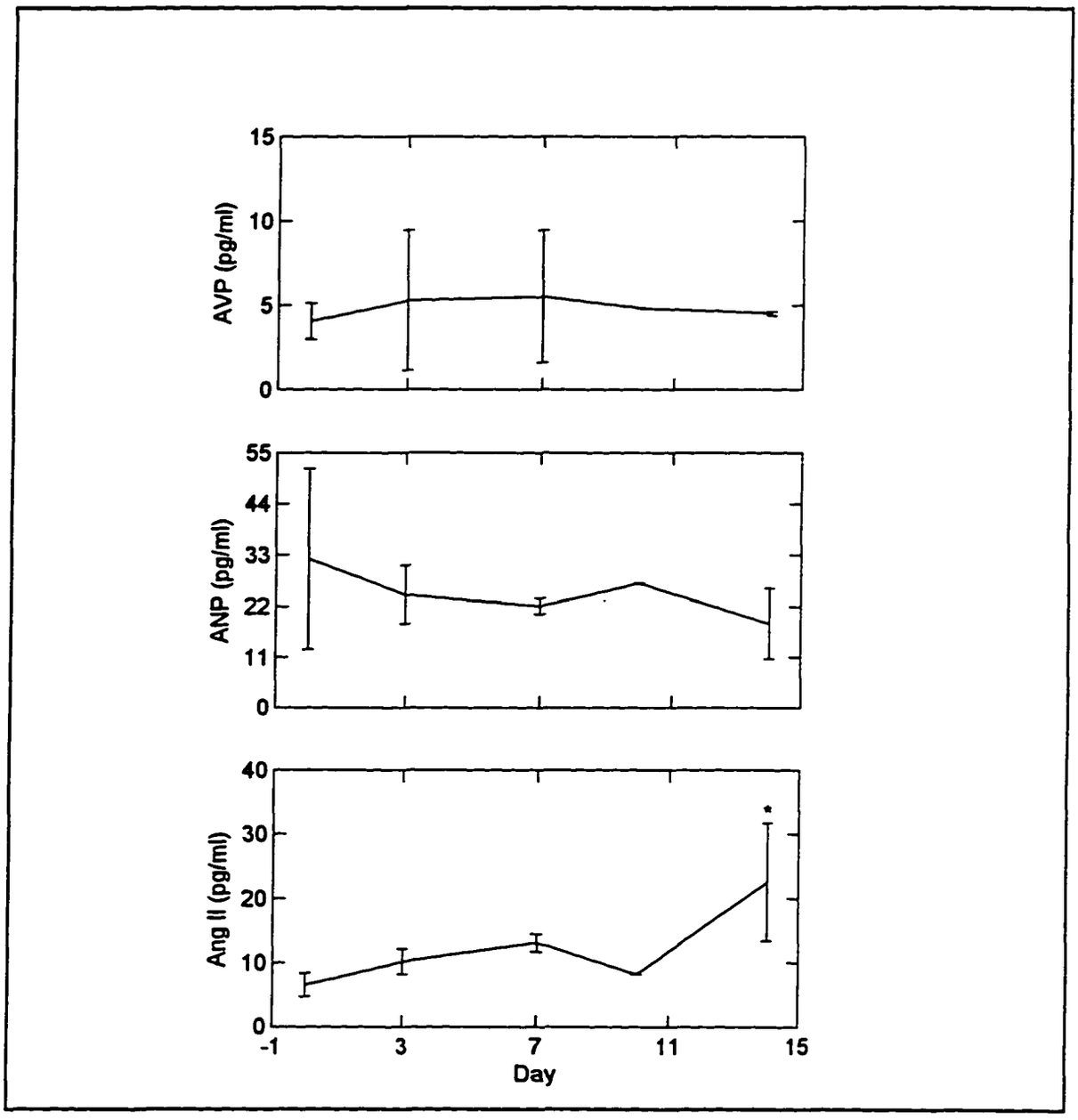

FIGURE 6.2. Plasma concentration of arginine vasopressin (AVP), atrial natriuretic peptide (ANP) and angiotensin II (Ang II) in two Steller sea lions under an experimental fasting regime. * $P < 0.1$ compared to initial values. Values shown are mean ± standard error.

There were no statistically significant differences in plasma hormone concentrations between food limitation and fasting at Day 0 (Table 6.1). By Day 7, plasma levels of Ang II-like ir material in fasted Steller sea lions were significantly higher than in food-limited animals ($P < 0.1$). This difference was also observed at Day 14.

6.4.3. *Free-ranging Steller Sea Lions*

Plasma levels of AVP-, ANP- and Ang II-like ir material in young Steller sea lions sampled in Alaskan waters are presented in Table 6.2 and Figure 6.3. The concentrations of AVP- and ANP-like ir material in Steller sea lions from the Aleutian Islands and the Gulf of Alaska were significantly higher than in animals from Southeast Alaska and in those held at the Vancouver Aquarium ($P < 0.1$). However, plasma Ang II-like ir material levels in Steller sea lions from the Gulf of Alaska and Southeast Alaska were significantly higher than those in sea lions from the Aleutian Islands and the Vancouver Aquarium ($P < 0.1$).

TABLE 6.1. Plasma concentrations of arginine vasopressin (AVP), atrial natriuretic peptide (ANP) and Angiotensin II (Ang II) in juvenile Steller sea lions under two different feeding regimes. Sample size is indicated in parentheses.

	AVP (pg/ml)	ANP (pg/ml)	Ang II (pg/ml)
FOOD LIMITATION (n=4)			
Day 0	7.4 ± 3.1	20.8 ± 2.8	5.7 ± 1.3
Day 7	4.1 ± 1.1	21.7 ± 2.8	7.9 ± 2.0
Day 14	7.2 ± 2.4	25.1 ± 3.0	9.2 ± 0.8
FASTING	(n=2)		
Day 0	4.1 ± 0.6	32.1 ± 10.6	6.5 ± 1.0
Day 7	5.5 ± 2.1	22.0 ± 0.9	13.1 ± 0.8 * +
Day 14	4.5 ± 0.1	18.2 ± 4.1	22.6 ± 5.0 * +

* $P < 0.1$ compared to values at Day 0. + $P < 0.1$ compared to food-limited Steller sea lions.

TABLE 6.2. Plasma concentration of vasopressin (AVP), atrial natriuretic peptide (ANP) and angiotensin II (Ang II) in Steller sea lions across their distribution range in Alaskan waters. Sample size is indicated in parentheses.

		AVP (pg/ml)	ANP (pg/ml)	Ang II (pg/ml)
Aleutian Islands	(46)	7.8 ± 0.5 *	59.8 ± 3.4 *	24.8 ± 2.9
Southeast Alaska	(41)	4.7 ± 0.4	29.1 ± 2.8	37.4 ± 4.6 *
Gulf of Alaska	(55)	8.7 ± 0.9 *	64.2 ± 3.7 *	36.5 ± 3.7 *
Vancouver Aquarium	(9)	5.9 ± 0.4	21.6 ± 0.6	23.8 ± 1.9

* $P < 0.1$ compared to concentrations in other areas.

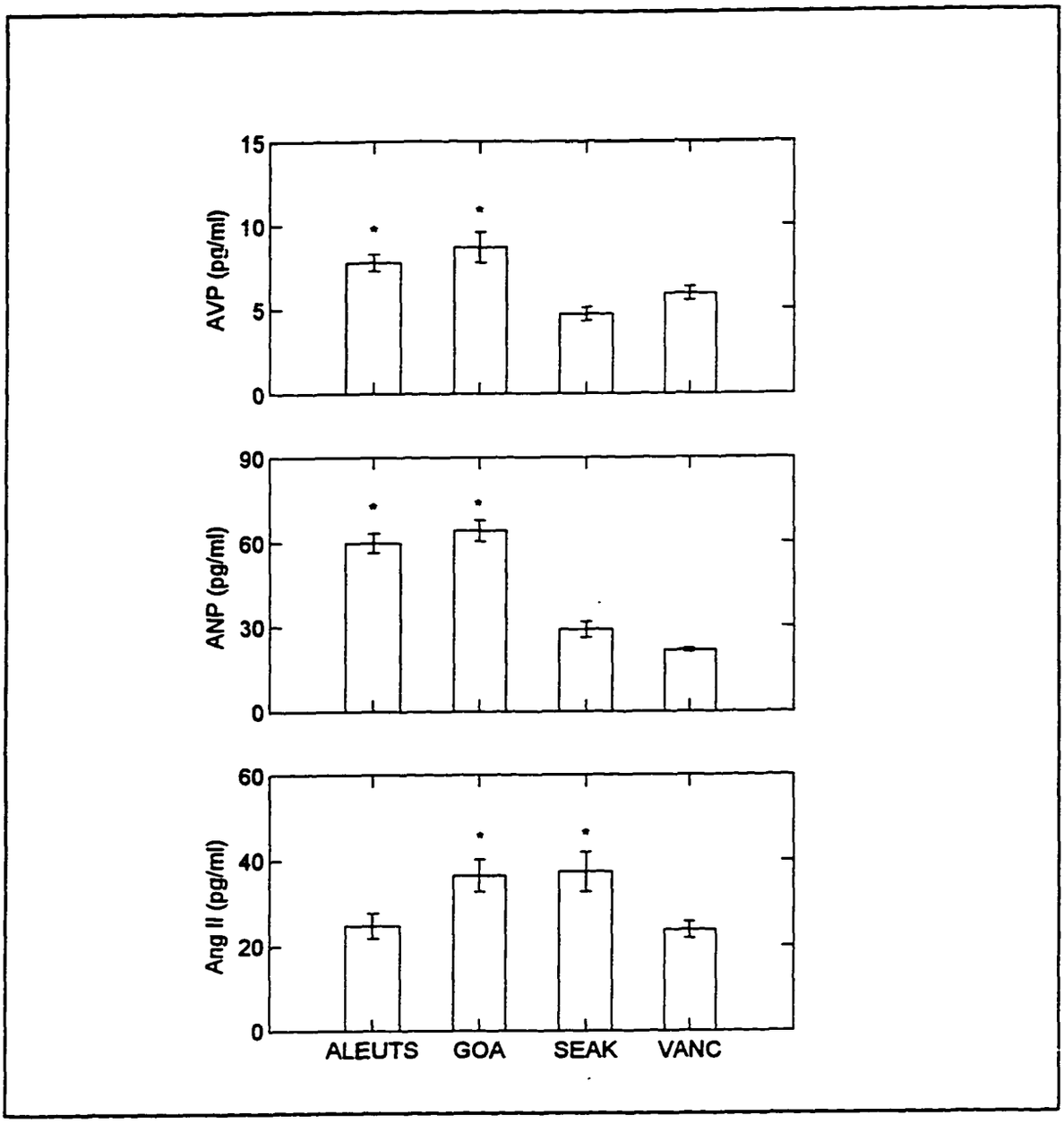

FIGURE 6.3. Plasma concentration of vasopressin (AVP), atrial natriuretic peptide (ANP) and angiotensin II (Ang II) in Steller sea lions across their distribution range in Alaskan waters. ALEUTS= Aleutian Islands; GOA= Gulf of Alaska; SEAK= Southeast Alaska; VANC= Vancouver. * $P < 0.1$ compared to concentrations in other areas.

6.5. *Discussion*

Plasma concentrations of AVP- and ANP-like ir material in samples from Steller sea lions at Day 0 of both experimental protocols were comparable to levels reported for a variety of free-ranging pinniped species at rest (42). However, levels of Ang II-like ir material in these captive Steller sea lions were lower than those previously reported for wild animals (42). Low plasma Ang II levels are associated with decreased plasma osmolarity and sodium concentration (14).

Neither short-term food limitation nor fasting affected the plasma AVP- or ANP-like ir material levels in Steller sea lions. However, at the end of both experimental regimes, plasma concentrations of Ang II-like ir material were significantly higher than initial values (Day 0). These findings are not entirely surprising and suggest that hydrosmotic balance was maintained in Steller sea lions during the experimental food limitation and fasting. Maintenance of plasma AVP- and ANP-like ir material levels during the imposed fasting may be, in part, due to access to fresh water and ice cubes. Plasma metabolite concentrations did change progressively during long periods of under-nutrition in Steller sea lions (Rea *et al.* unpublished), suggesting increased reliance on body fat stores for energy and metabolic water production from lipid catabolism. Adequate hydration state in fasting marine mammals is maintained by metabolically-derived water (26, 4). Maintenance of the plasma levels of thyroid hormones, corticosterone and insulin in fasting emperor penguins (*Aptenodytes forsteri*) and king penguins (*A. patagonicus*) (13), and of plasma concentrations of AVP- and ANP-like ir material in fasting Steller sea lions (this study) suggest a remarkable achievement of hydrosmotic and endocrine balance in fasting-adapted species.

During phase II (protein sparing phase) fasting, there is an increase in plasma levels of corticosterone and aldosterone in king penguins (6, 19). These results, and the fact that Ang II induces aldosterone secretion in mammals (30), are in agreement with our finding increasing plasma levels of Ang II-like ir material during food-limitation and

experimental fasting in Steller sea lions. Humans suffering from anorexia nervosa accompanied by dehydration and low plasma levels of potassium and sodium also had an enhanced activity of the RAAS (11, 40), which may be related to an excessive amount of sodium excreted during the first week of fasting (36).

Correlations found between plasma levels of Ang II- and ANP-like ir material and MCHC, plasma specific gravity and plasma and serum water during food limitation and fasting in Steller sea lions suggest that transvascular fluid shifting may also be a mechanism whereby these animals maintain hydrosmotic balance. Both ANP and Ang II in terrestrial mammals are involved in modulating vascular permeability and extracellular fluid partitioning (17, 24, 38), either by modifying pre- and post-capillary vascular resistance (39) or by altering tissue-specific protein transport (37, 43).

Differences in plasma concentrations of AVP-, ANP- and Ang II-like ir material were found over the geographic range of Steller sea lions in Alaskan waters. Although the average concentrations of AVP-like ir material in Steller sea lions sampled in the Aleutian Islands and Gulf of Alaska were significantly higher than those in the animals sampled in Southeast Alaska and those held in the Vancouver Aquarium, the ranges overlapped, suggesting these differences may be associated with physiological states rather than pathological conditions.

Interestingly, ANP-like ir material concentrations in Steller sea lions from the Aleutian Islands and the Gulf of Alaska, both areas where sea lion populations are declining, were significantly higher than concentrations in sea lions from Southeast Alaska and the Vancouver Aquarium. Elevated concentrations of ANP have been found in humans with congestive lung disease (1) and in patients with anorexia nervosa (2, 25).

We hypothesized that if Steller sea lions in Alaska were suffering nutritional deficiencies, secretion of all three hormones (Ang II, ANP and AVP) would be impaired leading to abnormal circulating hormone levels, similar to findings in rats and humans (12, 21, 25). The only evidence that might support this hypothesis are the significantly elevated

levels of plasma ANP-like ir material in the declining populations in the Aleutian Islands and the Gulf of Alaska. It is possible that different biochemical and physiological mechanisms operate during short-term and chronic food limitation, compensating for changes in blood volume, blood osmolality and blood pressure, and lead to distinct levels of endocrine balance.

Research on the effects of prolonged and repeated fasts on pinniped endocrinology should increase our understanding of the biochemical and physiological mechanisms involved in long-term survival. The results generated from this research may be useful in enabling researchers and wildlife managers to identify, in comparative studies, the nutritional status and general health of wildlife populations.

6.6. *Acknowledgments*

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7. CONCLUSIONS

This thesis explored several inter-related aspects of the endocrinology and cardiovascular physiology of pinnipeds. Baseline plasma levels of AVP-, ANP- and Ang II-like ir material were determined in Weddell seals, harbor seals, northern elephant seals, ringed seals, California sea lions and Steller sea lions, and were similar to values reported for other vertebrate species. However, age, species and geographic differences were found. Inherent physiological differences between species and developmental stages, the post-weaning or molting fast, or a combination of these factors could account for the observed differences in plasma levels of AVP-like ir material with age and among species. At this time, there is not a satisfactory explanation for the higher levels of ANP- and AVP-like ir material in Steller sea lions from the Aleutian Islands and the Gulf of Alaska and of AVP- and Ang II-like ir material in harbor seals from Prince William Sound, all areas where the pinniped populations are declining. A number of pathologic states in humans are associated to elevated concentrations of these hormones; among them, congestive lung disease, hypertension, heart failure, pericarditis and some types of anemia.

Changes in vasoactive hormones and heart rate during sleep-associated apnea were studied in northern elephant seal and Weddell seal pups. In both seal species, heart rate and circulating levels of Ang II- and AVP-like ir material were lower while concentrations of ANP-like ir material were higher during periods of apnea. These data support the hypothesis that plasma levels of ANP increase and levels of AVP and Ang II decrease in response to apnea-driven bradycardia, lowered cardiac output, and increased central blood volume. Whether the observed changes in vasoactive hormone concentrations during sleep-associated apnea also occur during breath-hold diving needs to be established.

Differences between apneic and eupneic heart rates among age groups suggest development of respiratory sinus arrhythmia (RSA) to a higher degree in the 14 week old than in the 8 week old northern elephant seal pups. RSA was greater in Weddell seal pups (6 weeks) than in the northern elephant seal pups of either age group. Similarly,

differences in vasoactive hormone concentrations and apnea duration with age suggest that the cardiorespiratory and hormonal functions are under post-natal development. Differences between the species may reflect the fact that the Weddell seal pups start diving at a younger age than northern elephant seal pups, suggesting that the respiratory, cardiovascular and hormonal systems develop in parallel to diving behavior.

The contribution of the autonomic nervous system to heart rate in response to sleep-associated apnea was also studied in northern elephant seal and Weddell seal pups. During normal ventilation, heart rate variability in all seal pups was strongly driven by respiration, while apneic heart rate was characterized by peaks in the lower frequencies with no peaks in the higher (respiratory) frequencies. A higher index of parasympathetic nervous system activity during apnea in both species and lower index of sympathetic nervous activity during apnea in elephant seal pups were found. The results indicate that in these seal pups the prevailing heart rate was preferentially influenced by the sympathetic nervous system during eupnea, and that the apneic bradycardia is mostly due to vagal input. The results are consistent with the expected increases in cardiac parasympathetic nervous activity in response to lower respiratory rates.

The combined results from these projects provide evidence of differential contribution of the endocrine and autonomic nervous systems to cardiovascular function in Weddell seal and northern elephant seal pups, dependent on the respiratory input. In these seal pups, sinus arrhythmia, apnea duration, heart rate and autonomic nervous activity are apparently correlated and under developmental influence. Furthermore, it is possible that the intervention of the vasoactive hormones maintains the functional integrity of the cardiovascular system in seals during repetitive breath-holding cycles. The significance of the role of the hormones during apneic episodes becomes apparent when one keeps in mind that seals maintain a relatively constant blood pressure even when heart rate, cardiac output and vessel resistance are changing, and that the actions of AVP, ANP and Ang II, directly or indirectly, modify blood pressure. In this sense, it would be interesting to

determine if the intervention of AVP, ANP and Ang II in resetting the baroreflex set point and altering vagal activity, as observed in terrestrial mammals, also occurs in seals.

The results of these projects have potential applications in biomedicine and cardiovascular biology. In contrast to marine mammals, humans do not tolerate long duration breath-holding. Sleep apnea syndrome, respiratory distress syndrome, and sudden infant death syndrome (SIDS) are examples of how apnea is maladaptive in humans. Power spectral signatures of heart rate of seals can be compared to those from humans in studies of pathological sleep apnea in adults and infants. Delineating hormonal profiles in humans, similar to the AVP, ANP, and Ang II profiles we have produced in seals, may prove to be of diagnostic and therapeutic value at different stages of various cardiovascular and pulmonary diseases. Studying the factors that control cardiorespiratory function in seal pups and how they develop with age will shed light on the integration of respiratory and cardiovascular systems in newborn mammals, and could point out ways in which failure to achieve such functional integration leads to SIDS.

Conversely, wildlife biology and management of pinniped species would benefit from studies in laboratory mammals. For example, since secretion rates and metabolism of ANP, AVP and Ang II are disrupted in response to weight cycling in rats and eating disorders (anorexia nervosa, bulimia) in humans, these hormones may be useful in exploring the hypothesis that the population decline of Steller sea lions and harbor seals in Alaskan waters is due to nutritional deficiencies.

To address the hypothesis that ANP-, AVP- and Ang II-like ir material in pinnipeds in Alaska may be related to malnutrition, Steller sea lions kept in the Vancouver Aquarium were subjected to a restricted diet for 28 days and an experimental fast for 14 days. Neither short-term food limitation nor fasting affected the plasma AVP- or ANP-like ir material levels in Steller sea lions, suggesting that hydrosmotic balance was maintained. At the end of both experimental regimes, plasma concentrations of Ang II-like ir material were significantly higher than initial values, although they were within the range reported

for healthy mammals under resting conditions. More importantly, significant correlations found in Steller sea lions between Ang II- and ANP-like ir material and MCHC, plasma specific gravity and plasma and serum water during food limitation and fasting suggest that transvascular fluid shifting may have occurred. The only evidence that Steller sea lion populations in Alaskan waters are affected by long-term food limitation that we can provide at this time are the significantly elevated levels of plasma ANP-like ir material in the declining populations at the Aleutian Islands and the Gulf of Alaska.

Clearly, further research is needed to evaluate interactions among hormones and physiological events pertinent to the natural histories of seals and sea lions, such as molting, fasting and diving. Little is known about the biosynthesis rate, half-life, control of secretion, cellular mechanisms of action, receptors, second messengers and signal transduction of the major hormones in marine mammals. Insufficient information is available regarding renal blood flow, pulmonary circulation, control of blood pressure and baroreflex set-point and sensitivity during diving and sleep-apnea in marine mammals. Neither fetal nor postnatal development of these functions have been studied in these animals. The increasing availability of diverse techniques in endocrinology, molecular biology, immunology, biochemistry and physiology may provide tools to compensate for the difficulties posed by the marine environment and the natural history of marine mammals, and allow us to answer some of these questions.

To provide an overall view of the mechanisms discussed in this thesis, schematic representation of the interactions of the respiratory, cardiovascular, hormonal and autonomic nervous systems during periods of breath-holding and spontaneous breathing in seal pups are shown in Figures 7.1 and 7.2, respectively. Seals hold their breath on the expiratory part of the breathing cycle. In mammals, this leads to stimulation of the trigeminal nerves and sympathetic vasomotor neurons, which in turn results in decreased heart rate and cardiac output, increased hematocrit and peripheral vasoconstriction (Figure 7.1). With vasoconstriction, blood is displaced from the peripheral tissues to the lungs and

heart in terrestrial mammals and the abdominal vena cava in marine mammals (Daly *pers. com.*). This increased "central" blood volume is a known stimulus for secretion of ANP. The actions of ANP will oppose any tendency for pressure to increase following peripheral vasoconstriction, thus maintaining an almost constant blood pressure. Additional effects of ANP include inhibition of AVP and the RAAS; cardioinhibition and increased activity of the PNS, enhancing bradycardia and lower cardiac output; and increased capillary permeability and contraction of the spleen, leading to increased hematocrit.

Filling of the lungs with the first breath after an episode of apnea stimulates central inspiratory neurons and pulmonary stretch receptors (Figure 7.2). As a result, heart rate increases, while total peripheral resistance and blood pressure decrease. When the peripheral circulation opens again, blood flow returns to the pre-apneic normal distribution. This re-shifting of blood volume stimulates secretion of AVP and Ang II, resulting in increased blood pressure. In addition, AVP resets the baroreflex to a higher heart rate, Ang II stimulates SNS and inhibits vagal tone to the heart, and both AVP and Ang II stimulate ventilation and breathing movements.

Both AVP and Ang II stimulate contraction of vascular smooth muscle in mammals. However, neither plasma levels of AVP nor Ang II increased in seal pups during periods of sleep apnea, when strong vasoconstriction is expected to occur. It is possible that stimulation of sympathetic vasomotor neurons and hypothalamically directed reflexes involving increased vagal outflow are sufficient to induce vasoconstriction; perhaps blood vessels of seals are not as sensitive to the effects of ANP, AVP and Ang II as in terrestrial mammals, or the actions of other vasoactive hormones override the effects of these. The studies described here show that we are only at the beginning of understanding the complex interactions in these unique mammals.

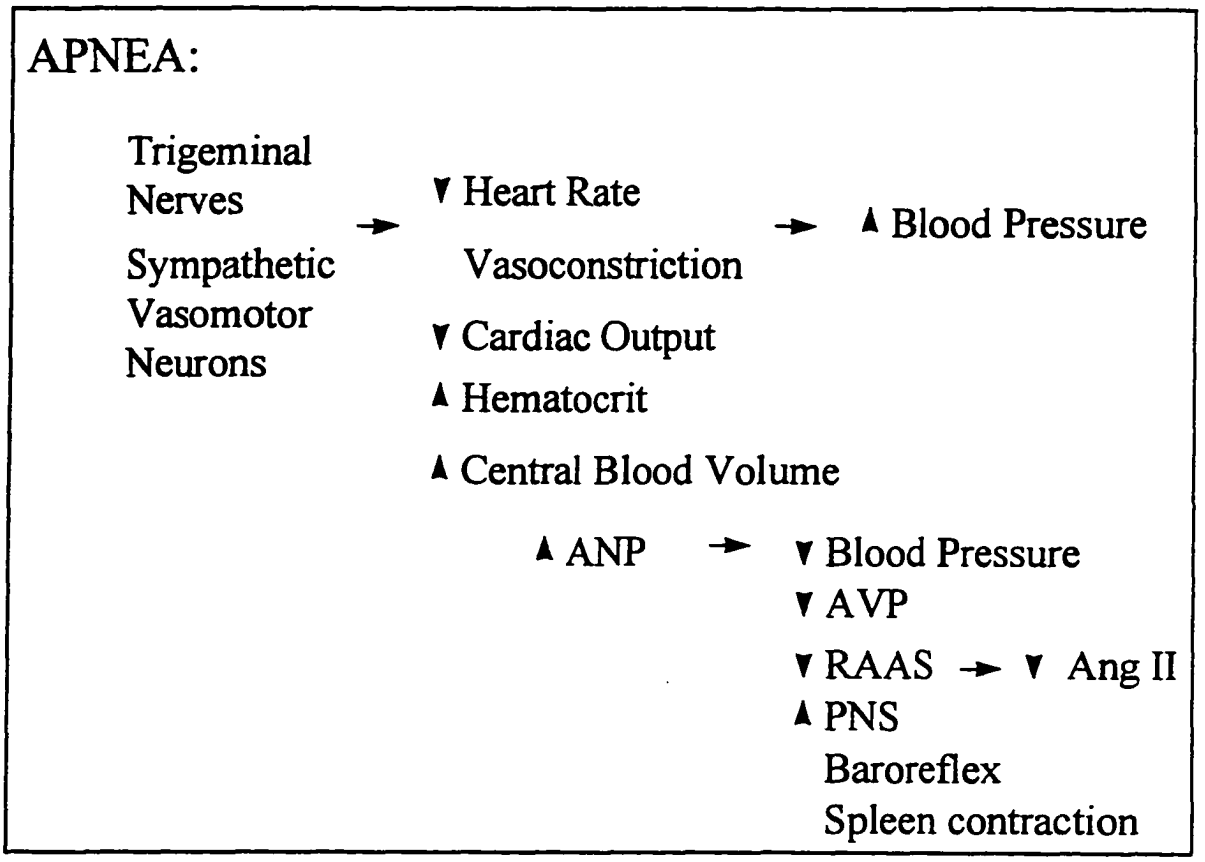

FIGURE 7.1. Schematic representation of the integrated function of the respiratory cardiovascular, hormonal and nervous systems in seal pups during sleep apnea.

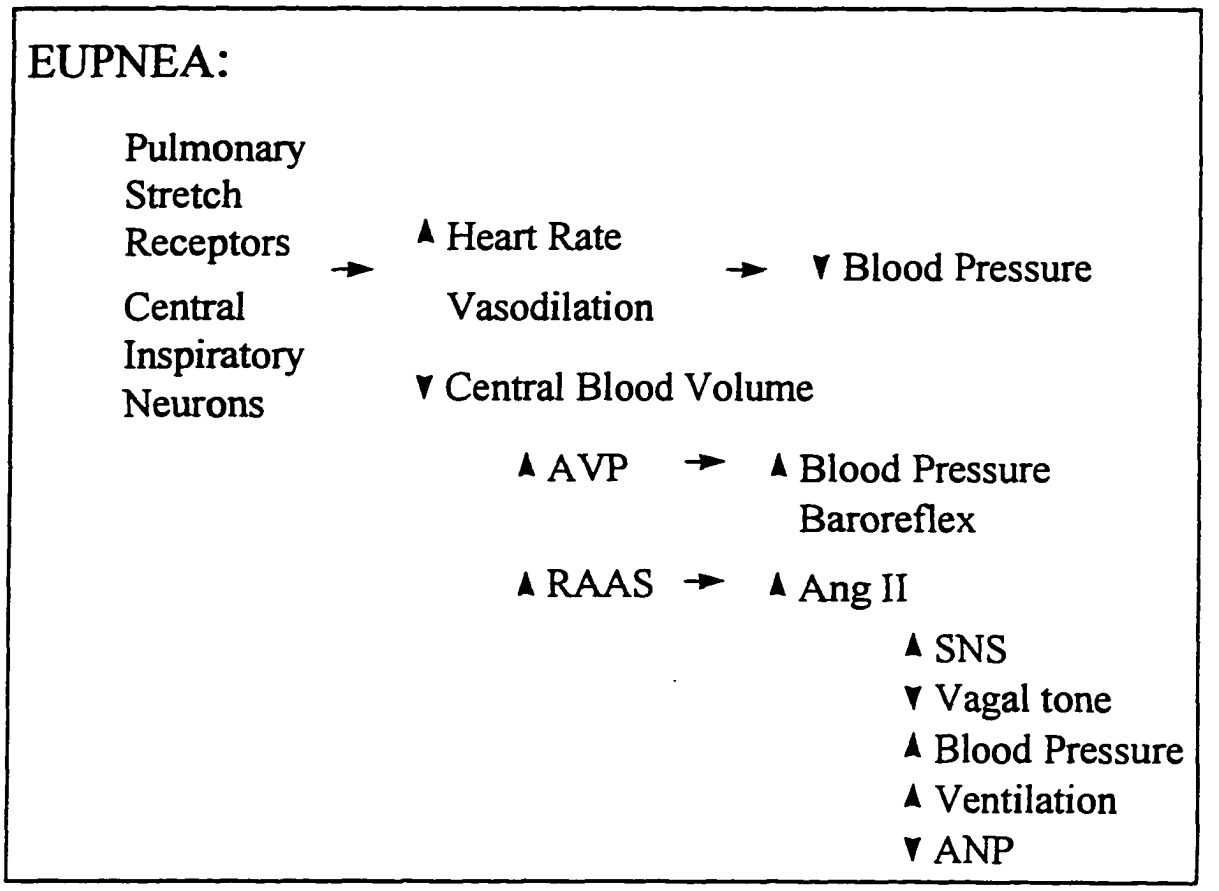

FIGURE 7.2. Schematic representation of the integrated function of the respiratory cardiovascular, hormonal and nervous systems in seal pups during spontaneous breathing.