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Plasma and urine levels of electrolytes, urea and steroid hormones involved in osmoregulation of cetaceans

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ABSTRACT

Cetaceans are well adapted to their hyperosmotic environment by properly developed osmoregulatory ability. A question here is how they regulate water and mineral balances in marine habitats. In the present study, we determined blood and urine levels of various chemicals involved in osmoregulation, compared them with those in artiodactyls, and characterized the values in the whales. Blood and urine samples obtained from baleen whales of common minke (*Balaenoptera acutorostrata*), sei (*B. borealis*), and Bryde's whales (*B. brydei*), and toothed whales of sperm whales (*Physeter macrocephalus*) were analyzed for osmolality, major electrolytes, urea, steroid hormones and glucose. The urine osmolality and Na⁺ concentrations in the cetaceans were much higher than those in the cattle. Furthermore, the cetaceans had 5 to 11-fold urea in plasma than the cattle, and 2 to 4-fold urea in urine. There were no significant difference in the plasma concentrations of corticosteroids between the cetaceans and the cattle. The present results indicate that the osmoregulatory parameters seem to be not affected by the reproductive stage and sex steroid hormones. The concentrations of urea in plasma and urine of the baleen whales were higher than those of the sperm whales, indicating a possibility that their osmoregulatory mechanisms may be correlated to their feeding habits. The present results suggest that cetaceans have unique osmoregulatory mechanisms by which they excrete strongly hypertonic urine to maintain fluid homeostasis in marine habitats.

Key Words: osmolality, electrolytes, urea, steroid hormones, cetaceans

INTRODUCTION

Marine mammals, whose blood compositions are similar to terrestrial mammals, can survive in seawater, despite the large difference in salinity between their internal fluid and the ambient environment. This fact implies that they should obtain water to maintain the water and mineral homeostasis. There are, however, only a few possibilities to obtain water for marine mammals: extraction from seawater, preformed dietary sources, and metabolic oxidation of food. Occasional seawater drinking is unusual in marine mammals (Elsner, 1999). Their predominant source of water is considered to be their food (Costa, 2002), so that they face different osmoregulatory problems depending on the type of prey that they consume.

The fossil data and genetic information indicated that transition of cetaceans from terrestrial to aquatic life is inferred to be a relatively recent evolutionary event (Graur and Higgins, 1994; Gatesy and O'Leary, 2001; Gingerich *et al.*, 2001). According to the sequence analyses of mitochondrial DNA, the divergence between hippopotamuses and cetaceans was dated to nearly 55 million years ago, while the divergence of basal artiodactyls was dated to 65 million years ago (Arnason *et al.*, 2000). Analyses of retroposon showed that whales, hippopotamuses and ruminants form a monophyletic group (Shimamura *et al.*, 1997; Nikaido *et al.*, 1999; Nikaido *et al.*, 2001a). Extant cetaceans are systematically divided into two suborders: Mysticeti (baleen whales) and Odontoceti (toothed whales), and their divergence was dated to 28-34 million years ago (Nikaido *et al.*, 2001b). Osmoregulation in aquatic ancestors of cetaceans probably relied on physiological mechanisms already present in the kidney of their terrestrial counterparts to maintain the water and electrolyte homeostasis in seawater. In artiodactyls, hippopotamuses live in aquatic environments, whereas a camel is a desert dweller. Comparative analyses of plasma and urine chemicals among cetaceans and artiodactyls that dwell in the different osmotic environments thus yield useful information to understand osmoregulation in cetaceans.

The composition of cetacean blood indicates that the osmotic pressure of whale blood is similar to that of terrestrial mammals (Medway, 1965; Kjeld and Theodorsdottir, 1991; Koopman *et al.*, 1995; Kjeld, 2001; Ortiz, 2001; Reidarson *et al.*, 2001; St. Aubin *et al.*, 2001). Some marine mammals can produce highly concentrated urine (Kjeld and Olafsson, 1987; Beuchat, 1990; Kjeld and Theodorsdottir, 1991; Koopman *et al.*, 1995; Beuchat, 1996; Kjeld, 2001; Ortiz, 2001; St. Aubin *et al.*, 2001), indicating their ability to tolerate drinking of seawater. Recently, using allometry of endogenous creatinine clearance in mammals,

urine-production rates, average daily krill ingestion, and seawater ingestion of fin (*Balaenoptera physalus*) and sei whales were estimated (Kjeld, 2003). However, because of their large size and the conservation of cetaceans under treaties and laws, the number of studies which investigated water and electrolyte balance was limited. There are few comparative studies on blood and urine values of chemicals among marine and terrestrial mammals, and also among baleen and toothed whales, which have different feeding habits.

In the present study, fundamental information of blood and urine chemistry was collected to understand the conditions of body fluid compositions in the cetaceans. We determined the values of osmolality, electrolytes (Na^+ , K^+ , Cl^- , Mg^{2+} and Ca^{2+}) and glucose in blood and urine samples. The same samples were also analyzed to determine the plasma concentrations of steroid hormones, such as aldosterone, cortisol, corticosterone, testosterone, estradiol and progesterone. The values of chemicals in plasma and urine were compared among the cetaceans and the artiodactyls, and also between the baleen and the sperm whales. On the basis of data obtained in the present study, we discussed the possible osmoregulatory mechanisms to maintain such a fluid condition.

MATERIALS AND METHODS

Samples

In the present study, samples were obtained by courtesy of several institutions as described below. The treatments of subjects from which plasma and urine were sampled were under the guidelines correspondent with the Guide for Care and Use of Laboratory Animals of Hokkaido University. The sizes and the numbers of animals used in the present study are shown in Table 1.

Whales. Common minke (*Balaenoptera acutorostrata*), sei (*B. borealis*), Bryde's (*B. brydei*) and sperm whales (*Physeter macrocephalus*) were captured during the 2002 research cruise of the Japanese Whale Research Program under Special Permit in the western North Pacific-Phase II (JARPN II), carried out from June to September (Fujise *et al.*, 2003). In this research program, baleen and sperm whales have been captured to obtain biological information for the management of the whale population.

Whale carcasses were carried by the sighting and sampling vessels to the research base vessel, Nisshin-maru, where biological measurements and sampling of tissues were carried out. About 5-10 ml of blood was collected from the flipper or the tip of the upper jaw or

sometimes the tail vessels of the captured whales into blood collection tubes without anticoagulant immediately after the captured whales were carried to the mother ship. Despite a particular effort to get the fresh postmortem samples as rapid as possible, the delay between death and beginning of blood sampling was about an hour or less. Blood samples were centrifuged at 1500 g for 10 min on board within a couple of minutes after collected. Urine samples were collected from the urinary bladder within a few hours. These samples were transferred into 2 ml plastic tubes, and stored at -80°C. The volume and contents of four stomachs of the whales were examined on board. Male reproductive status was determined by the testis weight criteria. Common minke whales having one testis weighing 350 g or over were considered to be sexually mature (Kato, 1986). Similarly, sei whales having one testis weighing 900 g or over and Bryde's whales having a pair of testes weighing 1500 g or over were considered sexually mature (Nishiwaki *et al.*, 1954; Masaki, 1976). Females were classified into four reproductive groups on the basis of anatomical indicators. They were divided as: sexually immature, mature resting, mature ovulating and mature pregnant distinguished by the presence or absence of a corpus luteum (CL) and/or corpus albicans (CA) in the ovaries (Lockyer, 1984). Some of females were regarded as sexually mature if any CL was found in their ovaries. Female whales with neither CL nor CA in their ovaries were considered immature. The resting group was non-estrous, non-lactating but sexually mature females. Females having ovarian CL without fetus were classified as ovulating females. Pregnancy of females was assessed by the presence of small sized embryos or a fetus in the uterus and a large CL in the ovaries.

Dromedary. Samples of a male dromedary (*Camelus dromedarius*, 7-year-old, normally maintained) were kindly offered by Dr. Hideki Endo (National Science Museum, Tokyo, Japan) in May 2003. The dromedary was euthanized in deep anesthesia by an intravenous injection of 2 ml/kg of ketamine/xylazine and 2500 mg of pentobarbital sodium, and then blood was collected from the vein of the side of the nose into blood collection tubes without anticoagulant. Urine samples were collected from the bladder. After centrifugation at 5200 g for 10 min, plasma and urine samples were obtained and stored at -20°C.

Bactrian camel. Samples were obtained from a female Bactrian camel (*Camelus bactrianus*, 11-year-old, 450 kg) that was normally maintained in Asahiyama zoo, Hokkaido, Japan in April 2003. Following general anesthesia by an intravenous injection of 850 mg of xylazine and 1860 mg of ketamine, blood was collected from the vein of right hind limb into blood collection tubes treated with EDTA-2K, heparin, and without anticoagulant. Urine was

collected during urination when the camel was awaking. Plasma sample was obtained after centrifugation at about 1900 g for 30 min, and stored at -20°C for further analyses. The urine sample was centrifuged for a few minutes and stored at -20°C.

Dairy cattle. Dairy cattle (*Bos taurus*) were maintained in the experimental farms of Hokkaido University. They were given free access to water and regular hay. Blood and urine samples were collected from ten dairy cattle in March 2003. Half of them were lactating cows (3 to 5-year-old, av. 650 kg) and the rest was heifer (2-year-old, av. 463 kg). Blood samples were collected from the tail-base into blood collection tubes containing EDTA-2K. Plasma samples were collected after centrifugation at about 1900 g for 30 min, and stored at -20°C. Urine samples were collected during urination, centrifuged for a few minutes, and stored at -20°C.

Analyses of osmolality and plasma and urine levels of electrolytes, urea and glucose

Osmolality. Plasma and urine osmolality were measured by a vapor pressure osmometer 5520 (Wescor, Inc., Logan, UT, USA) with fresh 290 mmol/kg and 1000 mmol/kg standard solutions. A single Whatman filter paper disc (Wescor, ss-033) was placed in the central depression of the holder by metal forceps, and 10 µl of the samples were expelled onto the disc. Saturated discs were rapidly transferred to the vapor pressure osmometer sample holder, and osmolality was determined. All samples were analyzed in duplicate.

Electrolytes. Plasma and urinary concentrations of Na⁺, K⁺ and Cl⁻ were determined by an electrolyte analyzer (AVL 9130, AVL-Scientific, Graz, Austria) with electrolyte controls. The detectable ranges were 40-205 mM for Na⁺, 1.5-15 mM for K⁺, and 50-200 mM for Cl⁻ when analyzed by the serum mode. The diluted samples were analyzed according to the manufacture's instruction. The concentrations of Mg²⁺ and Ca²⁺ in blood and urine were determined by a Polarized Zeeman Atomic Absorption Spectrophotometer (Z-5300, Z-8000, Hitachi Ltd., Tokyo, Japan). The plasma samples that showed the lower levels of Na⁺ than the detectable limit of an electrolyte analyzer were also analyzed by this spectrophotometer. The diluted samples and standard solutions were analyzed according to the manufacture's instruction. The absorbances of the samples, standard, and control plasma were determined in duplicate.

Urea. Concentrations of urea in blood and urine samples were determined by a Urea N B kit (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) that adopts the Urease-Indophenol method. Color A solution which was prepared fresh daily contained 0.25 M sodium salicylate, 6.7 mM sodium pentacyanonitrosyl ferrate (III) dihydrate and urease solution (19

U/ml) in 90 mM phosphate buffer, pH 7.0. Twenty μ l of samples, standards, controls, and distilled water as blank were added to 2 ml of Color A solution in 14-ml plastic tubes. The tubes were incubated in 37°C water bath for 15 min, and 2 ml of Color B solution was added. They were then incubated in 37°C water bath for 10 min. The absorbances of the samples, standards, and control plasma at 595 nm were measured against the blank tube to determine net absorbance using a microplate reader (MTP-300, Corona Electric Co., Ltd., Hitachinaka, Japan) and a NanoDrop[®] ND-1000 Spectrophotometer (NanoDrop Technologies, Inc. Rockland, DE, USA). All samples were analyzed in duplicate, and the concentrations of urea in blood and urine samples were estimated.

Glucose. Plasma glucose concentrations were determined using a Glucose CII-Test Wako kit (Wako Pure Chemicals Industries, Ltd.) which adopts the mutarotase-GOD method. Enzyme solution contains mutarotase (0.13 U/ml), glucose oxidase (9.0 U/ml), peroxidase (0.65 U/ml), ascorbate oxidase (2.7 U/ml), 0.5 mM 4-aminoantipyrine, and 5.3 mM phenol in 60 mM phosphate buffer, pH 7.1. Seven μ l of samples, standards, and control plasma were added to 1 ml of enzyme solutions, followed by incubation at 37°C for 15 min. The absorbances at 505 nm of the samples, standards, and control plasma were determined against the blank tube using a NanoDrop[®] ND-1000 Spectrophotometer. All samples were analyzed in duplicate.

Effects of different anticoagulants

When we collected blood and urine samples, blood collection tubes without anticoagulant were preferably used to minimize effects of different anticoagulants on the values of determined parameters. In the case of blood sampling from the cattle, however, blood collection tubes treated with EDTA-2K were used, since determination of the plasma levels of peptide hormones were considered of the same blood samples. Effects of different anticoagulants on the levels of chemicals were thus tested in plasma of the Bactrian camel treated with EDTA-2K, heparin, and without anticoagulant. Coefficients of variation (CVs) of electrolyte levels in the plasma samples were 1.3-6.6 %, except for K⁺, which were not determined. Nonetheless, different anticoagulants might not seriously affect the results, since the intra-assay CVs of the same plasma samples were sometimes larger than those in the plasma samples treated with different anticoagulants (data not shown). These results indicate that different anticoagulants did not distort the profiles of the plasma electrolyte levels in the present study.

EIA (aldosterone, cortisol, corticosterone, testosterone, estradiol and progesterone)

Plasma levels of steroid hormones were determined by enzyme immunoassays (EIA) basically as described in the previous studies (Asahina *et al.*, 1995; Onuma *et al.*, 2003). Steroid hormones were extracted from 0.5 ml of plasma samples twice with 3 ml of diethyl ether, evaporated by gentle flow of nitrogen gas, and reconstituted with assay buffer containing 0.2% bovine serum albumin (BSA) and 0.01% thimerosal in 0.05 M borate buffer, pH 7.8. Microtiter plates (MS-8496F, Sumitomo Bakelite Co. Ltd., Tokyo, Japan) were coated with 15 µg/ml anti-rabbit IgG (ICN Pharmaceuticals, Inc., Aurora, OH, USA), 0.05 M carbonate buffer, pH 9.7, and further coated with blocking solution containing 0.1% BSA and 3% sucrose in 0.05 M phosphate buffer, pH 7.4. The standards and samples were incubated with anti-steroid antiserum and HRP-labeled steroid (FKA 428-E, FKA 427 for aldosterone, FKA 404-E, FKA 403 for cortisol, FKA 420-E, FKA-419 for corticosterone, FKA 102-E, FKA 101 for testosterone, FKA 236-E, FKA 235 for estradiol, and FKA 302-E, FKA 301 for progesterone, Cosmo Bio Co. Ltd, Tokyo, Japan) at 4°C overnight. After washing with 0.9% NaCl, a substrate solution (0.5 mg/ml *o*-phenylenediamine, 0.01% H₂O₂ in 0.2 M citrate buffer, pH 4.5) was added and incubated at room temperature for 30 min. The reaction was stopped by addition of 0.6 N H₂SO₄ and the absorbance at 492 nm was measured with a microplate reader (MTP-300, Corona Electric Co. Ltd.). All samples were analyzed in duplicate.

In aldosterone assay, corticosterone showed cross-reactivity of 0.01% and cortisol less than 0.002%. In the cortisol assay, 11-deoxycortisol showed cross-reactivity of 11.5%, cortisone 4.0%, corticosterone 2.0%, and other structurally related steroids 0.2% or less. In corticosterone assay, DOC showed cross-reactivity of 8.0%, progesterone 2.1%, and other structurally related steroids 0.2% or less. In testosterone assay, 5 α -dihydrotestosterone showed cross-reactivity of 7.3%, androsterone 2.1%, 4-androstenedione 0.82%, and other steroids 0.15% or less. In estradiol assay, estradiol-3-glucuronide showed cross-reactivity of 56.3%, estrone-3-sulfate 26.8%, estrone-3-glucuronide 1.2%, estrone-3-sulfate 0.86%, estrone 0.8%, estriol 0.5%, and testosterone 0.05%. In progesterone assay, 5 α -pregnanedione showed cross-reactivity of 12.5%, 11 α -OH-progesterone 5.3%, pregnenolone 2.0%, and other steroids less than 0.2%.

Validation of EIA

The detection limit of the assay was 300 pg/ml, except for testosterone (30 pg/ml) and estradiol (10 pg/ml). A recovery of each hormone after extraction was checked by addition of standard solution to the mixed and pooled plasma samples of several cetacean species and

cattle. The recovery of each hormone was 78.9-126.8%. Both intra- and inter-assay CVs were within 15% for each hormone assay. Parallelisms between the extracted steroid hormones and the standard solutions were also examined by the parallel line assay. Serial dilution of the samples showed parallelism to the standard curves (data not shown). These results indicate that the present assay system is appropriate to determine the plasma levels of steroid hormones in the cetaceans, camels and cattle.

Statistical analyses

Values were presented as means \pm standard error of the means. One-way ANOVA followed by Tukey's test was applied for differences among species. Comparisons between gender were performed by Student's *t*-test. Correlations between the biological and chemical values, the plasma levels of steroid hormones, and time lag from death to beginning of blood and urine sampling were determined by Pearson's correlation coefficient. Because of difficulty to obtain sufficient numbers of samples from sperm whales, dromedary and Bactrian camel, we could not perform statistical analyses to detect the significant differences among them. In the comparison of the plasma concentrations of sex steroid hormones among different reproductive status in the cetaceans, we could not apply statistical analyses for the same reason. The values of lactating cow and heifer were combined, since the significant differences between them were not detected in most parameters.

RESULTS

The plasma osmolality in the cetaceans was slightly higher than that in the cattle, whereas the cetacean urine osmolality was obviously higher (Fig. 1). The concentrations of Na⁺ and Cl⁻ in plasma and urine also showed similar tendency (Tables 2, 3, Fig. 2). The differences in the osmolality and the levels of most electrolytes between the cetaceans and the artiodactyls were greater in urine than in plasma (Tables 2, 3 and Fig. 1-3). The plasma concentrations of urea in the cetaceans were 5 to 11-fold those in the cattle, and the urine concentrations were 2 to 4-fold (Fig. 4). Although the plasma concentrations of aldosterone in most of the cetaceans were higher than those in the camels and the cattle (Fig. 5), there were no significant differences in the levels of corticosteroids between the cetaceans and the cattle (Fig. 6). The baleen whales showed higher concentrations of most electrolytes and urea in plasma and urine than the sperm whales (Tables 2, 3 and Fig. 1-4). Some positive and negative correlations of variables showing physiologically interesting are listed in Table 5.

Osmolality (Fig. 1)

The osmolality of urine was 3 to 4-fold that of plasma in all species. The female baleen whales showed slightly but significantly higher plasma osmolality than the cattle, and the osmolality of cetacean urine was obviously higher than that of the cattle urine. In the cetaceans, the osmolalities of plasma and urine in the baleen whales were higher than those in the sperm whales. The osmolalities of plasma and urine in the whales showed positive correlations with the plasma and urine levels of most electrolytes, respectively ($p < 0.05$, Table 5).

Electrolytes

Na⁺ (Fig. 2) and Cl⁻ (Tables 2, 3). The plasma levels of Na⁺ in the baleen whales were 1.2-fold those in the cattle, whereas the urinary levels of Na⁺ in the baleen whales were about 7-fold those in the cattle. The ratio of urine Cl⁻ levels between the baleen whales and the cattle was also higher than that in the plasma. In the cetaceans, the plasma and urine concentrations of Na⁺ in the baleen whales were higher than those in the sperm whales, whereas the plasma and urine concentrations of Cl⁻ in the sperm whales were similar to those in the baleen whales.

K⁺ (Fig. 3). The plasma concentrations of K⁺ in the cattle could not be determined as blood samples from the cattle were treated with EDTA-2K. Plasma levels of K⁺ in the cetaceans were similar to those in the camels. Urinary levels of K⁺ in the cattle were much higher than those in the other animals. Significant correlations were found between the time lag from death to sampling and the plasma levels of K⁺ in the male minke and Bryde's whales ($p < 0.05$, Table 2, 3, Fig. 1-4).

Mg²⁺ and Ca²⁺ (Tables 2, 3). Although significant differences in the levels of plasma Mg²⁺ among species were not detected, the male Bryde's whales had higher levels of plasma Mg²⁺ than the females ($p < 0.05$). Sexual difference in the plasma Mg²⁺ levels was found only in this species. In the sei whales, the plasma levels of Ca²⁺ in the males were significantly higher than those in the females ($p < 0.05$). The urinary levels of Ca²⁺ in the cattle were much higher than those in the other species.

Urea (Fig. 4)

The plasma concentrations of urea in the cetaceans were 5 to 11-fold those in the cattle, and the urine concentrations were 2 to 4-fold. The levels of urinary urea in the camels were similar to those in the cetaceans. The Bryde's whales showed higher plasma levels of urea than the other baleen whales and the sperm whales. The concentrations of urea in plasma

and urine in the baleen whales were 50-90% higher than those in the sperm whales.

Steroid hormones and glucose

Aldosterone (Fig. 5), cortisol, corticosterone (Fig. 6) and glucose (Table 2). The plasma levels of aldosterone in the dromedary and most of the cattle could not be determined, because they were lower than the detectable limit (300 pg/ml), whereas those in the cetaceans could be determined. Although the plasma concentrations of aldosterone in most of the cetaceans were higher than those in the camels and the cattle, there were no significant differences in the levels of corticosteroids between the cetaceans and the cattle. There was a negative correlation between the urinary levels of Na^+ and aldosterone in the female sei whales ($p < 0.05$, Table 5). The camels showed much higher levels of cortisol, corticosterone and glucose than the cetaceans and the cattle. Positive correlations between corticosteroids were demonstrated in the male minke and sei whales ($p < 0.05$, Table 5). There was a significant correlation between the plasma levels of corticosterone and glucose in the female Bryde's whales ($p < 0.05$, Table 5).

Testosterone, estradiol and progesterone (Table 4). In the common minke and Bryde's whales, the levels of testosterone in the mature animals were higher than those in the immature whales. All female sei whales were pregnant, and the pregnant whales tended to have higher levels of progesterone than the other reproductive status in all species. There were no significant differences between the lactating cow and the heifer in the levels of estradiol and progesterone. There were positive correlations between body length or body weight and the plasma levels of progesterone in the female minke whales ($p < 0.05$, data not shown), however, no correlations were found between the high values of steroid hormones and the plasma concentrations of electrolytes.

DISCUSSION

In the present study, we showed plasma and urine chemistry in cetaceans and artiodactyls. We found that the cetaceans concentrate Na^+ and urea to excrete highly concentrated urine than the artiodactyls. There were no significant difference in the plasma concentrations of corticosteroids between the cetaceans and the cattle. The baleen whales showed higher concentrations of Na^+ and urea than the sperm whales in plasma and urine.

Effects of capture

The present results in the whales were obtained from the samples postmortally collected

and the time lag from death to beginning of blood and urine collection was about one hour. According to the previous study in dogs (Schoning and Strafuss, 1980), postmortem blood Na^+ and Cl^- values decreased slightly within 3 hours after death, whereas K^+ levels increased relatively fast with time. We consider that the present sampling protocol was not serious to the plasma and urinary levels of most electrolytes, since the length of time lag between death and sampling correlated only with the plasma levels of K^+ in the male whales ($p < 0.05$, Table 5). We were also afraid of a possibility of a contamination of samples with seawater, despite careful sampling of blood and urine. It was proposed that the levels higher than 4.5 mM of plasma Mg^{2+} indicated more than 5% contamination (Kjeld, 1987). In the present study, the plasma levels of Mg^{2+} in a few plasma samples were higher than 4.5 mM, however, the levels of other chemicals were similar to those in the other samples. Thus, we consider that the plasma samples in the present study were not seriously affected by artifacts and seawater contamination.

Osmolality and electrolytes

The plasma and urine levels of electrolytes in the whales reported here, except for K^+ , were consistent with those in the previous reports on the captive, free-ranging and rehabilitating cetaceans (Medway, 1965; Kjeld, 1987; Kjeld and Theodorsdottir, 1991; Koopman *et al.*, 1995; Ortiz and Worthy, 2000; Kjeld, 2001; Ortiz, 2001; Reidarson *et al.*, 2001; St. Aubin *et al.*, 2001; Kjeld, 2003). Hence, the values in the present whale samples were considered to be relatively close to those of living whales. The values in the cattle, except for the much higher values of urinary K^+ , were also almost the same with those reported previously (Kaneko *et al.*, 1997). In contrast, the plasma levels of K^+ in the whales were slightly higher than those samples taken from free-ranging bottlenose dolphins (*Tursiops truncatus*), harbor porpoises (*Phocoena phocoena*), and the rehabilitating California gray whale calf (*Eschrichtius robustus*) (Medway, 1965; Koopman *et al.*, 1995; Ortiz and Worthy, 2000; Reidarson *et al.*, 2001), indicating postmortem increase of the plasma values of K^+ in the whales. In the present results, the urine values of osmolality, Na^+ , and Cl^- in the female baleen whales were significantly higher than those in the cattle ($p < 0.05$), indicating that increased excretion of these electrolytes should be a critical factor for cetaceans to regulate the levels of body salts to adapt for their marine environment.

Urea

Urea plays a key role in the urine-concentrating mechanism in mammals (Sands, 2002). In the inner medullary collecting duct, a gradient of urea is established for passive NaCl

absorption in the absence of an osmotic gradient. There may be multiple mechanisms by which vasopressin regulates the different urea transporter proteins and mRNA isoforms to reabsorb and recycle urea in the kidney. The plasma concentrations of urea in the baleen whales were higher than those in the sperm whales in the present study, and also in some captive odontocetes and beluga whales (*Delphinapterus leucas*) (Malvin and Rayner, 1968; St. Aubin *et al.*, 2001). Furthermore, the baleen whales showed higher concentrations of urea in both plasma and urine than the cattle and camels in this study, and other land mammals (Kaneko *et al.*, 1997). The present study suggest that the cetaceans could maintain their body fluid to utilize urea in their kidney and that different actions of urea transporters between marine and terrestrial mammals, but there is only one report which investigated the function of urea transporters in cetaceans (Janech *et al.*, 2002).

Adrenocortical hormones

Aldosterone promotes Na^+ absorption and K^+ secretion across the renal distal tubule-collecting duct system in mammals. The plasma levels of aldosterone in the whales were higher than those in the other whales previously reported (Ortiz and Worthy, 2000; Kjeld, 2001; St. Aubin, 2001), so that the values of aldosterone may be valued slightly higher in our present assays. In the present study, the cetaceans showed the higher levels of plasma aldosterone than the artiodactyls. Nevertheless, only in the female sei whales, a negative correlation was found between the plasma levels of aldosterone and the urinary concentrations of Na^+ (Table 5). The high value of plasma aldosterone in the male sperm whales in Fig. 5 was due to abnormal values of one male sperm whale, which showed the higher values of most steroid hormones, however, it did not show the abnormal values of any other plasma chemicals.

Anesthesia with ketamine and xylazine has little effect on glucocorticoid levels in rabbit within 1 hour after treatment (Illera *et al.*, 2000). Since our blood samples were collected from the anesthetized camels within 1 hour after treatment, we consider that the levels of their plasma glucocorticoid were not affected by anesthesia but adrenocorticotropin-induced stimulation by stress response. In contrast, the plasma levels of corticosteroids and glucose in the whales and the cattle correspond with the reported values (Kaneko *et al.*, 1997; St. Aubin, 2001) respectively, and the plasma levels of corticosteroids in the cetaceans were similar to those in the cattle, indicating that they were seem to be not affected by stress response. In Indo-Pacific bottlenose dolphin and killer whales (*Orcinus orca*), diurnal changes in the cortisol levels were exhibited as diurnal terrestrial mammals (Suzuki *et al.*,

2003), however, in this study the cetaceans did not show any daily pattern of plasma cortisol levels in relation to the time of day (data not shown) as Kjeld showed in the fin whales (2001). There were no significant differences in the plasma concentrations of corticosteroids between the cetaceans and the cattle, suggesting that corticosteroids are not important for osmoregulation in cetaceans.

Sex steroid hormones

In the common minke and Bryde's whales, the levels of testosterone in the immature animals were lower than those in the mature whales and similar to those in the common and southern minke, Bryde's, and sei whales in feeding season reported previously (Yoshioka and Fujise, 1992; Suzuki *et al.*, 2001; Kjeld *et al.*, 2003; Kjeld *et al.*, 2004; Watanabe *et al.*, 2004). There were no correlations between the plasma levels of testosterone and the plasma and urine values of osmolality in the male whales.

The present study showed that the pregnant whales had the higher levels of progesterone than the animals of other reproductive status. These high levels in the pregnant whales were almost similar to those in common and southern minke whales reported previously (Yoshioka *et al.*, 1990; Yoshioka and Fujise, 1992; Iga *et al.*, 1996; Suzuki *et al.*, 2001; Kjeld *et al.*, 2003; Kjeld *et al.*, 2004; Muranishi *et al.*, 2004). It has been shown that pregnancy is characterized by an increase in extracellular fluid, plasma, and blood volume in different mammalian species (Phippard *et al.*, 1986; Schrier and Briner, 1991), and that vasopressin response to an osmotic challenge is affected by sex steroid hormones (Sladek *et al.*, 2000). However, there were no positive correlations between the high levels of plasma sex steroid hormones and the plasma and urine values of osmolality in the pregnant whales. The female minke whales showed significantly higher levels of most steroid hormones than the males and sometimes than the females of other whale species ($p < 0.05$), but no correlations were found between the high values of steroid hormones and the plasma concentrations of electrolytes in the female minke whales. The present results indicate that the osmoregulatory parameters seem to be not affected by the reproductive stage and sex steroid hormones.

The effects of different feeding habit

Since marine mammals appear to obtain source of water from their food (Costa, 2002), and the ratio of water to electrolytes is quite different between vertebrate and invertebrate prey, marine mammals face different osmoregulatory problems depending on the type of prey that they consume. Typically baleen whales feed on zooplankton, mainly euphausiids or copepod since they are enriched with essential amino acids, but their major food items are

specialized with area. According to the report of the present cruise (Fujise *et al.*, 2003), most common minke and sei whales fed on krill, copepod, and anchovies, whereas the Bryde's whale took anchovies, and the sperm whales mainly had squids.

We found that the baleen whales had the higher concentrations of Na^+ and urea in plasma and urine than the sperm whales. The levels found in the sperm whales are similar to those found in the other toothed whales like bottlenose dolphin and beluga (Malvin and Rayner, 1968; St. Aubin *et al.*, 2001), which feed mainly on fish. As mentioned earlier, the predominant source of water in marine mammals is considered to be their food (Costa, 2002) and then osmoregulatory mechanism could be influenced by the type of prey they consume. Therefore the differences in the concentrations of Na^+ and urea between the baleen and the sperm whales reported in this study can be explained by their different feeding habits. Further works that consider in more detail the different feeding habits between baleen and toothed whales could assist in understanding the osmoregulatory mechanisms in cetaceans.

Possible mechanisms of cetacean osmoregulation

Vasopressin, aldosterone, urea transporter and aquaporin are important to maintain water and electrolytes homeostasis in the mammalian kidney. The present study indicated a possibility of more efficient actions of urea transporters in cetacean than that in terrestrial mammals, since the whales could maintain high levels of urea in plasma and excrete concentrated urea in urine. Function of some types of urea transporters is regulated by vasopressin for urea reabsorption and also water reabsorption (Sands, 2002). The high levels of electrolytes and urea in the cetacean urine indicated that antidiuretic action, probably of vasopressin, is important for production of hypertonic urine. Studies on the function of vasopressin and urea transporter will help us to understand the mechanisms of cetacean osmoregulation.

Conclusion

The present study showed that the cetaceans conserve their body water by producing more concentrated urine than the terrestrial mammals. Although the plasma concentrations of aldosterone in the cetaceans were higher than those of the cattle, there were no significant difference in the plasma concentrations of corticosteroids between the cetaceans and the cattle, suggesting that corticosteroids are not important for osmoregulation in the cetaceans. Instead, vasopressin may have important roles to retain water in the cetacean kidney as shown in terrestrial mammals. Urea transporters in the kidney also appear to offer effective mechanisms to produce hypertonic urine. Cetaceans have unique osmoregulatory

mechanisms, which are correlated to their feeding habits, to excrete hypertonic urine and maintain fluid homeostasis to adapt seawater.

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Table 1. The numbers, means \pm SE of body length (BL) and body weight (BW) of animals used in the present study.

Sex	Order	Suborder	Common name	n	BL (m)	BW (t)
Males						
	Cetacea					
		Mysticeti	Common minke whale	14	7.33 \pm 0.22	4.40 \pm 0.27
			Sei whale	6	13.68 \pm 0.21	19.32 \pm 0.82
			Bryde's whale	7	11.61 \pm 0.37	12.37 \pm 0.98
		Odontoceti	Sperm whale	2	9.26 \pm 0.84	11.73 \pm 2.53
	Artiodactyla					
		Tylopoda	Dromedary	1	ND	ND
Females						
	Cetacea					
		Mysticeti	Common minke whale	9	6.26 \pm 0.53	3.55 \pm 0.85
			Sei whale	6	15.15 \pm 0.16	28.22 \pm 0.81
			Bryde's whale	6	12.67 \pm 0.46	16.79 \pm 1.70
		Odontoceti	Sperm whale	3	9.79 \pm 0.54	12.00 \pm 1.83
	Artiodactyla					
		Tylopoda	Bactrian camel	1	ND	0.45
		Ruminantia	Lactating cow	5	ND	0.65 \pm 0.14
			Heifer	5	ND	0.45 \pm 0.19

ND: not determined.

Table 2. Concentrations of chemicals in plasma (means \pm SE).

Sex	Order	Common name	n	Cl ⁻ (mM)	Mg ²⁺ (mM)	Ca ²⁺ (mM)	Glucose (mM)
Males							
Cetacea							
		Common minke whale	14	112.2 \pm 4.1	2.0 \pm 0.3	3.3 \pm 0.1	5.5 \pm 0.8
		Sei whale	6	107.9 \pm 3.1	2.1 \pm 0.2	3.6 \pm 0.1**	8.3 \pm 0.9
		Bryde's whale	7	128.0 \pm 10.7	4.5 \pm 2.0*	3.6 \pm 0.2	6.2 \pm 1.1
		Sperm whale	2	125.3 \pm 0.3	2.2 \pm 0.3	3.0 \pm 0.2	4.4 \pm 1.2
Artiodactyla							
		Dromedary	1	109.0	1.3	2.5	18.1
Females							
Cetacea							
		Common minke whale	9	120.1 \pm 4.0 ^b	2.7 \pm 0.7	3.2 \pm 0.1	6.0 \pm 0.8 ^b 5.4 \pm 0.4 ^a
		Sei whale	6	109.3 \pm 5.1 ^b	2.5 \pm 0.9	3.4 \pm 0.2	^b
		Bryde's whale	6	111.1 \pm 2.1 ^b	1.9 \pm 0.3	3.5 \pm 0.1	6.2 \pm 0.8 ^b
		Sperm whale	3	119.3 \pm 2.7	1.7 \pm 0.2	2.6 \pm 0.1	3.3 \pm 0.2
Artiodactyla							
		Bactrian camel	1	105.0	1.1	2.9	11.2
		Cattle	10	102.4 \pm 0.7 ^a	1.3 \pm 0.0	3.1 \pm 0.1	3.4 \pm 0.1 ^a

Significant differences among species are indicated with different letters ($p < 0.05$ by One-Way ANOVA followed by Tukey's-test). Significant differences between gender were indicated by * ($p < 0.05$) and ** ($p < 0.01$) by Student's *t*-test.

Table 3. Concentrations of chemicals in urine (means \pm SE).

	Order		Cl ⁻	Mg ²⁺	Ca ²⁺
Sex	Common name	n	(mM)	(mM)	(mM)
Males					
	Cetacea				
	Common minke whale	10	290.8 \pm 22.6	11.0 \pm 1.5 ^a	3.7 \pm 0.5 ^b
	Sei whale	5	374.9 \pm 28.8	21.2 \pm 4.5 ^b	2.6 \pm 0.8 ^{ab}
	Bryde's whale	7	266.3 \pm 28.6	11.2 \pm 2.0 ^a	1.4 \pm 0.4 ^a
	Sperm whale	2	375.3 \pm 47.8	19.5 \pm 0.5	2.4 \pm 0.4
	Artiodactyla				
	Dromedary	1	70.5	16.0	1.3
Females					
	Cetacea				
	Common minke whale	3	267.2 \pm 53.6 ^{bc}	20.7 \pm 3.9 ^{ab}	2.4 \pm 0.9
	Sei whale	5	350.1 \pm 27.7 ^c	13.5 \pm 3.2 ^{ab}	1.3 \pm 0.5
	Bryde's whale	5	224.9 \pm 31.8 ^b	10.0 \pm 2.5 ^a	1.4 \pm 0.4
	Sperm whale	2	475.5 \pm 8.5	13.8 \pm 0.5	0.7 \pm 0.1
	Artiodactyla				
	Bactrian camel	1	115.5	21.0	0.6
	Cattle	10	111.4 \pm 6.3 ^a	23.8 \pm 2.6 ^b	6.1 \pm 1.5

Significant differences among species are indicated with different letters ($p < 0.05$ by One-Way ANOVA followed by Tukey's-test).

Table 4. Plasma levels of testosterone, estradiol, and progesterone (means \pm SE) and reproductive status of experimental animals.

sex	Order Common name	Reproductive status	n	Testosterone (ng/ml)	Estradiol (ng/ml)	Progesterone (ng/ml)		
Male	Cetacea	Common minke whale	Immature	2	0.21 \pm 0.03			
			Mature	12	0.28 \pm 0.05			
		Sei whale	Immature	1	2.62			
			Mature	5	1.68 \pm 0.23			
		Bryde's whale	Immature	4	0.17 \pm 0.04			
			Mature	3	2.15 \pm 1.23			
		Sperm whale	ND ¹	2	1.62 \pm 0.03			
		Artiodactyla	Dromedary	Mature	1	0.68		
		Female	Cetacea	Common minke whale	Immature	6		0.57 \pm 0.26
Pregnant	2					0.13 \pm 0.03	12.1 \pm 0.26	
Resting	1					0.02	ND ²	
Sei whale	Pregnant			6		0.14 \pm 0.07	6.60 \pm 0.86	
Bryde's whale	Immature			1		0.03	ND ²	
	Pregnant			3		0.20 \pm 0.09	4.11 \pm 1.88	
	Lactating/Resting			2		0.03 \pm 0.00	0.11 \pm 0.00	
Sperm whale	Immature			1		0.03	0.18	
	Ovulating/Resting			2		0.02 \pm 0.01	0.35	
Artiodactyla	Cattle			Immature	5		0.04 \pm 0.01	5.38 \pm 1.27
				Lactating	5		0.03 \pm 0.01	2.83 \pm 0.75
	Bactrian camel			Mature	1		0.25	3.26

ND¹: not determined; ND²: not detected.

Table 5. Some positive and negative correlation coefficients (r) of variables.

Variables		Male			Female		
		Minke	Sei	Bryde's	Minke	Sei	Bryde's Cattle
Body length	Body weight	0.959**	0.902*	0.909**	0.985**		0.952**
Lag time	Plasma K ⁺	0.535*		0.933**			
Plasma							
osmolality	Plasma Na ⁺	0.968**		0.973**	0.942**	0.814*	0.840*
	Plasma Cl ⁻	0.932**		0.921**	0.832**		0.838*
	Plasma K ⁺	0.764**				0.872*	
	Plasma Mg ²⁺	0.637*		0.838*			
	Plasma Ca ²⁺	0.919**		0.980**		0.960**	
	Plasma urea	0.612*					
	Cortisol	0.741**					
Plasma Na ⁺	Aldosterone		-0.848*				
Plasma glucose	Corticosterone						0.820*
	Testosterone						0.935**
	Progesterone						0.651*
Urinary							
osmolality	Urinary Na ⁺						0.949*
	Urinary Cl ⁻		0.893*				0.954*
	Urinary K ⁺	0.769**				0.961**	
	Urinary urea			0.846*		0.981**	0.978** 0.840**
Urinary Na ⁺	Aldosterone						-0.971*
Aldosterone	Cortisol	0.817**					
	Corticosterone	0.647*	0.873*		0.758*		0.983*
	Testosterone	0.868**	0.909*				
	Estradiol	0.792**	0.989**		0.960**	0.901*	0.992**
Cortisol	Corticosterone	0.769**	0.986**				
	Testosterone	0.537*					
	Progesterone		0.990**				
	Estradiol			0.983**			

Significant correlations are indicated with * ($p < 0.05$) and ** ($p < 0.01$) determined by Pearson's correlation coefficient.

Figure Legends

Fig. 1. Plasma and urine osmolality (means \pm SE, mOsm/kg) in the common minke, sei, Bryde's and sperm whales, and dromedary, Bactrian camel and cattle. Significant differences among species are indicated with different letters ($p < 0.05$ by One-Way ANOVA followed by Tukey's-test).

Fig. 2. Concentrations of Na⁺ (mM, means \pm SE) in plasma (A) and urine (B) of common minke, sei, Bryde's and sperm whales, and dromedary, Bactrian camel and cattle. Significant differences among species are indicated with different letters ($p < 0.05$ by One-Way ANOVA followed by Tukey's-test).

Fig. 3. Concentrations of K⁺ (mM, means \pm SE) in plasma (A) and urine (B) of common minke, sei, Bryde's, and sperm whales and dromedary, Bactrian camel and cattle. Significant differences among species are indicated with different letters ($p < 0.05$ by One-Way ANOVA followed by Tukey's-test). ND: not determined.

Fig. 4. Concentrations of urea (mM, means \pm SE) in the plasma (A) and urine (B) of common minke, sei, Bryde's and sperm whales, and dromedary, Bactrian camel and cattle. Significant differences among species are indicated with different letters ($p < 0.05$ by One-Way ANOVA followed by Tukey's-test).

Fig. 5. Plasma concentrations (means \pm SE) of aldosterone in the common minke, sei, Bryde's and sperm whales and dromedary, Bactrian camel and cattle. Significant differences among species are indicated with different letters ($p < 0.05$ by One-Way ANOVA followed by Tukey's-test). Significant differences between gender were indicated by * ($p < 0.05$) by Student's *t*-test.

Fig. 6. Plasma concentrations (means \pm SE) of cortisol (A) and corticosterone (B) in the common minke, sei, Bryde's and sperm whales and dromedary, Bactrian camel and cattle. Significant differences among species are indicated with different letters ($p < 0.05$ by One-Way ANOVA followed by Tukey's-test). Significant differences between gender were indicated by ** ($p < 0.01$) by Student's *t*-test.

Fig. 1 Birukawa et al.

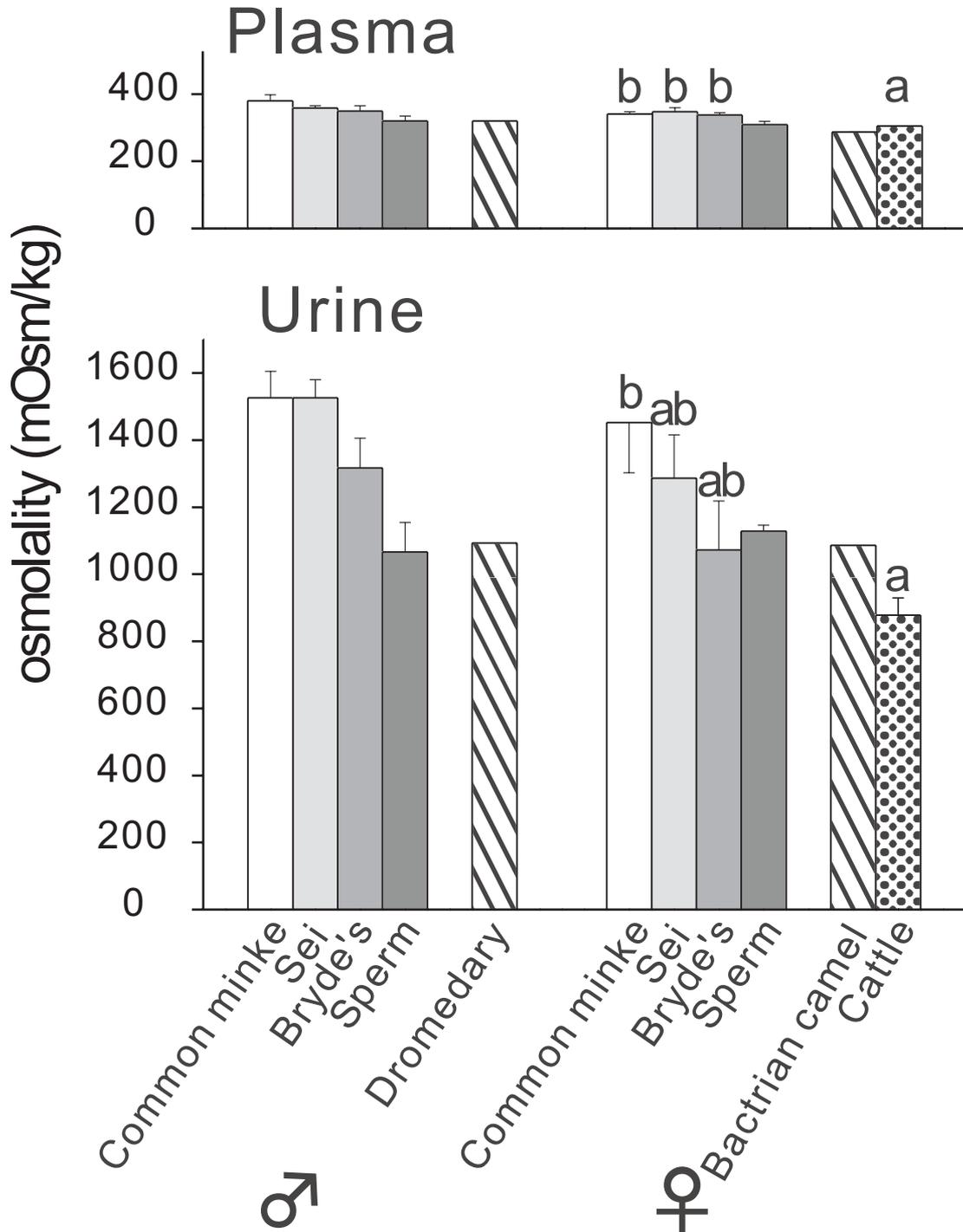


Fig. 2 Birukawa et al.

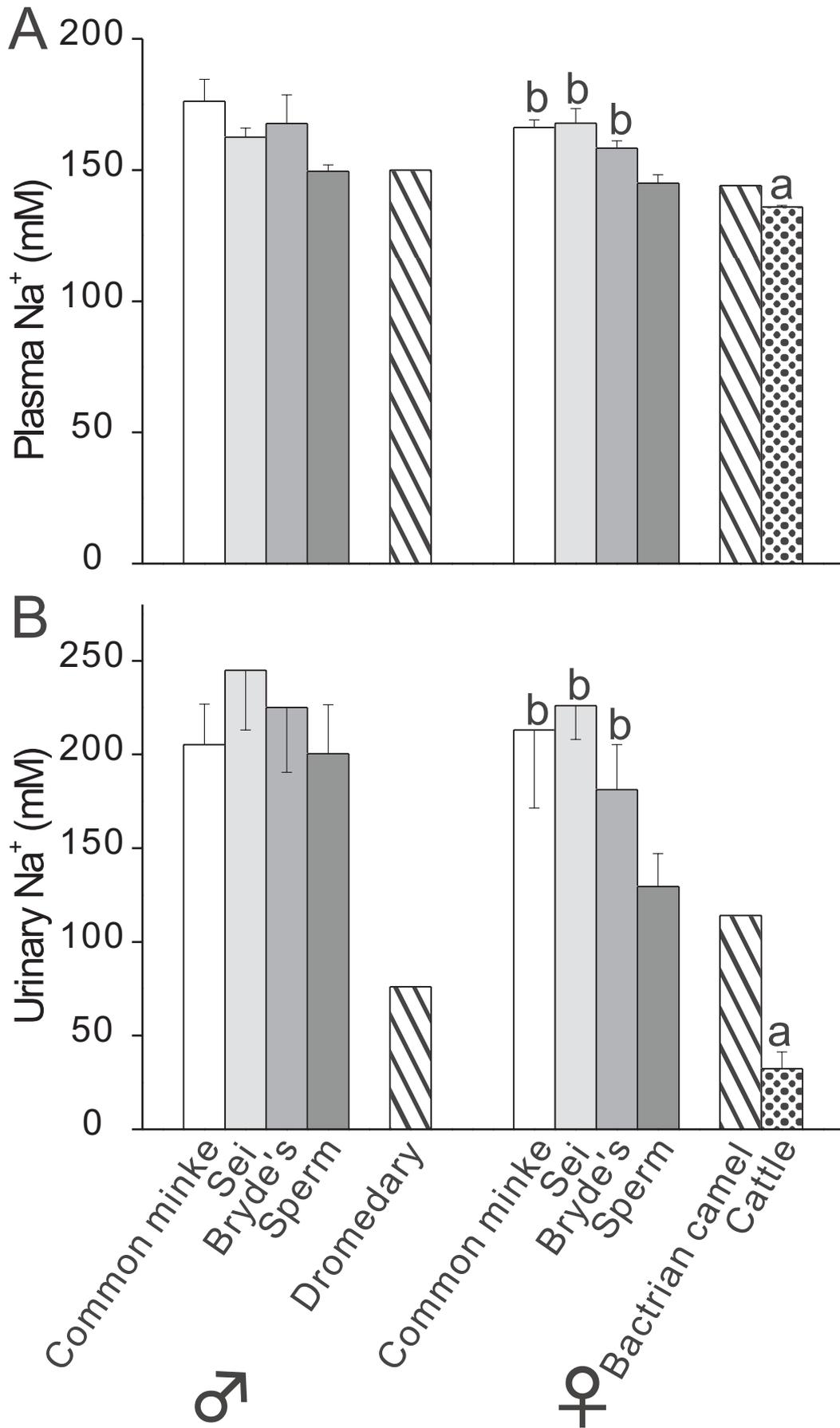


Fig. 3 Birukawa et al.

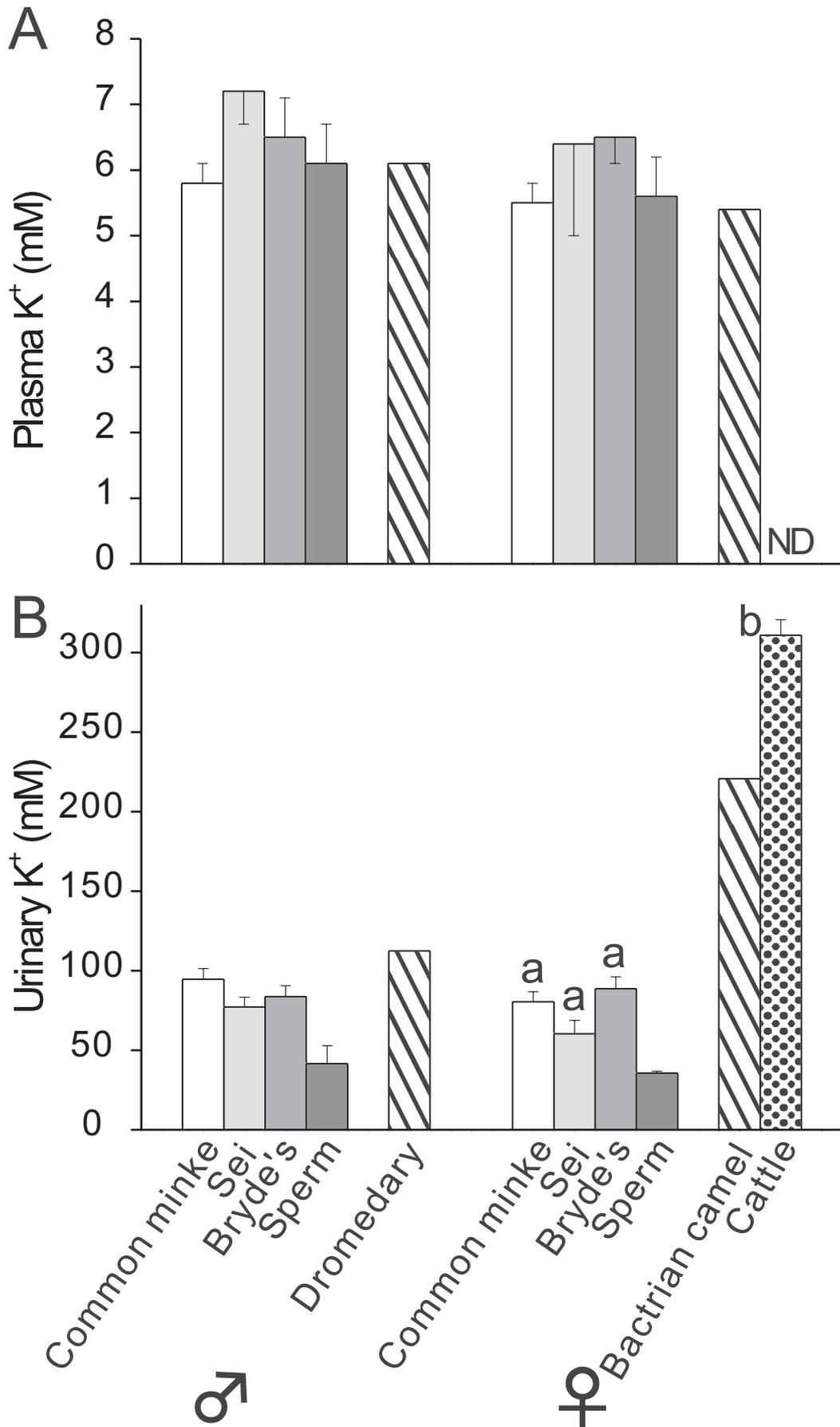


Fig. 4 Birukawa et al.

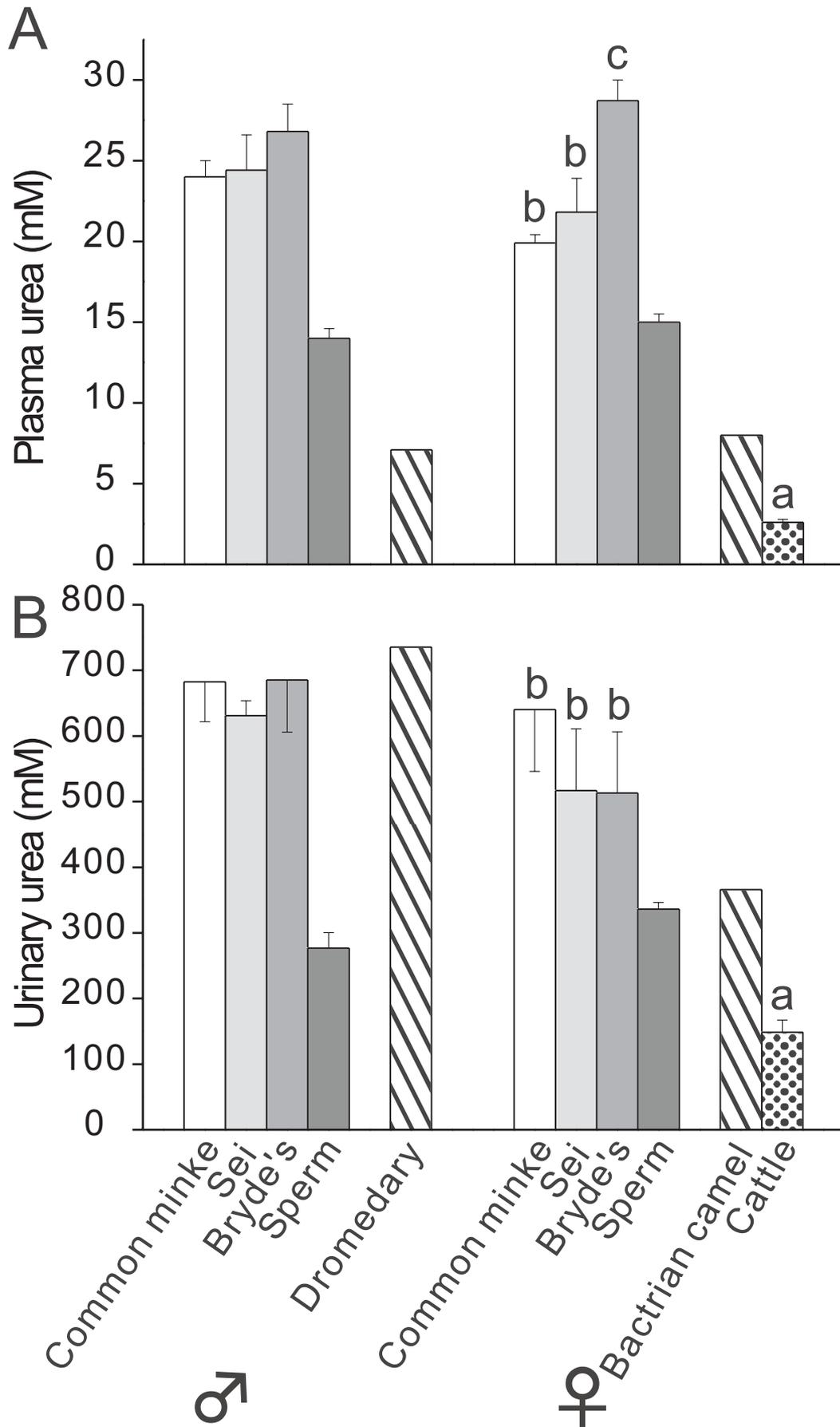


Fig. 5 Birukawa et al.

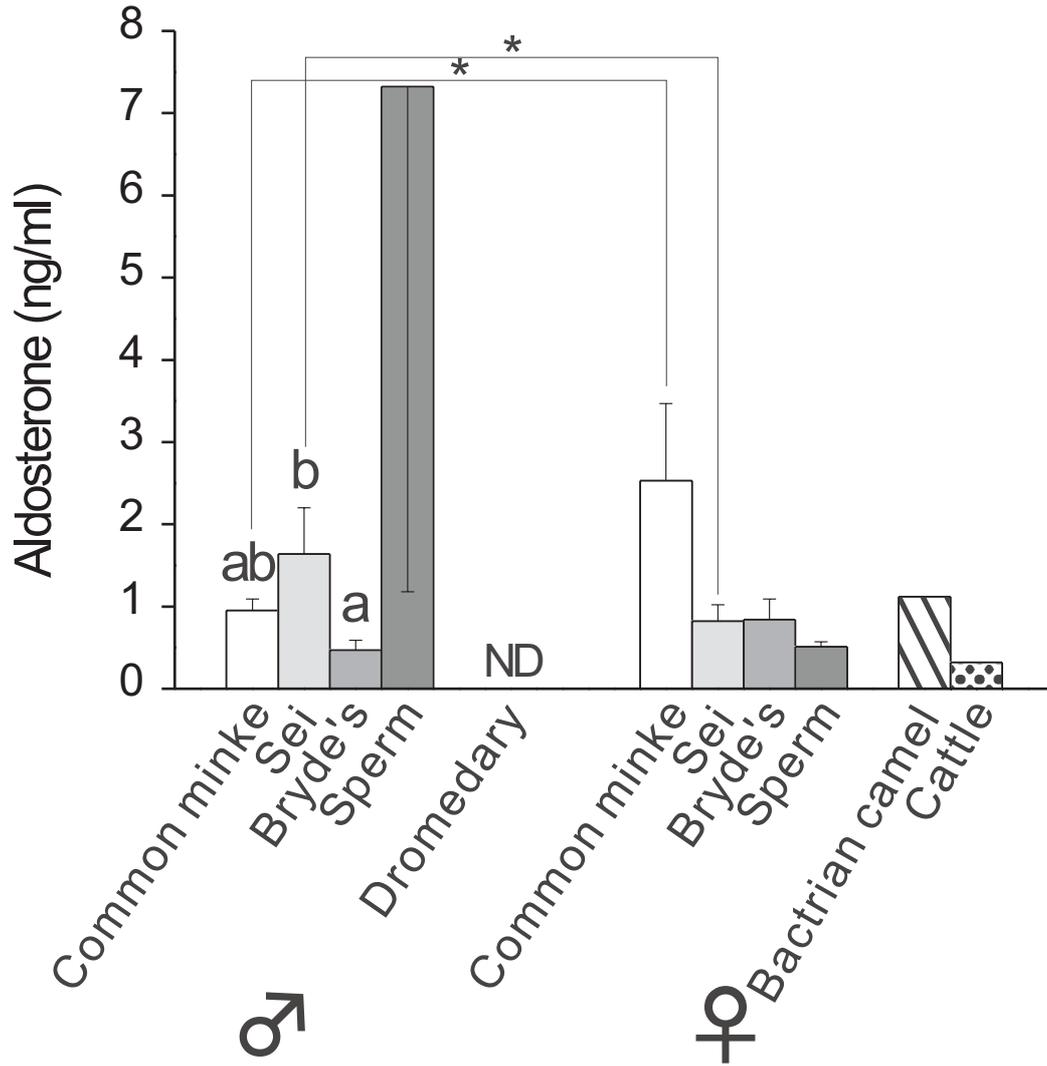


Fig. 6 Birukawa et al.

