Int Aquat Res (2019) 11:267–276
https://doi.org/10.1007/s40071-019-00236-0

ORIGINAL RESEARCH

Development of a system for measuring calcitonin in the stingray *Dasyatis akajei* (a cartilaginous fish): the possible involvement of stingray calcitonin in gonadal development

Takashi Takagi · Toshio Sekiguchi · Yuichi Sasayama · Atsuhiko Hattori · Takushi X. Watanabe · Yoichiro Kitani · Yoshiaki Tabuchi · Hajime Matsubara · Ajai K. Srivastav · Tran Ngoc Hai · Thumronk Amornsakun · Nobuo Suzuki

Received: 31 March 2019 / Accepted: 5 August 2019 / Published online: 16 August 2019

Abstract To elucidate the physiological role of calcitonin (CT) in stingrays (cartilaginous fish), an enzyme-linked immunosorbent assay (ELISA) system using a specific antibody against stingray CT has been developed. Synthetic stingray CT was subcutaneously injected into mice four times—once every 2 weeks—together with an adjuvant. We purified the IgG antibody fraction using the protein A affinity chromatography from collected antiserum. Evaluating the antibody titer, we found the antibody's optimum dilution ratio to be 600 times. Competitive ELISA has been developed using the antibody diluted 600 times. Our antibody did not cross-react with teleost CTs and muscle extraction, but cross-reacted with stingray plasma and the extract of the ultimobranchial gland, the secretary organ of stingray CT. Using this ELISA, we measured the plasma CT level in stingrays and examined its correlation with several mineral concentrations. Plasma CT did not show significant correlation to calcium, magnesium, inorganic phosphorus, sodium, chlorine, or urea, although there

T. Takagi
Department of Biology, Faculty of Science, University of Toyama, Gofuku, Toyama 930-8555, Japan

T. Sekiguchi · Y. Sasayama · Y. Kitani · N. Suzuki (✉)
Noto Marine Laboratory, Institute of Nature and Environmental Technology, Division of Marine Environmental Studies, Kanazawa University, Noto-cho, Ishikawa 927-0553, Japan
e-mail: nobuos@staff.kanazawa-u.ac.jp

A. Hattori
Department of Biology, College of Liberal Arts and Sciences, Tokyo Medical and Dental University, Ichikawa, Chiba 272-0827, Japan

T. X. Watanabe
Peptide Institute, Inc, Ibaraki, Osaka 567-0085, Japan

Y. Tabuchi
Life Science Research Center, University of Toyama, Sugitani, Toyama 930-0194, Japan

H. Matsubara
Noto Center for Fisheries Science and Technology, Kanazawa University, Noto-cho, Ishikawa 927-0552, Japan

A. K. Srivastav
Department of Zoology, D.D.U. Gorakhpur University, Gorakhpur 273-009, India

T. N. Hai
College of Aquaculture and Fisheries, Can Tho University, Can Tho City, Vietnam

T. Amornsakun
Fisheries Technology Program, Faculty of Science and Technology, Prince of Songkla University, Pattani 94000, Thailand

was a correlation among the factors involved in osmoregulation, such as sodium, chlorine, and urea. On the other hand, plasma CT was significantly correlated to body weight and length. Furthermore, there was a significant correlation between plasma CT and gonad weight. Since plasma CT was correlated with the weight of liver, which is involved in the synthesis of egg yolk protein, we examined the influence of 17β-estradiol (E_2) on CT secretion. After E_2 injection, the plasma CT level increased significantly. This is the first study to demonstrate that E_2 induced plasma CT secretion in cartilaginous fish.

Keywords Calcitonin · Sexual maturation · ELISA · Stingray · Cartilaginous fish

Introduction

Calcitonin (CT) is a peptide hormone that is produced in C-cells of the thyroid glands of mammals and in ultimobranchial glands (UBG) of non-mammalian vertebrates (Dacke 1979; Wendelaar Bonga and Pang 1991; Sasayama 1999). This hormone lowers the serum calcium (Ca) concentration when administered to some mammals, such as rats, rabbits, and humans, by inhibiting osteoclastic activity (Dacke 1979; Azria 1989). In teleosts as well as mammals, furthermore, it has been demonstrated that CT suppressed osteoclastic activity in both freshwater and seawater teleosts (Suzuki et al. 2000; Sekiguchi et al. 2009, 2017; Kase et al. 2017).

Cartilaginous fish, as well as other vertebrates, have been reported to possess UBG, and the administration of shark UBG extraction to rats induces hypocalcemia, as does homologous CT (Copp et al. 1967). We previously showed by immunohistochemical methods that the stingray, *Dasyatis akajei*, possesses a pair of large UBGs that contain abundant CT (Sasayama et al. 1984). Using the stingray, therefore, the amino acid sequence of CT has been determined (Takei et al. 1991). Stingray CT is more closely related structurally to the CT of the salmon lineage (sequence homology 65–78%) than those of mammals (31–38%) (Sasayama et al. 1992). In addition, the injection of synthetic stingray CT enables hypocalcemic activity in rats (Sasayama et al. 1992), similar to the effect of extraction from shark UBG (Copp et al. 1967). On the other hand, we examined the effects of ultimobranchialectomy (UBX) on plasma minerals in the stingray *Dasyatis akajei* (Suzuki et al. 1995). However, plasma Ca level did not influence by UBX in stingrays (Suzuki et al. 1995). Therefore, the function of CT in cartilaginous fish has not been clarified until now, although stingray and shark CTs can regulate the Ca balance in mammals. Cartilaginous fish such as sharks, skates, rays, and chimaeras accumulate urea and trimethylamine oxide in the body due to osmotic pressure adjustment as an organic osmolyte in marine and euryhaline elasmobranchs (Dacke 1979; Bentley 1998; Hazon et al. 2003). By this regulation, the osmotic pressure in the body is almost equal to that of seawater. The osmotic pressure in teleosts is around one-third that of seawater and is lower than that of cartilaginous fish (Dacke 1979; Bentley 1998). Considering these physiological conditions, the effect of CT on cartilaginous fish may be different from that on teleosts.

To elucidate the physiological role of CT in cartilaginous fish, we started with the stingray. The primary structure of CT of this cartilaginous fish has been determined (Takei et al. 1991); there is also synthesized CT, which causes physiological activity in rats (Sasayama et al. 1992). For these reasons, we used the stingray as an experimental material in the present study. Thus, we first developed an enzyme-linked immunosorbent assay (ELISA) system using a specific antibody against stingray CT. Using this ELISA, we measured the plasma CT level in the stingray and analyzed the correlation between CT and several mineral concentrations. Furthermore, the correlation of CT with body weight, body length, gonad weight, and liver weight were examined. Moreover, we examined the influence of female sex hormone, 17β-estradiol (E_2), on plasma CT secretion.

Materials and methods

Animals

Dasyatis akajei stingrays ranging from 100–3500 g (n = 30) in body weight were caught by fishermen with fixed nets or gill nets around the Yokata fishing port of Toyama Bay (Fig. 1). In the case of stingrays, sex distinctions can be made easily by the presence or absence of a clasper, a male feature. Collected stingrays were carried to the tank by car. Prior to the experiments, fish were acclimatized in an aquarium (120 cm ×

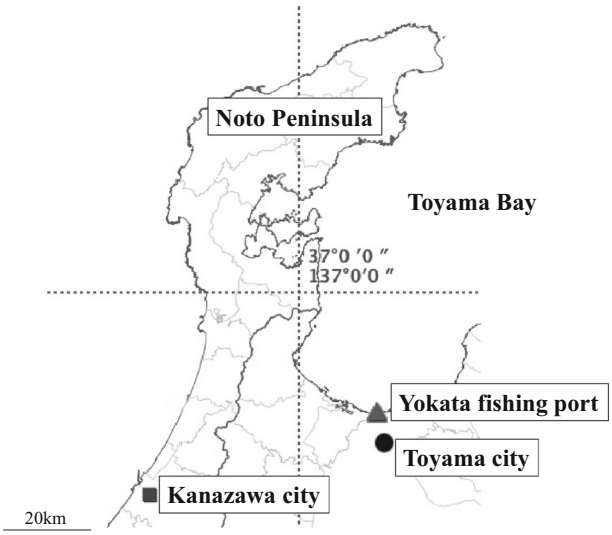

Fig. 1 Map of the area from which stingrays were collected for the present study. This map is based on Digital Map 25000 (Map Image) provided by Geospatial Information Authority of Japan. Filled triangle: Yokata fishing port; filled circle: Toyama city; filled square: Kanazawa city

60 cm × 45 cm) for 1–2 days after collection. Thereafter, the experiment was started. To avoid the effects of sex hormones, juvenile stingrays 100–200 g (n = 10) were used for the experiment regarding the influence of E_2 injections on plasma CT concentrations. Experiments with stingrays were performed according to the recommendations of the ethical guidelines of Kanazawa University and the University of Toyama.

Development of an ELISA system using a specific antibody against stingray CT

Synthetic stingray CT, provided by the Peptide Institute, Inc. (Osaka, Japan), was used in the present study. Stingray CT (100 μg/ml) diluted with 0.9% NaCl was prepared. Then, an equal volume of polyvinyl pyrrolidone (0.5 mg/ml) was added to the CT solution, and the mixture was stirred at room temperature for 2 h. After that, adjuvant (Ribi ImmunoChem Research Inc., Hamilton, MT, USA) was added and vigorously stirred. Finally, a CT solution (50 μg/ml) was prepared.

The prepared CT solution (200 μl/individual) was subcutaneously injected into five mice under ether anesthesia every 2 weeks for a total of four times. Blood was collected from the tail vein after the second and third injections, and the titer of antiserum against stingray CT was examined. On day 10, after four doses, blood was drawn from mice under ether anesthesia. The collected blood was centrifuged at 25,000×g for 10 min at 4 °C. We purified the antibody as an IgG fraction from the collected antiserum using affinity chromatography with protein A (Bio-Rad Laboratories, K.K., Tokyo, Japan). NaN_3 (0.1%) was added to the purified IgG solution. Experiments with mice were performed in accordance with the recommendations of the ethical guidelines of the University of Toyama.

Next, the purified antibody titer was examined. The ELISA procedures have been described in detail by Robertson (1981) and Suzuki (2001). The synthetic stingray CT was diluted in a carbonate/bicarbonate buffer (0.1 M, pH 9.6) at a concentration of 80 ng/ml. Then, aliquots of 100 μl were transferred to 96 wells of polystyrene microtiter plates (Corning Inc., New York, NY, USA). The plates were sealed and incubated at 25 °C for 20–28 h. The coated plates were washed four times with a washing solution (10 mM phosphate buffer solution containing 0.05% Tween 20: PBST, pH 7.4) just before incubation. The aliquots of diluted antibody (100 μl) were transferred to CT-coated plates and incubated at 4 °C for 24 h. After incubation, the plates were washed four times with PBST. Aliquots of 100 μl of 1:10,000 dilution of biotinylated goat IgG (E 432, Dako Japan Co., Ltd., Kyoto, Japan) with a diluting solution (PBS without 0.1% NaN_3) were added and incubated with constant agitation for 2 h at room temperature (around 25 °C). After incubation, biotinylated goat IgG was discarded, and the plates were washed four times with PBST. Then, aliquots of 100 μl of a 1:10,000 dilution of peroxidase-conjugated streptavidin (P 397, Dako Japan Co., Ltd., Kyoto, Japan) with a

diluting solution (PBS without 0.1% NaN_3) were added and incubated in wells with constant agitation for 1 h at room temperature. After incubation, the plates were washed four times with PBST. Just before color development, 8 mg of *o*-phenylenediamine dihydrochloride (Wako Co., Ltd., Osaka, Japan) was added to 12 ml of 0.1 M citric acid and 0.2 M Na_2HPO_4 solution (pH 4.5) containing 2.4 µl of hydrogen peroxide (30% solution, Wako Co., Ltd., Osaka, Japan). Aliquots of 100 µl of this solution were added and incubated in wells with constant agitation at room temperature for 10–15 min. After incubation, the reaction was stopped by the addition of 50 µl of 3 N sulfuric acid. The optical density of the reaction product in the plates was measured at 492 nm with a microplate reader (MTP-100, Corona Electric Co., Ltd., Tokyo, Japan).

Competitive ELISA has been developed using the determined dilution of the antibody as described in Robertson (1981) and Suzuki (2001). In separate glass tubes, 250 µl of the antibody with a diluting solution (10 mM phosphate buffer solution containing 0.1% bovine serum albumin and 0.1% NaN_3:PBS, pH 7.4) was pre-incubated with the same volume of the serial concentration of synthetic stingray CT (12.5–800 ng/ml) or diluted plasma samples for 3 days at 4 °C. Thereafter, the stingray CT-coated plates were washed four times with PBST. After pre-incubation, aliquots of 100 µl of diluted stingray CT or diluted plasma samples were transferred to CT-coated plates and incubated at 4 °C for 24 h. Then, a standard curve was made as described above. The plasma CT levels of stingrays were calculated using the standard curve.

The specificity of the antibody was checked using teleost CTs, such as synthetic salmon and eel CTs (Sigma-Aldrich, Inc., St. Louis, MO, USA). In addition, the extraction from the UBG or muscle of stingrays and the diluted stingray plasma were examined to evaluate the antibody specificity.

Measurement of plasma CT, mineral concentrations, body weight, body length, gonad weight, and liver weight

Under anesthesia with ethyl 3-aminobenzoate, methanesulfonic acid salt (Sigma-Aldrich) at a dilution of 1/3000, blood was collected from stingray hearts with heparinized syringes. The collected blood was centrifuged at 25,000×*g* for 10 min at 4 °C. The plasma samples were immediately frozen and kept at − 50 °C until analysis. Thereafter, the body weight, body length, gonad weight, and liver weight of each stingray were measured. The Ca, magnesium (Mg), and sodium (Na) levels in stingray plasma samples were determined by atomic absorption spectrophotometry (180–70 type; Hitachi-Zeeman; Hitachi Co., Ltd., Tokyo, Japan). The chlorine (Cl) levels in stingray plasma were measured with a chloridemeter (C-50 type, Jookoo Sangyo Co., Ltd., Tokyo, Japan). Inorganic phosphorus (Pi) levels in plasma samples were measured using the method of Fiske and Subbarow (1925). Urease-indophenol (Searcy et al. 1967) was used to measure plasma urea in the stingray.

Influence of E_2 injection on plasma CT levels in the stingray

Juvenile stingrays (100–200 g) were weekly anesthetized with ethyl 3-aminobenzoate, methanesulfonic acid salt (Sigma-Aldrich) in chilled seawater (4 °C) at a dilution of 1/10,000. Thereafter, blood was initially sampled from the tail vein. E_2 was melted by ethanol and then mixed with oil. The prepared E_2 (1 µg/g body weight) was injected intraperitoneally into six stingrays. The control stingrays ($n = 4$) were injected intraperitoneally with vehicle solution in the same manner. During the experimental period of 5 days, seawater was exchanged at least once a day to prevent the accumulation of NH^{4+}. Five days after injection, both groups were anesthetized again, and blood was collected from the tail vein. The collected blood was centrifuged at 25,000×*g* for 10 min at 4 °C. Plasma samples were immediately frozen and kept at − 50 °C until analysis. Plasma CT and Ca levels were measured as described above.

Statistical analysis

The statistical significance of the correlation was examined using linear regression analysis (Figs. 5, 6, and Table 1). The statistically significant difference from the initial values in the control or experimental group was assessed by paired *t* test (Figs. 7 and 8). In all data, the selected significance level was $p < 0.05$.

Table 1 Relationships among plasma calcitonin (CT), calcium (Ca), magnesium (Mg), inorganic phosphorus (Pi), sodium (Na), chlorine (Cl), and urea

	CT	Ca	Mg	Pi	Na	Cl	Urea
CT	–						
Ca	0.136						
Mg	0.176	0.346					
Pi	0.084	0.348	0.192				
Na	0.154	0.451*	0.328	0.263			
Cl	0.055	0.407*	0.383*	0.216	0.695***		
Urea	0.246	0.204	0.124	0.541**	0.659***	0.608***	–

Data indicate each r value

*, **, and *** indicate statistically significant differences at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively

Results

The titration curve of a specific antibody against stingray CT

Using the purified IgG by an affinity chromatography with protein A, the antibody titration curve against stingray CT was examined. The data of the antibody titration curve are shown in Fig. 2. Since it is known that around 40% of the maximum color development is the optimum dilution concentration, we decided to use a 600-time dilution in the following experiment.

Analysis of the antibody specificity

The antibody specificity was examined using salmon (Fig. 3a) and eel CTs (Fig. 3b). This antibody was not cross-reacted with teleost CTs. The antibody was cross-reacted with both the extraction of UBG (Fig. 4a) and plasma (Fig. 4b) in the stingray but was not cross-reacted with the extraction from muscle in the stingray (Fig. 4a). The variances of intra- and inter-assays were within 5% and 10%, respectively.

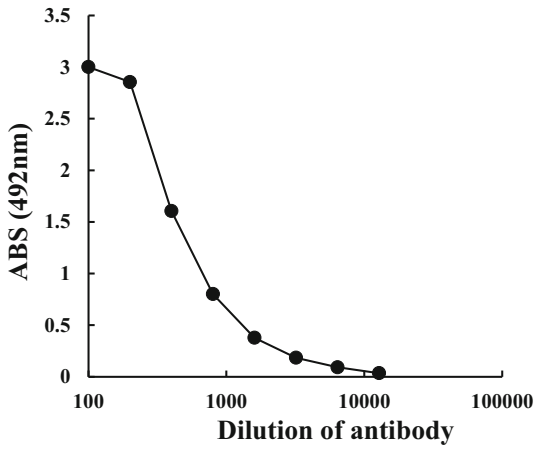

Fig. 2 The titration curve of the specific antibody against stingray CT. Synthetic stingray CT was subcutaneously injected into mice, together with an adjuvant. We purified the IgG antibody fraction using protein A affinity chromatography from the collected antiserum. Thereafter, the antibody titration curve against stingray CT was examined. The data indicate the mean of triplication

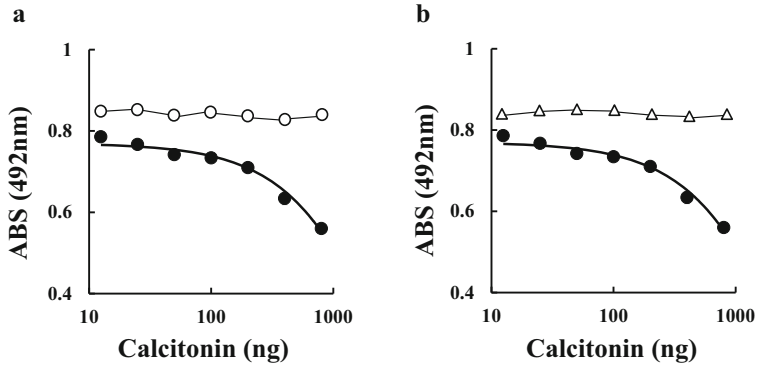

Fig. 3 Cross-reaction of the antibody against stingray CT (filled circle) to salmon (unfilled circle) (**a**) and eel (unfilled triangle) (**b**) CTs. Our purified antibody did not cross-react with salmon and eel CTs. The standard curve shows mean ± SE (n = 3). The data for salmon and eel CTs indicate the mean of triplication

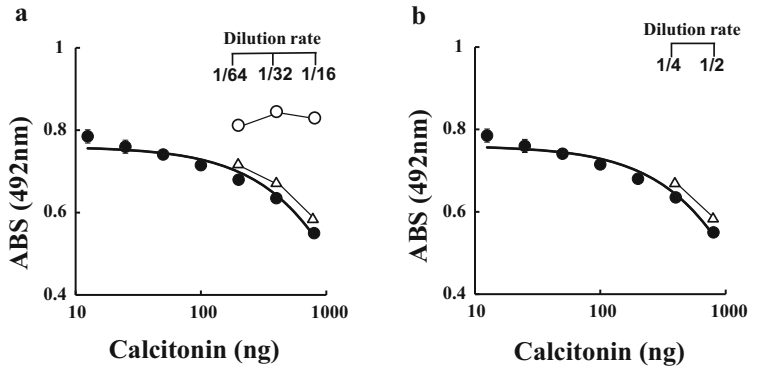

Fig. 4 Analysis of the specificity of the antibody. The extraction from the ultimobranchial gland (UBG) (unfilled triangle) or muscle (unfilled circle) of the stingray (**a**), and the diluted stingray plasma (unfilled triangle) (**b**) were examined to evaluate the specificity of the antibody. The standard curve (filled circle) shows mean ± SE (n = 3). The data for the UBG, muscle extraction, and plasma of the stingray indicate the mean of triplication

Correlation of plasma CT with mineral concentrations, body weight, body length, gonad weight, and liver weight

The data for the correlation between plasma CT and mineral concentrations are shown in Table 1. There was no significant difference in the plasma CT concentration between males and females. Therefore, we analyzed the data of both sexes together. Plasma CT did not have a significant correlation to Ca, Mg, Pi, Na, Cl, urea, although there was a close correlation among the factors involved in osmoregulation, such as Na, Cl, and urea. Plasma Ca had a significant correlation with plasma Na and Cl. Also, a significant correlation was obtained between plasma Mg and Cl. Plasma Pi had a significant correlation with plasma urea.

On the other hand, plasma CT had a significant correlation with body weight (r = 0.625, Fig. 5a) and body length (r = 0.543, Fig. 5b). In addition, there was a significant correlation between plasma CT and gonad weight (r = 0.471, Fig. 6a). Plasma CT was also significantly correlated with liver weight (r = 0.514, Fig. 6b).

Effect of E_2 injection on plasma CT levels in the stingray

Five days after the injection of E_2, the plasma CT level increased significantly (Fig. 7). In the case of vehicle injection, there was no significant difference between the initial and final values (Fig. 7).

On the other hand, plasma Ca levels did not change after E_2 injection, at least in present conditions (Fig. 8).

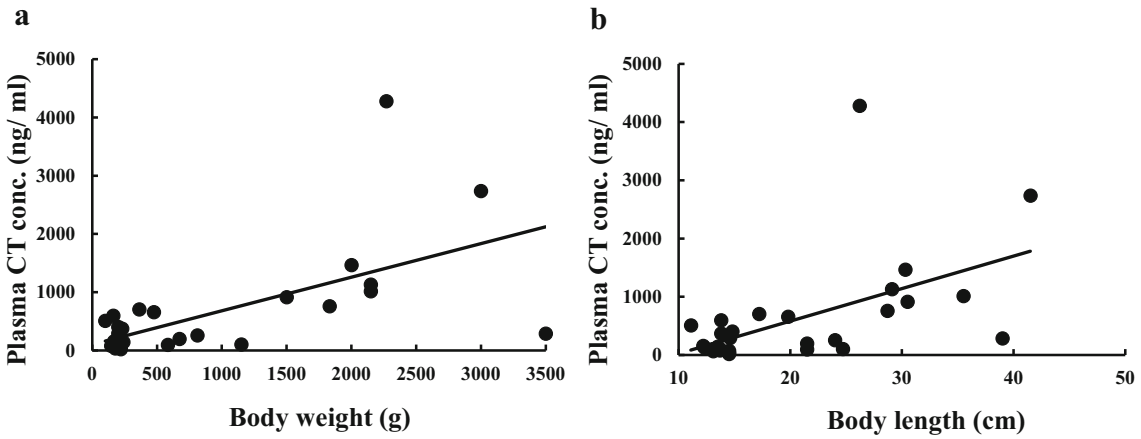

Fig. 5 Correlation of plasma CT with body weight (**a**) or body length (**b**). Plasma CT has a significant correlation with body weight (n = 30, r = 0.625, $p < 0.001$) and body length (n = 30, r = 0.543, $p < 0.01$)

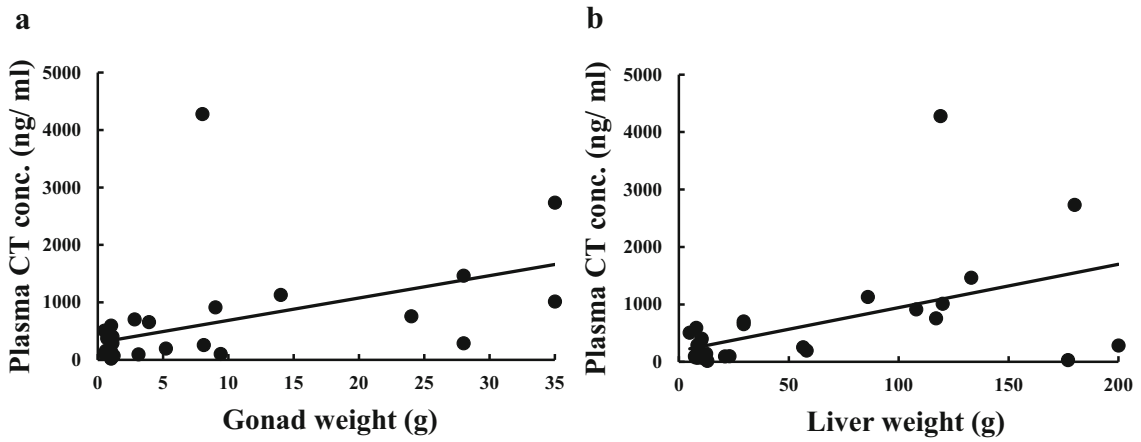

Fig. 6 Correlation of plasma CT with gonad weight (**a**) or liver weight (**b**). Plasma CT has a significant correlation with gonad weight (n = 30, r = 0.471, $p < 0.01$) and liver weight (n = 30, r = 0.514, $p < 0.01$)

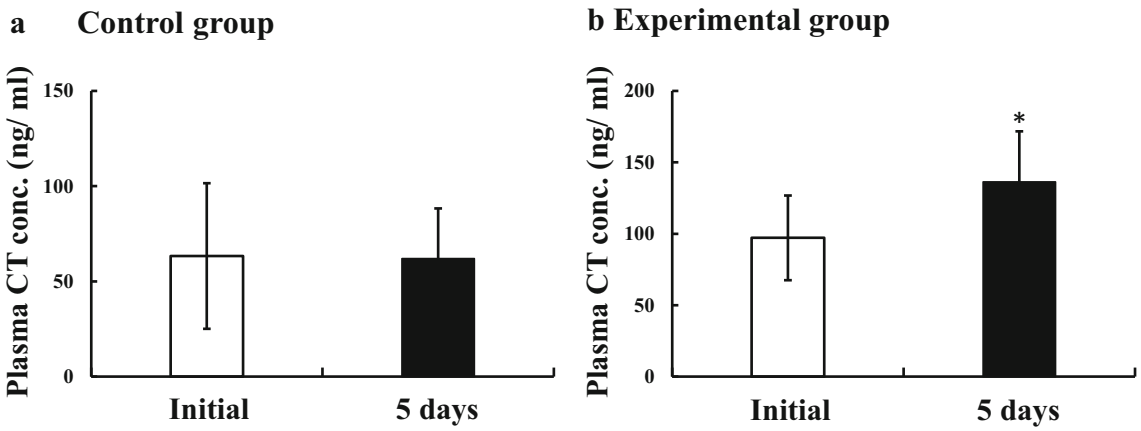

Fig. 7 Effect of vehicle (**a**) or E_2 (**b**) injection on plasma CT levels in the stingray. E_2 (1 μg/g of body weight) was injected intraperitoneally after initial blood sampling. Stingrays in the control group were injected with vehicle in the same manner as experimental stingrays. Blood sampling was then performed on day five. The asterisk indicates a statistically significant difference, at $p < 0.01$, from the initial values in the control (n = 4) or experimental group (n = 7). The data show mean ± SE

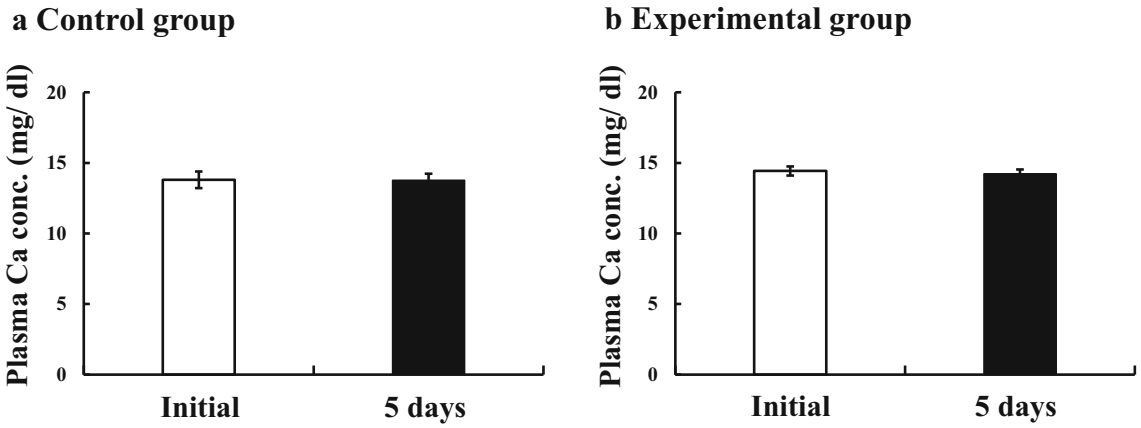

Fig. 8 Effect of vehicle (**a**) or E_2 (**b**) injection on plasma Ca levels in the stingray. E_2 (1 μg/g of body weight) was injected intraperitoneally after initial blood sampling. Stingrays in the control group (n = 4) were injected with vehicle in the same manner as experimental stingrays (n = 7). Blood sampling was then performed on day five. There were no significant differences between initial and final values. The data show mean ± SE

Discussion

We developed an ELISA system using a specific antibody against stingray CT. This antibody did not cross-react with teleost CTs because the amino acid identities of stingray CT to salmon CT and eel CT were 66 and 75%, respectively (Suzuki et al. 1999a). In addition, our antibody cross-reacted with the stingray plasma and the extraction of UBG. Thus, this ELISA system is useful for analyzing stingray plasma CT levels.

Using this ELISA system, we measured stingray plasma CT concentrations. As a result, plasma CT did not show a significant correlation with Ca, Mg, Pi, Na, Cl, or urea. We previously reported that the effects of UBX on plasma minerals and urea concentrations were examined in the stingray *Dasyatis akajei* (Suzuki et al. 1995). Our previous study indicated that plasma Ca, Mg, Pi, Na, Cl, and urea concentrations were not influenced by UBX in stingrays (Suzuki et al. 1995). In eels, *Anguilla japonica* (Suzuki et al. 1999b), and goldfish, *Carassius auratus auratus* (Suzuki et al. 2004), we reported that plasma CT had a significant correlation with plasma Ca. Therefore, the function of CT in stingrays (cartilaginous fish) may be different from that in teleosts. On the other hand, divalent salts, such as Ca and Mg, correlated with Cl because they exist in the form of $CaCl_2$ or $MgCl_2$ in seawater. Furthermore, there was a very strong correlation among Na, Cl, and urea directly related to osmotic pressure. The correlation between phosphorus and urea is unknown. We think that there may be new regulatory mechanisms.

On the other hand, we found that plasma CT levels in large stingrays were higher than those in small stingrays, and that plasma CT has a significant correlation with body weight and body length. These results indicate that CT may play some role related to body growth. In addition, there was a significant correlation between plasma CT and gonad weight. Since plasma CT was correlated with liver weight and involved in the synthesis of egg yolk protein, vitellogenin (Hiramatsu et al. 2015), we examined the influence of E_2 on CT secretion in an in vivo experiment. Five days after E_2 injections, plasma CT levels had increased. There was a significant difference between the initial and final values. Previously, using Scatchard analysis, immunoblot analysis, and gel chromatography, we demonstrated that two forms of estrogen receptor protein were present in the cytosol of stingray UBG (Yamamoto et al. 1996). Therefore, we believe that E_2 directly functions in stingray UBG and promotes CT secretion from UBG. It has been reported that CT plays some roles in the reproductive physiology of quail (Dacke et al. 1976), rats (Lu et al. 1998), and mice (Wang et al. 1998). In female fish, also, the plasma CT level increases in sockeye salmon (*Oncorhynchus nerka*) (Watts et al. 1975), eels (*Anguilla japonica*) (Yamauchi et al. 1978), rainbow trout (*Salmo gairdneri*) (Björnsson et al. 1986), and brown trout (*Salmo trutta*) (Norberg et al. 1989) during the reproductive period. In the case of goldfish (*Carassius auratus auratus*), three subtypes of estrogen receptor were expressed in the UBG (Suzuki et al. 2004). After E_2 administration, plasma CT levels increased in goldfish (*Carassius auratus auratus*) (Suzuki et al. 2004) as well as stingrays. Thus, CT has some significant function in the reproductive physiology of stingrays as well as other vertebrates.

We previously reported that plasma CT levels of teleosts such as eels (*Anguilla japonica*) and goldfish (*Carassius auratus auratus*) ranged from undetectable to 2599 pg/ml (Suzuki et al. 1999b, 2004). The plasma CT level in the stingray was higher than those of eels (*Anguilla japonica*) and goldfish (*Carassius auratus auratus*). In the bonnethead shark (*Sphyrna tiburo*), a high level of plasma CT has been also reported (Nichols et al. 2003). In this shark, the developing embryo is the principal target of maternally produced CT during gestation (Nichols et al. 2003). During lactation, plasma CT levels increased in mammals such as humans (Greer et al. 1984) and deer (Chao et al. 1985). As stingrays are ovoviviparous fish, CT may be involved in an action (fetal growth) different from that in oviparous teleosts such as eels (*Anguilla japonica*) and goldfish (*Carassius auratus auratus*).

In female teleosts, E_2 promoted the synthesis of vitellogenin, which is a major component of egg protein, and a Ca-binding protein (Kwon et al. 1993; Tinsley, 1985). At the same time, E_2 activated osteoclasts in their scales and promoted Ca resorption from the scales (Persson et al. 1995; Suzuki et al. 2000). Consequently, plasma vitellogenin and Ca levels increased in a manner corresponding to the increase in the E_2 level (Norberg et al. 1989). In female goldfish (*Carassius auratus auratus*) and nibbler fish (*Girella punctate*), CT has a significant function in protecting scales from the excess degradation of Ca at vitellogenesis (Suzuki et al. 2000). However, our present data indicated that plasma Ca levels did not change after E_2 injection. Since the stingray itself does not possess hard bones and osteoclasts as do teleosts, the function of CT may be different from that of teleosts. The purpose of this study is to investigate the secretagogue action of CT by estrogen. Therefore, small individuals were used in order to eliminate the influence of endogenous hormones, whereas it is necessary to use mature females to investigate the correlation among vitellogenin, CT, and Ca. Furthermore, in the case of leopard sharks (*Triakis semifasciata*), Glowacki et al. (1985) reported that the administration of salmon CT produced hypercalcemia, although the injection of salmon CT to bass (teleost) produced hypocalcemia. Therefore, these results indicate that CT has some function in cartilaginous fish under several physiological conditions.

Acknowledgements This study was supported in part by grants to N.S. (Grant-in-Aid for Scientific Research [C] No. 16K07871 by JSPS and Kanazawa University CHOZEN project), to T.S. (Grant-in-Aid for Scientific Research [C] No. 18K06312 by JSPS), and to A.H. (Grant-in-Aid for Scientific Research [C] No. 18K11016 by JSPS). This work was partly supported by the cooperative research program of the Institute of Nature and Environmental Technology, Kanazawa University, Accept Number 19010.

Compliance with ethical standards

Conflict of interest The authors have no competing interests to declare.

References

Azria M (1989) The calcitonins: physiology and pharmacology. Karger, Basel

Bentley PJ (1998) Hormones and osmoregulation. In: Bentley PJ (ed) Comparative vertebrates endocrinology, 3rd edn. Cambridge University Press, Cambridge, pp 337–378

Björnsson BTh, Haux C, Förlin L, Deftos LJ (1986) The involvement of calcitonin in the reproductive physiology of the rainbow trout. J Endocrinol 108:17–23

Chao CC, Brown RD, Deftos LJ (1985) Metabolism of calcium and phosphorus during pregnancy and lactation in white-tailed deer. Acta Endocrinol (Copenh) 109:269–275

Copp DH, Cockcroft DW, Kueh Y (1967) Calcitonin from ultimobranchial glands of dogfish and chickens. Science 158:924–925

Dacke CG (1979) Calcium regulation in the chondrichthyes. In: Dacke CG (ed) Calciumn regulation in sub-mammalian vertebrates. Academic Press, London, pp 96–98

Dacke CG, Furr BJA, Boelkins JN, Kenny AD (1976) Sexually related changes in plasma calcitonin levels in Japanese quail. Comp Biochem Physiol Part A 55:341–344

Fiske CH, Subbarow Y (1925) The colorimetric determination of phosphorus. J Biol Chem 66:375–400

Glowacki J, O'Sullivan J, Miller M, Wilkie DW, Deftos LJ (1985) Calcitonin produces hypercalcemia in leopard sharks. Endocrinology 116:827–829

Greer FR, Lane J, Ho M (1984) Elevated serum parathyroid hormone, calcitonin, and 1,25-dihydroxyvitamin D in lactating women nursing twins. Am J Clin Nutr 40:562–568

Hazon N, Wells A, Pillans RD, Good JP, Anderson WG, Franklin CE (2003) Urea based osmoregulation and endocrine control in elasmobranch fish with special reference to euryhalinity. Comp Biochem Physiol Part B 136:685–700

Hiramatsu N, Todo T, Sullivan CV, Schilling J, Reading BJ, Matsubara T, Ryu YW, Mizuta H, Luo W, Nishimiya O, Wu M, Mushirobira Y, Yilmaz O, Hara A (2015) Ovarian yolk formation in fishes: molecular mechanisms underlying formation of lipid droplets and vitellogenin-derived yolk proteins. Gen Comp Endocrinol 221:9–15

Kase Y, Ikari T, Sekiguchi T, Sato M, Ogiso S, Kawada T, Matsubara S, Satake H, Sasayama Y, Endo M, Kitamura K, Hattori A, Watanabe TX, Maruyama Y, Watanabe Y, Funahashi H, Kambegawa A, Suzuki N (2017) Sardine procalcitonin amino-terminal cleavage peptide has a different action from calcitonin and promotes osteoblastic activity in the scales of goldfish. Comp Biochem Physiol Part A 211:77–83

Kwon HC, Hayashi S, Mugiya Y (1993) Vitellogenin induction by estradiol-17β in primary hepatocyte culture in the rainbow trout, *Oncorhynchus mykiss*. Comp Biochem Physiol Part B 104:381–386

Lu CC, Tsai SC, Wang SW, Tsai CL, Lau CP, Shih HC, Chen YH, Chiao YC, Liaw C, Wang PS (1998) Effects of ovarian steroid hormones and thyroxine on calcitonin secretion in pregnant rats. Am J Physiol 274:E246–E252

Nichols S, Gelsleichter J, Manire CA, Cailliet GM (2003) Calcitonin-like immunoreactivity in serum and tissues of the bonnethead shark, *Sphyrna tiburo*. J Exp Zool Part A 298:150–161

Norberg B, Björnsson BTh, Brown CL, Wichardt U-P, Deftos LJ, Haux C (1989) Changes in plasma vitellogenin, sex steroids, calcitonin, and thyroid hormones related to sexual maturation in female brown trout (*Salmo trutta*). Gen Comp Endocrinol 75:316–326

Persson P, Takagi Y, Björnsson BTh (1995) Tartrate resistant acid phosphatase as a marker for scale resorption in rainbow trout, *Oncorhynchus mykiss*: effects of estradiol-17β treatment and refeeding. Fish Physiol Biochem 14:329–339

Robertson DR (1981) A competive inhibition enzyme-linked immunosorbent assay for frog calcitonin. Gen Comp Endocrinol 45:12–20

Sasayama Y (1999) Hormonal control of Ca homeostasis in lower vertebrates: considering the evolution. Zool Sci 16:857–869

Sasayama Y, Oguro C, Yui R, Kambegawa A (1984) Immunohistochemical demonstration of calcitonin in ultimobranchial glands of some lower vertebrates. Zool Sci 1:755–758

Sasayama Y, Suzuki N, Oguro C, Takei Y, Takahashi A, Watanabe TX, Nakajima K, Sakakibara S (1992) Calcitonin of the stingray: comparison of the hypocalcemic activity with other calcitonins. Gen Comp Endocrinol 86:269–274

Searcy RL, Foreman JA, Ketz A, Reardon J (1967) A new automated method for urea nitrogen analysis. Tech Bull Regist Med Technol 37:107–111

Sekiguchi T, Suzuki N, Fujiwara N, Aoyama M, Kawada T, Sugase K, Murata Y, Sasayama Y, Ogasawara M, Satake H (2009) Calcitonin in a protochordate, *Ciona intestinalis*: the prototype of the vertebrate Calcitonin/Calcitonin gene-related peptide superfamily. FEBS J 276:4437–4447

Sekiguchi T, Shiraishi A, Satake H, Kuwasako K, Takahashi H, Sato M, Urata M, Wada S, Endo M, Ikari T, Hattori A, Srivastav AK, Suzuki N (2017) Calcitonin-typical suppression of osteoclastic activity by amphioxus calcitonin superfamily peptides and insights into the evolutionary conservation and diversity of their structures. Gen Comp Endocrinol 246:294–300

Suzuki N (2001) Calcitonin-like substance in the plasma of Cyclostomata and its putative role. Comp Biochem Physiol Part B 129:319–326

Suzuki N, Takagi T, Sasayama Y, Kambegawa A (1995) Effects of ultimobranchialectomy on the mineral balances of the plasma and bile in the stingray (Elasmobranchii). Zool Sci 12:239–242

Suzuki N, Ueda K, Sakamoto H, Sasayama Y (1999a) Fish calcitonin genes: primitive bony fish genes have been conserved in some lower vertebrates. Gen Comp Endocrinol 113:369–373

Suzuki N, Suzuki D, Sasayama Y, Srivastav AK, Kambegawa A, Asahina K (1999b) Plasma calcium and calcitonin levels in eels fed a high calcium solution or transferred to seawater. Gen Comp Endocrinol 114:324–329

Suzuki N, Suzuki T, Kurokawa T (2000) Suppression of osteoclastic activities by calcitonin in the scales of goldfish (freshwater teleost) and nibbler fish (seawater teleost). Peptides 21:115–124

Suzuki N, Yamamoto K, Sasayama Y, Suzuki T, Kurokawa T, Kambegawa A, Srivastav AK, Hayashi S, Kikuyama S (2004) Possible direct induction by estrogen of calcitonin secretion from ultimobranchial cells in the goldfish. Gen Comp Endocrinol 138:121–127

Takei Y, Takahashi A, Watanabe TX, Nakajima K, Sakakibara S, Sasayama Y, Suzuki N, Oguro C (1991) New calcitonin isolated from the ray, *Dasyatis akajei*. Biol Bull 180:485–488

Tinsley D (1985) A comparison of plasma levels of phosphoprotein, total protein and total calcium as indirect indices of exogenous vitellogenesis in the Crucian carp, *Carassius carassius* (L.). Comp Biochem Physiol Part B 80:913–916

Wang J, Rout UK, Bagchi IC, Armant DR (1998) Expression of calcitonin receptors in mouse preimplantation embryos and their function in the regulation of blastocyst differentiation by calcitonin. Development 125:4293–4302

Watts EG, Copp DH, Deftos LJ (1975) Changes in plasma calcitonin and calcium during the migration of salmon. Endocrinology 96:214–218

Wendelaar Bonga SE, Pang PKT (1991) Control of calcium regulating hormones in the vertebrates: parathyroid hormone, calcitonin, prolactin, and stanniocalcin. Int Rev Cytol 128:139–213

Yamamoto K, Suzuki N, Takahashi N, Sasayama Y, Kikuyama S (1996) Estrogen receptors in the stingray (*Dasyatis akajei*) ultimobranchial gland. Gen Comp Endocrinol 101:107–114

Yamauchi H, Orimo H, Yamauchi K, Takano K, Takahashi H (1978) Increased calcitonin levels during ovarian development in the eel, *Anguilla japonica*. Gen Comp Endocrinol 36:526–529

Publisher's Note