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ORIGINAL RESEARCH

Possible involvement of *Calcitonin* I and II in calcium metabolism of the female reproductive physiology of goldfish (*Carassius auratus*)

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Abstract Two types of *Calcitonin* (*Calcitonin I* (*CtI)*, *Calcitonin II* (*CtII)*) were previously determined in goldfish. The present study examined the expression levels of *CtI* and *CtII* in the ultimobranchial glands (UBGs) using quantitative PCR with a TaqMan probe and their correlation with the reproductive physiology. The results showed that the mRNA expression of *CtI* and *CtII* in females had a significant relationship with their plasma Ca concentrations, while those in males did not correlate with their plasma Ca concentrations. Furthermore, there was a significant co-relationship between plasma CALCITONIN (CT) and Ca levels or gonad somatic index in female but not male goldfish. Additionally, the plasma CT and Ca levels in females were significantly higher than those in males. The *calcium-sensing receptor* mRNA expression in the UBGs of female goldfish was significantly higher than that in male goldfish, indicating that the UBGs in female goldfish respond sensitively to changing plasma Ca conditions during the reproductive period. On the other hand, it has been considered that the target of the secreted CT is osteoclasts in the fish scales, while it is bone in mammals. Next, we examined the *CtI* and *CtII* mRNA expressions in the scales of goldfish. In the scales of both sexes, we found that *CtII* mRNA was expressed, but *CtI* was not detected. *CtII* expression was immunohistochemically observed in the osteoblasts. Furthermore, *CtII* mRNA expression in scales increased significantly with E_2 treatment. Thus, in the present study, we demonstrate that CTI and CTII are involved in Ca metabolism, particularly in female goldfish reproductive physiology. CTII expressed in the ectopic organs

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such as scales has an ectopic function regarding Ca metabolism through paracrine or autocrine mechanisms during the female reproductive period.

Keywords Calcitonin . Estrogen . Calcium . Ultimobranchial glands . Scales . Reproductive physiology . Goldfish

Introduction

CALCITONIN (CT) is a peptide hormone composed of 32 amino acid residues. This hormone is secreted from the C cells of thyroid glands in mammals and the ultimobranchial glands (UBGs) in non-mammalian vertebrates (Dacke 1979; Wendelaar Bonga and Pang 1991; Sasayama 1999). CT is associated with calcium (Ca) metabolism. The serum or plasma Ca concentrations decrease when CT is administered to some mammals—such as rats, rabbits, and humans—as it inhibits osteoclastic activity (Dacke 1979; Azria 1989). In teleosts as well as mammals, CT inhibited osteoclastic activity in both freshwater and seawater teleosts in *in vitro* studies (Suzuki et al. 2000; Sekiguchi et al. 2009; Sekiguchi et al. 2017; Kase et al. 2017). Therefore, CT functions in Ca metabolism in fish the same as it does in mammals (Suzuki et al. 1999a). Particularly during specific stages—such as the reproductive stage—plasma CT levels in goldfish and salmon were also higher in females than in males (Watts et al. 1975; Suzuki et al. 2004). Furthermore, estrogen receptors are detected in the UBGs, and estrogen directly acts on the UBGs and promotes CT secretion (Suzuki et al. 2004; Takagi et al. 2019). The significant increase in CT has been reported before ovulation in eels (Yamauchi et al. 1978) and salmonid fishes (Björnsson et al. 1986; Norberg et al. 1989). The above results indicate that CT is related to reproductive physiology, especially in female teleosts (Sekiguchi et al. 2021).

We previously determined two types of *Ct* (*CtI* and *CtII*) in goldfish (Suzuki et al. 1999b). CTI has been isolated from the UBGs of goldfish, and their amino acid sequence was determined (Sasayama et al. 1993). The other *Ct* type (*CtII*) has been determined from the genomic DNA of the goldfish liver (Suzuki et al. 1999b). However, there has been no study regarding the *CtI* and *CtII* expression in the UBGs associated with reproductive physiology.

In the present study, we used a quantitative PCR with a TaqMan probe to examine the expression levels of *CtI* and *CtII* in the UBGs and their relation to reproductive physiology. On the other hand, it is known that estrogen promotes osteoclast activity and elutes Ca from fish scales during the reproductive period (Persson et al. 1995; Suzuki et al. 2000; Sekiguchi et al. 2021). In fish scales, the activated osteoclasts are considered to be suppressed by CT secreted from their UBGs (Suzuki et al. 2000; Sekiguchi et al. 2021). Therefore, we focused on fish scales and analyzed the mRNA expression of *CtI* and *CtII* in goldfish scales as a target organ for CT. Furthermore, the influence of estrogen on *Ct* mRNA expression was examined in goldfish scales. To the best of our knowledge, the present study is the first report demonstrating that CTII, as well as CTI, is involved in Ca metabolism, particularly in the female reproductive physiology, and that CTII is expressed in the ectopic organs such as scales and has an ectopic function in a paracrine or autocrine manner in goldfish scales.

Materials and methods

Animals

Goldfish (*Carassius auratus*) were purchased from Higashikawa Fish Farm (Nara, Japan). Female (n = 10) (128.51 ± 3.75 g) and male (n = 9) (88.18 ± 2.61 g) goldfish with gonads at various stages of maturation were used to investigate the relationship between CT expression and reproductive physiology. The maturation stages were determined by the gonad somatic index (GSI%) (gonadal weight/body weight × 100). Additionally, the *Ct* mRNA expression in estrogen-treated scales was measured in the female goldfish (n = 7) (21.51 ± 1.59 g) using an *in vitro* bioassay of the scales (Suzuki et al. 2000). Because this bioassay was performed using scales in a 96-well plate, smaller goldfish were used. All experimental procedures were conducted to avoid causing pain to the goldfish while they were anesthetized and were performed in accordance with Kanazawa University's Guide for the Care and Use of Laboratory Animals.

Collection of blood, UBGs, and scales from goldfish

Goldfish were anesthetized using 0.03% ethyl 3-aminobenzoate, methanesulfonic acid salt (Sigma-Aldrich, Inc., St. Louis, MO, USA) neutralized with 0.03% sodium bicarbonate. After measuring the body weight, blood samples (around 1 ml) were collected from the caudal vessels of each individual goldfish (female, n = 10; male, n = 9) into a heparinized syringe and centrifuged at 25,000 × g for 10 min at 4°C. The plasma was immediately frozen and kept at −80°C until plasma Ca and CT were analyzed. Thereafter, the goldfish were dissected, and their gonadal weight was measured to obtain the GSI for each fish. Then, the UBGs present on the ventral side of the esophagus were cut and put into RNALater® (Sigma-Aldrich) and stored at −80°C until used for mRNA expression analysis. Eight scales from each individual were collected from one row above the left lateral line and stored in the same manner as the UBGs.

Measurement of plasma Ca and CT levels

Using the collected plasma samples, the plasma Ca and CT concentrations were measured. Plasma Ca levels were determined using a commercial kit (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) with a microplate reader (Sato et al. 2017). Plasma CT levels were determined by using an enzyme-linked immunosorbent assay (ELISA). Details of the ELISA method were previously described by Suzuki (2001). The detection limit was 25 pg/ml (Suzuki 2001). The specificity of the antiserum was checked using peptide hormones (N-terminal fragment of the 1-34 bovine parathyroid hormone and human calcitonin gene-related peptide). This antiserum did not cross-react with these peptide hormones.

The mRNA expression of *CtI*, *CtII*, and *Calcium-sensing receptors*

We examined the mRNA expression of *CtI*, *CtII*, and *Calcium-sensing receptors* in the UBGs, which are the CT secretory organ. Total RNA was prepared from the dissected UBGs or scales using a total RNA isolation kit (NucleoSpin RNA II, Takara Bio, Inc., Shiga, Japan) and treated with DNase I (RNase-Free DNase Kit, Takara Bio, Inc.) for 15 min at room temperature to remove residual genomic DNA. Complementary DNA synthesis was also performed using a commercial kit (PrimeScript™ II 1st Strand cDNA Synthesis Kit, Takara Bio, Inc.).

The specific primers and TaqMan probes for the *CtI* and *CtII* are shown in Fig. 1. The specificity of primer sets for *CtI* and *CtII* was checked using the *CtI* and *CtII* cDNA, respectively. A primer set for *Calcium-sensing receptor* (forward: 5′-TCTAAATGCCCGAACAACTCC-3′; reverse: 5′-CACAAAAACACCCAACACGA-3′) was reported in a previous study (Kakikawa et al. 2012). The amplification of *β-Actin* cDNA was performed using specific primers (forward: 5′-CGAGCGTGGCTACAGCTTCA-3′; reverse: 5′-GCCCGTCAGGGAGCTCATAG-3′) (Azuma et al. 2007). The PCR amplification was analyzed using a real-time PCR apparatus (Mx3000p, Agilent Technologies, Santa Clara, CA, USA) (Suzuki et al. 2011). The conditions for the qPCR amplification consisted of 2 min of initial denaturation at 95°C followed by 40–50 cycles of denaturation at 95°C for 30 s, annealing and

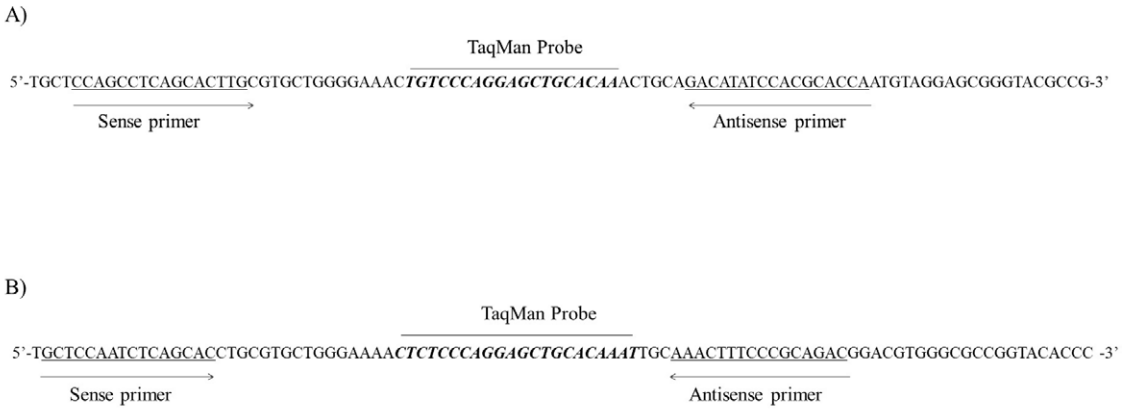

Fig. 1 Location of primers and TaqMan probe for (A) *CtI* (LC744803) and (B) *CtII* (LC744804)

extension at 60°C for 40 s, and a single cycle for dissociation curve analysis. The mRNA levels were normalized to the *β-Actin* mRNA level. *CtI*, *CtII*, and *Calcium-sensing receptor* mRNA expression levels were analyzed in the UBGs, while *CtI* and *CtII* mRNA expression levels were examined in the scales. In the case of scales, an agarose electrophoresis using 3% NuSieve GTG agarose gel (FMC BioProducts, Rockland, ME, USA) was performed to confirm the size of the PCR product after PCR amplification. Thereafter, the direct sequence of the PCR products amplified in the scales was analyzed, and the nucleotide sequence of *CtII* was confirmed.

Effect of 17β-estradiol on *Ct* mRNA expression in the scales of goldfish

It is known that teleost scales regenerate after being removed. In our previous study, we reported that the response of osteoblasts to 17β-estradiol (E_2) in regenerating scales was higher than that in normally developed scales (Yoshikubo et al. 2005). Therefore, we used regenerating scales to analyze the influence of E_2 on *Ct* mRNA expression. In order to prepare the regenerating scales, female goldfish were anesthetized with 0.03% ethyl 3-aminobenzoate and methanesulfonic acid salt (Sigma-Aldrich) neutralized with 0.03% sodium bicarbonate. Thereafter, normally developed scales were removed from both sides (one row above the lateral line) of the body to allow for the regeneration of scales. On day 14, goldfish were anesthetized again, and the regenerating scales were removed. The scales were put into Leibovitz's L-15 medium (Thermo Fisher Scientific Inc., Grand Island, NY, USA) containing a 1% penicillin–streptomycin mixture (Thermo Fisher Scientific Inc.) and pre-incubated at 15°C for 1 hr. The other regenerating scales were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PBS) (pH 7.4) and used for immunohistochemical analysis. After pre-incubation to acclimate the scales to the medium, the influence of E_2 on the *Ct* mRNA expression in the scales of goldfish was studied. The scales were moved to the L-15 medium containing a 1% penicillin–streptomycin mixture (Thermo Fisher Scientific Inc.) supplemented with or without E_2 (100 µg/ml, water soluble type, Sigma-Aldrich). After 6 h of incubation, the scales were transferred into RNALater® (Sigma-Aldrich) and stored at -80°C until use. The total RNA isolation and cDNA synthesis were performed as described above. The *Ct* mRNA expression analysis was carried out by real-time PCR with the specific primers and TaqMan probes shown in Fig. 1. The mRNA levels were normalized to the *β-Actin* mRNA level as described above.

Immunohistochemical analysis for CT in goldfish scales

For the immunohistochemical analysis, the fixed regenerating scales were analyzed as whole mount samples or cryosections in accordance with the methods detailed by Yamamoto et al. (2020). After rinsing with PBS, the scales were used as whole-mount samples or embedded in Tissue-Tek® optimal cutting temperature compound for the preparation of cryosections. They were incubated with a blocking solution containing 0.1% Tween 20, 0.3% glycine, 10% normal goat serum, and 1% bovine serum albumin for 1 h at room temperature, and then with primary antibody: anti-salmon CT serum (×50,000) overnight at 4°C (Suzuki 2001). For the secondary antibody, Alexa Fluor® 488-labeled anti-rabbit IgG (A11034, Molecular Probes; × 1000) was used. After 1 h of incubation at room temperature, scales were then rinsed in PBS and stained with 4′, 6-diamidino-2-phenylindole to visualize the nuclei. For the negative control, immunostaining was also performed using anti-CT that had been pre-absorbed with 5 µg/ml of salmon CT.

Whole-mount regenerating scales were stained for alkaline phosphatase (ALP) activity with NBT/BCIP solution (Roche Applied Science, Mannheim, Germany) as previously reported (Yachiguchi et al. 2014). Additionally, semi-thin sections of regenerating scales embedded in an epoxy resin were stained with toluidine blue to confirm the cell morphology.

Statistical analysis

The statistical significance of the correlation was examined using linear regression analysis (Figs. 4–7). The statistically significant difference between two groups was assessed by independent sample *t*-test (Figs. 2, 3, 8, and 9) or paired *t*-test (Fig. 11). In all data, the statistical significance level was $P < 0.05$.

Results

The mRNA expression of *CtI* and *CtII* in UBGs

Using specific primers and a TaqMan probe, *CtI* and *CtII* were amplified in the UBGs of female and male goldfish. The results are shown in Fig. 2.

CtI mRNA expression was observed in the UBGs of both female and male goldfish. However, there was no significant difference between the expression levels in the females and males in *CtI* mRNA, although the *CtI* mRNA expression in females tended to be higher than that in males. There was no significant difference between the female and male expression levels of *CtII* mRNA as with the *CtI* mRNA expression.

Plasma CT and Ca levels in female and male goldfish

The plasma CT and Ca concentrations are shown in Fig. 3. Plasma CT levels in female goldfish were three times higher than those in males (Fig. 3A). Plasma Ca concentrations as well as plasma CT were significantly higher in female than in male goldfish (Fig. 3B).

Correlation of *CtI* and *CtII* with reproductive physiology

The correlations between the *CtI* and *CtII* mRNA expression in UBGs and plasma Ca concentrations are shown in Figs. 4 and 5, respectively. In female goldfish, both *CtI* and *CtII* mRNA expressions were signifi-

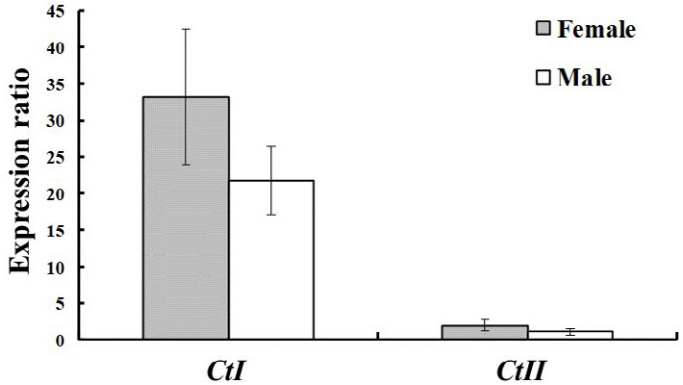

Fig. 2 The mRNA expression of *CtI* and *CtII* in the ultimobranchial glands of female and male goldfish
There was no significant difference between the expression levels of female and male goldfish in either *CtI* or *CtII*. All data are expressed as the mean ± SE. Female, n = 10; male, n = 9.

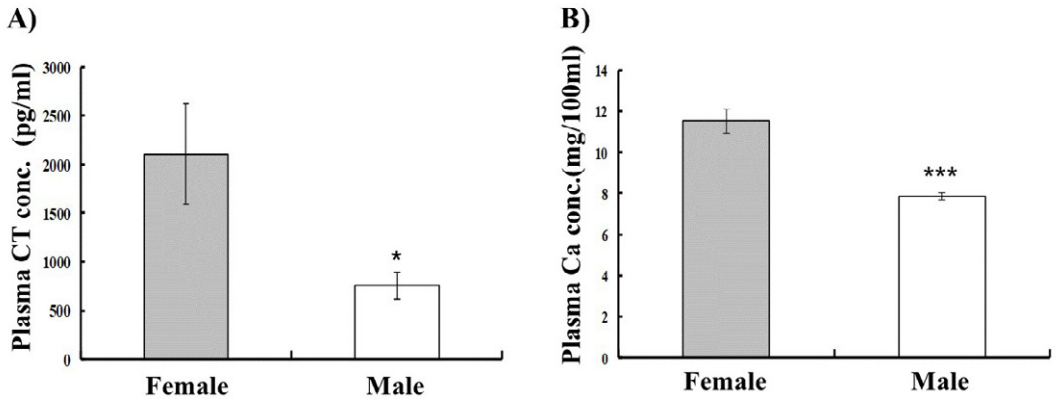

Fig. 3 Plasma CT (A) and Ca (B) concentrations in female and male goldfish
* and *** denote statistically significant differences at $P < 0.05$ and $P < 0.001$, respectively. All data are expressed as the mean ± SE. Female, n = 10; male, n = 9.

cantly correlated with the plasma Ca concentration levels. In the case of male goldfish, however, there was no significant correlation between the *CtI* or *CtII* mRNA expression and plasma Ca concentrations.

Furthermore, plasma CT levels in female goldfish were significantly related to their plasma Ca levels (Fig. 6A) or GSI (Fig. 7A). In males, however, the plasma CT concentrations were not associated with their plasma Ca levels (Fig. 6B) or GSI (Fig. 7B).

The mRNA expressions of *Calcium-sensing receptors* in the UBGs of female and male goldfish.

The results are shown in Fig. 8. The expression of *Calcium-sensing receptor* mRNA was detected in both female and male goldfish. We found that *Calcium-sensing receptor* mRNA expression in the UBGs of female goldfish was significantly higher than that in the UBGs of male goldfish.

Detection of CT in the scales of goldfish

To detect ectopic CT in the scales, *CtI* and *CtII* mRNA expressions were examined in the scales. Only *CtII* was detected in the scales of both female and male goldfish (Fig. 9A). However, there was no significant difference in *CtII* expression levels between female and male goldfish. The PCR products of *CtII* in the scales were the same size as those amplified in the UBGs (Fig. 9B). The *CtII* sequence in the scales was confirmed by the direct sequencing of PCR products amplified from the scales.

CT immunoreactivity was detected in the scale osteoblasts in the fibrous layer (Fig. 10A) and disappeared with an absorption test (Fig. 10B). Many of these scale osteoblasts were ALP positive (Fig. 10C) and were cuboidal in shape (Fig. 10D).

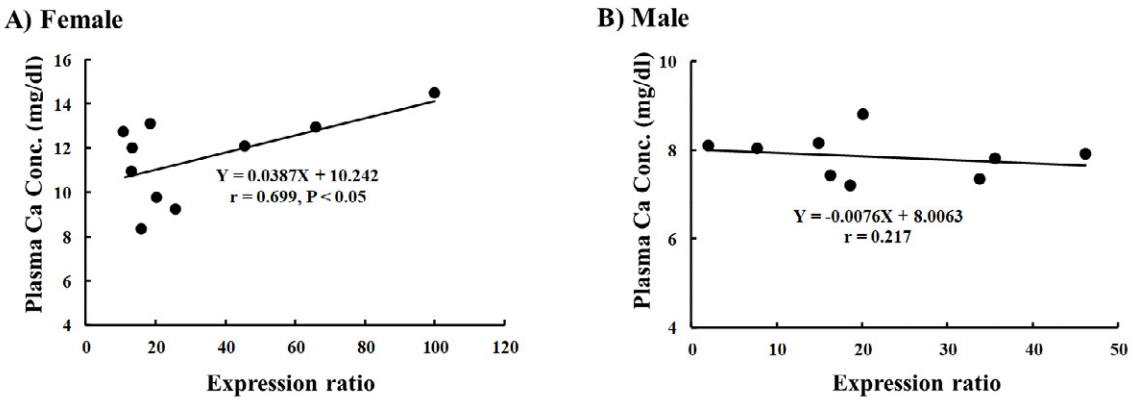

Fig. 4 Correlation of *CtI* mRNA expression in ultimobranchial glands with the plasma Ca concentrations in female (A) and male (B) goldfish
CtI mRNA expression has a significant correlation with plasma Ca concentration levels in females (n = 10) but not males (n = 9).

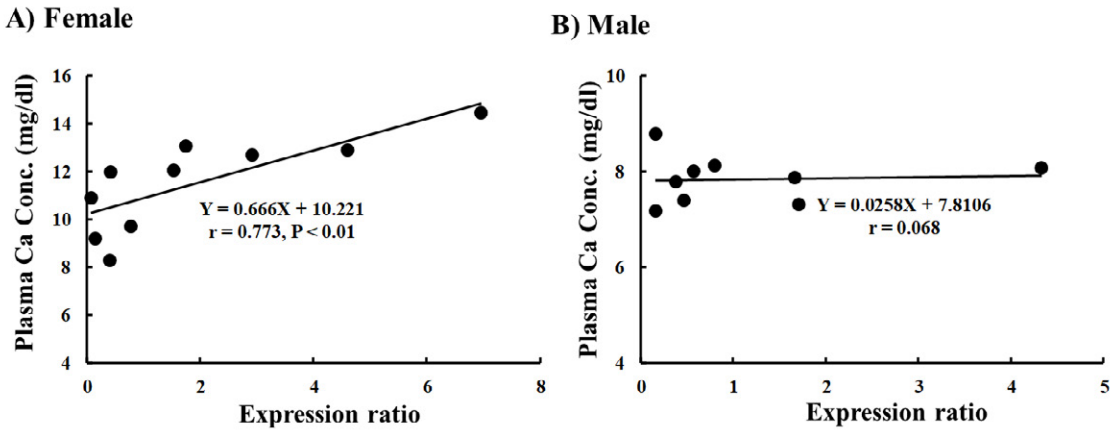

Fig. 5 Correlation of *CtII* mRNA expression in ultimobranchial glands with the plasma Ca concentrations in female (A) and male (B) goldfish
CtII mRNA expression has a significant correlation with plasma Ca concentration levels in females (n = 10) but not males (n = 9).

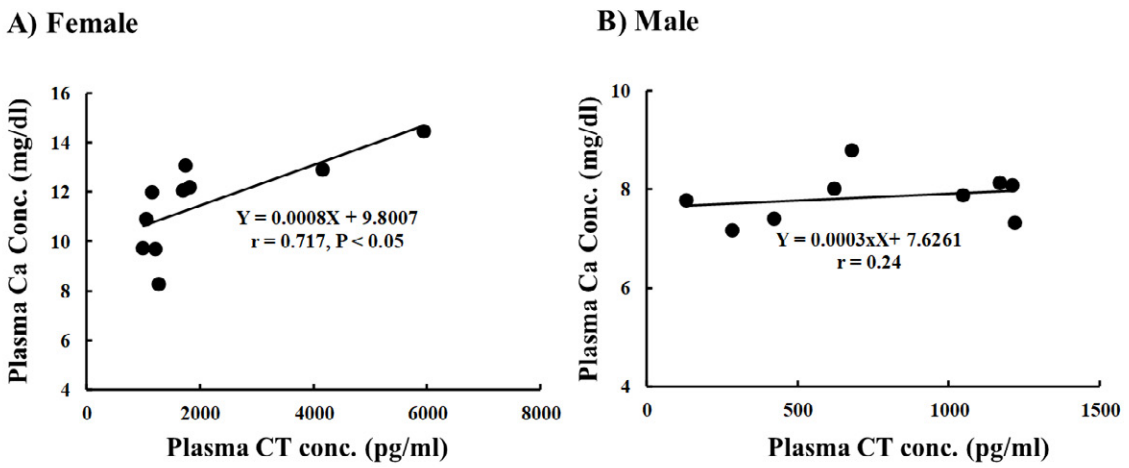

Fig. 6 Correlation of the plasma CT concentrations with the plasma Ca concentrations in female (A) and male (B) goldfish
Plasma CT concentrations have significant correlation with the plasma Ca concentration levels in females (n = 10) but not males (n = 9).

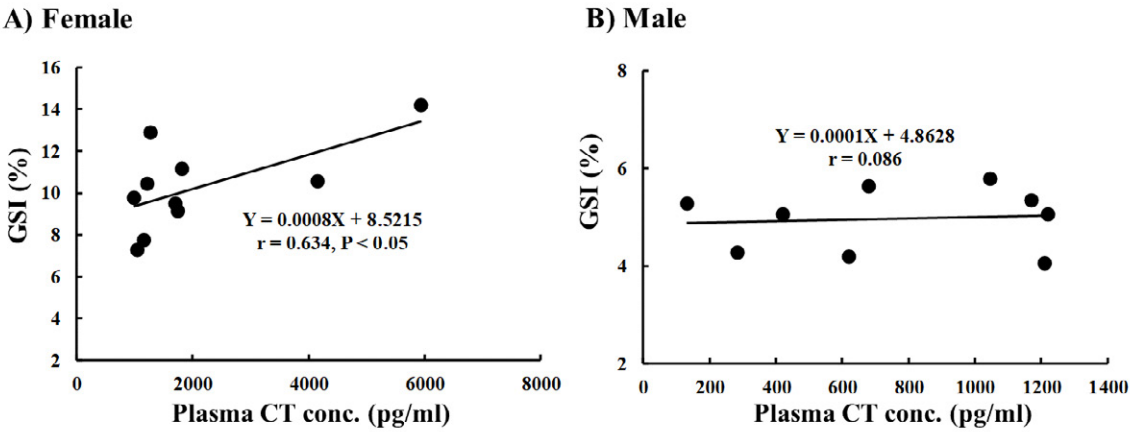

Fig. 7 Correlation of the plasma CT concentrations with the gonad somatic index (GSI) (%) in female (A) and male (B) goldfish
Plasma CT concentrations are significantly correlated with the GSI (%) in females (n = 10) but not males (n = 9).

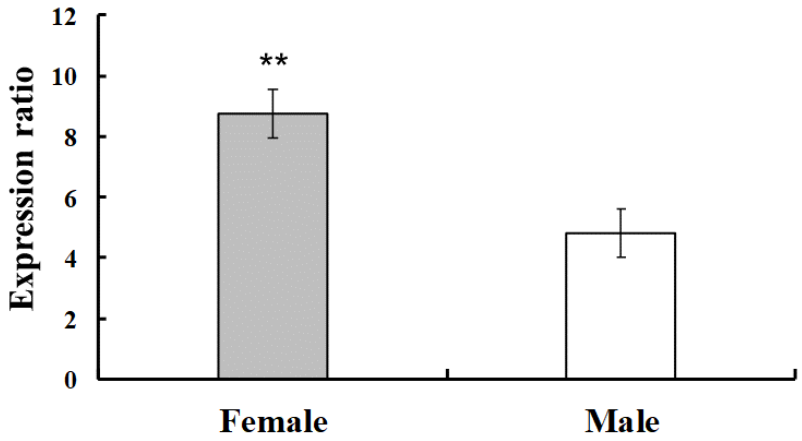

Fig. 8 The mRNA expression of *Calcium-sensing receptors* in the ultimobranchial glands of female and male goldfish
** denotes a statistically significant difference at P < 0.01. All data are expressed as the mean ± SE. Female, n = 10; male, n = 9.

Effect of E_2 on *CtII* mRNA expression in the scales of goldfish

As described in the previous section, we found that only *CtII* was expressed in the scales of goldfish. Therefore, we examined the influence of E_2 on the *CtII* mRNA in the goldfish scales. The results show that *CtII* mRNA expression in the E_2-treated scales increased significantly as compared with that in the control scales (Fig. 11).

Discussion

We previously reported that *CtII* was identified from the genomic DNA of the liver in goldfish (Suzuki et al. 1999b). The present study demonstrated that *CtI* and *CtII* mRNA expressions were detected in the UBGs using quantitative PCR with a TaqMan probe. Additionally, the mRNA expression of *CtI* and *CtII* in females had a significant correlation with their plasma Ca concentrations in females, while those in males were not associated with their plasma Ca concentrations. Therefore, *CtII*, as well as *CtI*, has a significant

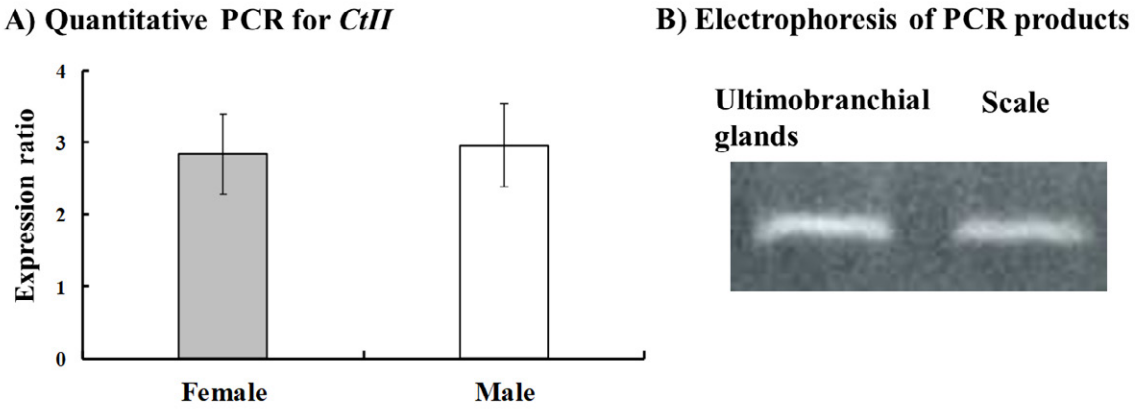

Fig. 9 The mRNA expression of *CtII* in goldfish scales
CtII expression was detected in the scales of female and male goldfish using quantitative PCR with a TaqMan probe (A) and electrophoresis (B). There was no significant difference in *CtII* expression levels between female and male goldfish. All data are expressed as the mean ± SE. Female, n = 10; male, n = 9.

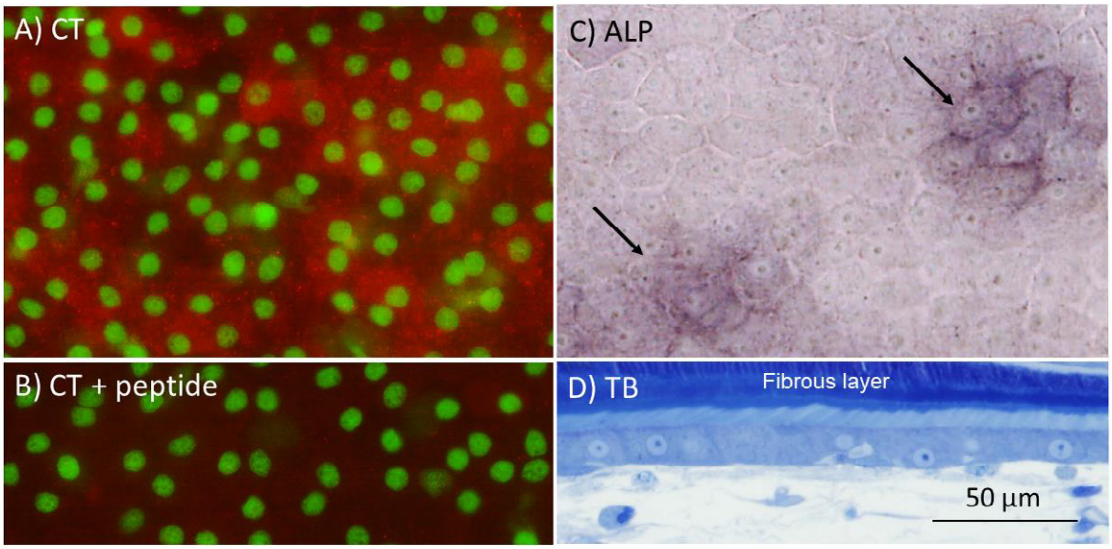

Fig. 10 Detection of CT immunoreactivity in regenerating scales
(A) CT immunoreactivity (red) was detected in the mononuclear cells on the surface of the fibrous layer. (B) No immunoreactivity was detected with the primary antibody against CT, which was pre-incubated with an excess amount of the antigen peptide. (C) Positive staining for alkaline phosphatase (ALP) activity (purple) was detected in some of the mononuclear cells (arrows). (D) Light microscopy image of the cells on the fibrous layer observed on a semi-thin plastic section stained with toluidine blue.

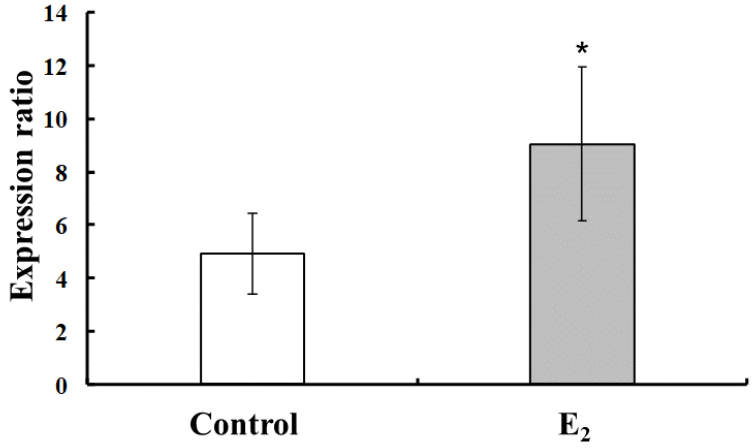

Fig. 11 Effect of E_2 on *CtII* mRNA expression in the scales of goldfish
* denotes a statistically significant difference by paired t-test at $P < 0.05$. All data are expressed as the mean ± SE (n = 7).

function in Ca metabolism in females but not in males during reproductive periods. Additionally, we measured the plasma CT concentrations in both females and males. We found a significant co-relationship between plasma CT and Ca levels in female but not male goldfish. Furthermore, the plasma CT and Ca levels in females were significantly higher than those in males. Judging from these obtained results, CT synthesis and the secretion of CT in the UBGs of females seem to respond more sensitively to plasma Ca than do those of males. To further analyze the difference in Ca metabolism between females and males, we investigated *Calcium-sensing receptor* mRNA expression in UBGs.

In mammals, *Calcium-sensing receptors* have been cloned and detected from the C cells of the thyroid gland (Garrett et al. 1995). This receptor has been demonstrated to monitor the increase of blood Ca and promote CT secretion (Freichel et al. 1996). In teleosts, *Calcium-sensing receptors* have been determined and expressed in several tissues associated with osmo-regulative tissues and endocrine glands (Loretz et al. 2009). However, there have been no reports on the expression of *Calcium-sensing receptors* in the UBGs. To the best of our knowledge, the present study is the first report demonstrating that *Calcium-sensing receptors* were expressed in the UBGs of goldfish. Furthermore, we found that *Calcium-sensing receptor* mRNA expression in the UBGs of female goldfish was significantly higher than that in the UBGs of male goldfish during reproductive periods. Therefore, the UBGs in female goldfish respond sensitively to changing plasma Ca levels in female goldfish. Then, CT was secreted from UBGs in female goldfish according to the relative changes in the elevated plasma Ca concentrations. However, the UBG sensitivity of male fish to plasma Ca was low. Plasma CT levels were not correlated with the plasma Ca in male goldfish. This result regarding the correlation between plasma CT and plasma Ca in males agreed with that of the freshwater teleost, *Mastacembelus armatus*, during the reproductive period (Verma and Alim 2015). On the other hand, in male eels, we previously reported that the relationship between CT secretion and plasma Ca has been observed (Suzuki et al. 1999a). Namely, the plasma Ca concentration was more than doubled when a high concentration of $CaCl_2$ dissolved in a consommé solution with several amino acids was orally administered to male eels. In accordance with the remarkably elevated plasma Ca, the plasma CT concentration was then significantly increased in male eels as compared with eels administered only the consommé solution. Therefore, the secretion of CT in males and females may be different in terms of physiological phenomena.

Most teleosts have an acellular bone structure, which is characterized by the absence of osteoblasts and osteoclasts within the vertebral bone (Weiss and Watabe 1979; Ekanayake and Hall 1988), while teleost scale is a calcified tissue that contains osteoclasts and osteoblasts that are similar to those found in avian and mammalian bone (Suzuki et al. 2007; Thamamongood et al. 2012; Yano et al. 2013). Therefore, it is a functional internal Ca reservoir rather than a vertebral bone during periods of increased Ca demand, such as sexual maturation and starvation (Mugiya and Watabe 1977; Bereiter-Hahn and Zylberberg 1993; Suzuki et al. 2008). We previously reported that estrogen receptors are detected in UBGs, and that estrogen acts directly on UBGs and promotes CT secretion (Suzuki et al. 2004). We considered that the secreted CT functioned in the osteoclasts of the fish scales (Sekiguchi et al. 2021) because the correlation between

CT and E_2 in goldfish (a freshwater teleost) and nibbler fish (a seawater teleost) was demonstrated in their cultured scales (Suzuki et al. 2000). Namely, in goldfish scales, CT inhibited the osteoclastic activity which was enhanced by E_2 treatment in a dose-dependent manner. Similar results were observed with the scales of female nibbler fish. Therefore, CT has the potential to protect scales from excessive Ca degradation by E_2 during teleost reproduction. In the present study, we found that *CtII* mRNA was expressed in the scales of both sexes, but *CtI* was not expressed in the scales. CTII expression was immunohistochemically observed in osteoblasts and the *CtII* expressed in the scales increased significantly with E_2 treatment. This suggests that *CtII* expression in scales is associated with the reproductive physiology in female goldfish.

It has been reported that CT-immunoreactive cells or CT-like substances are also present in organs other than the thyroid glands and UBGs (Azria 1989; Okuda et al. 1999). In goldfish, we detected both CT-immunoreactive cells and *Ct* mRNA expression in the intestines (Okuda et al. 1999). The sequence of this *Ct* completely coincided with *CtI* but not *CtII*, although the function has not yet been elucidated (Okuda et al. 1999). The present study indicated that *CtII* was expressed in the ectopic organs such as scales. In addition, we demonstrated that *CtII* mRNA expression was enhanced by treatment with E_2. This indicates that CTII expressed in scales has a functional role in the Ca metabolism of female goldfish.

Procalcitonin (PCT), the precursor of CT, undergoes successive cleavages to form three distinct molecules: CT, C-terminal fragment, and N-terminal fragment called the amino-terminal procalcitonin (N-terminal procalcitonin) (Azria 1989; Kase et al. 2017). Because both CT and N-terminal procalcitonin are produced from common precursor PCT, our finding of the existence of CTII in goldfish scales indicates that N-terminal procalcitonin may potentially exist in the scales as well. Our previous study reported that the N-terminal procalcitonin of the sardine (*Sardinops melanostictus*) activated osteoblasts, and that CT suppressed osteoclastic activity (Kase et al. 2017). These facts, taken together with our finding of E_2-induced *CtII* expression in goldfish scales, indicate that N-terminal procalcitonin might activate osteoblasts, and CTII might suppress osteoclasts in goldfish scales, which promotes bone formation after bone resorption by E_2.

Conclusion

The present study examined the expression levels of *CtI* and *CtII* in the UBGs using quantitative PCR with a TaqMan probe and their correlation with reproductive physiology. The mRNA expression of both *CtI* and *CtII* in females had a significant relationship with their plasma Ca concentration levels, while those in males did not correlate with their plasma Ca concentrations. Furthermore, there were significant co-relationships between the plasma CT and Ca levels or GSI in female but not male goldfish. Since *Calcium-sensing receptor* mRNA expression in the UBGs of female goldfish was significantly higher than that in the UBGs of male goldfish, the UBGs in female goldfish respond sensitively to changing plasma Ca levels in female goldfish during the reproductive period. We also found that *CtII* mRNA was expressed in the scales of both sexes, but that *CtI* was not expressed in the scales. Furthermore, *CtII* mRNA expressions in scales were enhanced by the addition of E_2, indicating that the CTII expressed in the scales possesses an ectopic function in a paracrine or autocrine manner.

Author contributions NS, KK, AKS, TS, and HM: Design and conceptualization of the study; JH and AH: finalization of the draft; AF and YS: Collection of the samples; KH, RK, YT, YF, MI, TF, SU, and UK: Analysis of the samples and data acquisition and interpretation; NS. and JH.: Coordination of the study and finalization of the manuscript.

Competing interests The authors have no competing interests to declare.

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