Dissertation

EFFECTS OF ENVIRONMENTAL POLLUTANTS ON OSTEOCLASTS AND OSTEOBLASTS OF TELEOST SCALES

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CONTENTS

Ⅰ. General Introduction

Environmental pollution due to a toxic substance has become a problem in recent years. Namely, some traces of polluting chemicals, such as polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins and furans (PCDD/Fs), hexachlorocyclohexanes (HCHs), 2,2-bis-(*p*-chlorophenyl)-1,1,1-trichloroethane (DDT), and polycyclic aromatic hydrocarbons (PAHs), have accumulated in animals and higher up the food chain. The concentrated chemicals influence marine mammals (Tanabe, 2003), birds (Ricardo et al., 2006), and humans (Mori and Todaka, 2011). In heavy metals as well as polluting chemicals, similar results have been reported (David et al., 1990; Storelli and Marcotrigiano, 2003).

On the other hand, 80% of marine pollution is caused by pollution on land (Omori and Thorne-Miller, 2006). Chemical pollutants that are difficult to degrade are dispersed into rivers and then into the ocean, even if the chemicals are used on land. The hydrosphere, the world of water on the surface layer of the earth, which includes seas and rivers, occupies 75% of the earth. Fish belong to the vertebrate group and comprise the largest animal group among the vertebrates. Fish live everywhere in this very large hydrosphere and are influenced by the polluted water. In fish, actually, deformity of the bone has been reported in polluted areas of the sea (Olufsen and Arukwe, 2011; Ju et al., 2012).

In mammals, vertebral bone is an active organ which puts calcium in and out, and has an important role as a mineral store. In many bony fish, however, particularly teleosts, which form the great majority of living fish, their vertebral bone came to be inactive organ to prevent an outflow of calcium. Modern bony fish have developed other methods for regulating calcium and phosphate (Dacke, 1979). Namely, teleosts develop a new regulating system using their scales. It has been reported that teleost scales are more important for storing calcium than are the vertebra, jaw, and otolith, judging from a study of ^{45}Ca-prelabeled scales, vertebrae, jaws, and otoliths of goldfish and killifish (Mugiya and Watabe, 1977). Vertebral bone has an important role in swimming. If the vertebral bone is broken, the fish cannot swim. Most teleosts use their scales as a calcium source. A novel *in vitro* assay system was developed (Suzuki et al., 2000; Suzuki and Hattori, 2002) using goldfish scales, which contain osteoclasts and osteoblasts (Bereiter-Hahn and Zylberberg, 1993; Suzuki et al., 2000; Yoshikubo et al., 2005; Suzuki et al., 2007). In the present study, the influence of environmental chemical pollutants on fish bone metabolism was examined using this assay method.

In the present study, I noted both mercury and PCB as environmental chemical pollutants and examined their influence on

osteoclasts and osteoblasts of teleost scales; I chose these particular chemicals because they are pollutants of global concern due to their widespread occurrence, long-term persistence, strong resistance, long-range transportation, and high bioaccumulation and will disturb biological diversity and the ecosystem.

This thesis is demonstrated through the following 3 experiments. First, the effects of inorganic mercury (InHg) on osteoclasts and osteoblasts of freshwater fish were studied *in vitro*. Mercury has been recognized as an environmental contaminant since the Minamata disaster in the late 1950s. Minamata disease was caused by the consumption of marine fish severely polluted with mercury from local industrial discharge due to the Minamata disaster (Takeuchi et al., 1978; Takeuchi, 1982). The effect of mercury on the central nervous system has been studied widely, revealing that mercury is a neurotoxic material; its poisoning effect is characterized by damage to discrete portions of the brain, such as the visual cortex and the granule layer of the cerebellum (Castoldi et al., 2001). As bioaccumulation of mercury in bone is lower than that in neural tissues (Boyer et al., 1978; Doyle, 1979; Berglund et al., 2000), little attention has been given to bone in this area of research. Recently, Lake et al. (2006) reported that the correlation between the total mercury concentration of the scales and that

of the muscles was high (r = 0.89) and suggested that assessing available mercury in fish scales was suited for predicting the mercury in muscle tissue. In the present study, therefore, we first examined the effect of InHg on the scale osteoclasts and osteoblasts in fresh water teleosts, goldfish (*Carassius auratus*).

Second, evaluation of the effects of InHg and methylmercury (MeHg) on osteoclasts and osteoblasts in the scales of marine teleosts was studied. In the present study, we examined the effect of InHg and MeHg on the scale osteoclasts and osteoblasts using the assay system with the scales of nibbler fish. To confirm the effects of InHg and MeHg on osteoclasts and osteoblasts, the mRNA expressions of osteoclastic markers and osteoblastic markers were partially sequenced and investigated using a quantitative PCR method. In addition, the mRNA expression of metallothionein (MT), which is a metal-binding protein that protects the organism from heavy metal (Hamer, 1986; Klaassen et al., 1999; Jin et al., 2002), was also examined.

Third, the influence of PCB (118) on bone metabolism was examined. In 1968, several thousand people who consumed rice bran oil polluted with PCBs in the northern Kyushu area of Japan experienced various symptoms throughout their bodies involving pigmentation, liver

dysfunction, and numbness of hands and feet. For more than 30 years, PCBs have been detected everywhere of the earth, even though using PCBs was prohibited in most countries by the early 1970s. Because PCBs are fat soluble, they accumulate in body fat (Mori and Todaka, 2011). In humans, changes in bone metabolism associated with exposure to PCBs have also been investigated (Hodgson et al., 2008). However, the direct effects of PCBs on osteoclasts and osteoblasts have not yet been elucidated in any animals. We, therefore, analyzed the effect of PCB (118) on scale osteoclastic and osteoblastic activities, as well as plasma calcium levels, in goldfish scales because PCB (118) is the highest congener as compared with PCB-105, -156, -167, -123, -157, -114, -189, -77, -126, -81, or -169 in fish (Bhavsar et al., 2007).

The present study is the first to demonstrate the direct effect of environmental pollutants such as mercury and PCB on osteoclasts and osteoblasts in teleost fish.

Ⅱ. Effects of inorganic mercury (InHg) on osteoclasts and osteoblasts of the goldfish scales *in vitro*

Introduction

Heavy metals such as mercury, cadmium and copper are known to be extremely toxic to organisms. Mercury has been recognized as an environmental contaminant since the Minamata disaster in the late 1950s. Minamata disease which was caused by the consumption of marine fishes severely polluted with mercury from local industrial discharge due to this Minamata disaster (Takeuchi et al., 1978; Takeuchi, 1982). This extremely adverse situation occurred because of mercury, a highly toxic compound, was severely bio-accumulated (in case of long-finned eels: approx. 1,000,000 times higher than environmental water) by fish (Redmayne et al., 2000).

The effect of mercury on the central nervous system has widely studied and revealed that mercury is a neuro-toxic material, and its poisoning effect is characterized by the damage in discrete portions of the brain, such as the visual cortex and the granule layer of the cerebellum (Castoldi et al., 2001). As bio-accumulation of mercury in bone is lower than that in neural tissues (Boyer et al., 1978; Doyle, 1979; Berglund et al., 2000), much attention has not been given to bone in this area of research.

Recently, Lake et al. (2006) reported that the correlation between

the total mercury concentration of the scales and that of the muscles was high (r = 0.89), and suggested the suitability for prediction of muscle tissue by the assessment of available mercury in the fish scales. It is known that the scales are calcified tissue which contains osteoclasts and osteoblasts (Bereiter-Hahn and Zylberberg, 1993; Suzuki et al., 2000; Yoshikubo et al., 2005; Suzuki et al., 2007) and is reported that the scales are a better potential internal calcium reservoir than the body skeletons, jaws and otolithes, examined by the ^{45}Ca-labelling study for the calcified tissues of goldfish and killifish (Mugiya and Watabe, 1977). In fishes, thus, the scale accumulates mercury and seems to be a sensitive tissue for mercury.

Recently, we have developed a novel in vitro assay system using goldfish scale (Suzuki et al., 2000; Suzuki and Hattori, 2002) because the scale is a very active tissue of calcium regulation in fish described above. In the present study, therefore, we examined the effect of InHg on the scale osteoclasts and osteoblasts. To confirm the effects of InHg on osteoclasts and osteoblasts, the mRNA expressions of osteoclastic markers (tartrate -resistant acid phosphatase: TRAP and cathepsin K) and osteoblastic marker (insulin-like growth factor-1: IGF-1) were investigated using reverse-transcription polymerase chain reaction (RT-PCR). Furthermore, the mRNA expression of metallothionein (MT), which is a

metal-binding-protein that protects the organism from heavy metal (Hamer, 1986; Klaassen et al., 1999), was also examined using RT-PCR.

Materials and Methods

Animals

Our previous study (Suzuki et al., 2000) indicated that the sensitivity for calcemic hormone such as estrogen and calcitonin was higher in mature female than mature male in goldfish (*Carassius auratus*). Therefore, mature female goldfish (n = 12, 35.50 ± 1.30 g) were purchased from commercial source (Higashikawa Fish Farm, Yamatokoriyama, Japan) and used in the scale in vitro assay. All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of Kanazawa University.

Effect of $HgCl_2$ (InHg) on TRAP and ALP activities in the cultured scales of goldfish

A 1% penicillin-streptomycin mixture (ICN Biomedicals Inc., OH, USA) was added to Eagle's minimum essential medium (MEM; ICN Biomedicals Inc.). HEPES (Research Organics Inc., OH, USA) (20 mM) was added into MEM and adjusted to pH 7.0. After filtration, MEM was used in this experiment for analyzing the effect of $HgCl_2$ on TRAP and alkarine phosphatase (ALP) activities in the cultured goldfish scales. Scales

collected from goldfish under anesthesia with ethyl 3-aminobenzoate, methanesulfonic acid salt (MS-222, Sigma-Aldrich, Inc., MO, USA) and incubated for 6 hrs in MEM supplemented with 10^{-8}-10^{-3} M InHg (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and compared with control (InHg-free medium). To evaluate the effect of InHg on osteoclasts and osteoblasts, furthermore, scales were incubated with InHg (10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M) for comparatively longer exposure times, namely 18, 36, and 64 hrs. After incubation, scales were fixed in 10% formalin in a 0.05 M cacodylate buffer (pH 7.4) followed by a storage in a 0.05 M cacodylate buffer at 4°C until analysis.

The measurement methods of TRAP and ALP activities have been described by Suzuki and Hattori (2002). We detected the respective enzyme activity from one scale by transferring each scale into a 96-well-microplate and directly incubating it with the substrate in each well. The procedure of TRAP measurement was as follows. Each scale was transferred to its own well in a 96-well microplate after measurement of the scale weight. An aliquot of 200 µl of 10 mM *para*-nitrophenyl-phosphate and 20 mM tartrate in a 0.1 M sodium acetate buffer (pH 5.3) was added to each well. This plate was then incubated at 20°C for 30 min while being shaken. After incubation, the reaction was stopped by adding 50 µl of 2 N

NaOH. One hundred and fifty µl of a colored solution was transferred to a new plate, and the absorbance was measured at 405 nm. The absorbance was converted into the amount of produced *para*-nitrophenol (pNP) using a standard curve for pNP. The results are shown as means ± SEM of eight scales.

ALP activities were measured using an alkaline buffer (100 mM Tris-HCl, pH 9.5; 1 mM $MgCl_2$; 0.1 mM $ZnCl_2$). Other conditions were the same as those for the measurement of TRAP activity.

Changes of TRAP, cathepsin K, IGF-1, and MT mRNA expression in $HgCl_2$-treated scales for 18 hrs of culture

Scales were collected from goldfish under anesthesia with MS-222. To examine changes in TRAP, cathepsin K, IGF-1, and MT mRNAs that responded to $HgCl_2$, these scales were incubated for 18 hrs in MEM (containing antibiotic and 20 mM HEPES) supplemented with InHg (10^{-4} M) and compared with the control (without metals). We previously reported that IGF-1 mRNA expression decreased at 18 hrs of incubation (Suzuki and Hattori, 2003). Therefore, this incubation period was adopted. After incubation, the scales were frozen at -80 °C for mRNA analysis.

Total RNAs were prepared from the goldfish scales using a total

RNA isolation kit (Nippon Gene, Tokyo, Japan). RT-PCR was performed using Oligotex-dT 30 Super (Takara Bio Inc., Otsu, Japan) as an oligo dT primer to prevent genomic DNA contamination (Suzuki et al., 1997). The gene-specific primers (TRAP 5': AACTTCCGCATTCCTCGAACAG; TRAP 3': GGCCAGCCACCAGGAGATAA; cathepsin K 5': GCTATGGAGCCACACCAAAAGG; cathepsin K 3': CTGCGCTTCCAGCTCTCACAT) reported by Azuma et al. (2007) were used. IGF-1 and MT cDNAs were also amplified using gene specific primers (IGF-1 5': GGAGACGCTGTGCGGG; IGF-1 3': CCTCAGCTCACAGCTCTG; MT 5': ATGGATCCGTGCGAATGC; MT 3': CTCCTCATTGACAGCAGCT). These were designed from the nucleotide sequences of respective cDNA (IGF-1: Kermouni et al., 1998; MT: Chan, 1994). β-actin cDNA using a primer set (5':CACTGTGCCCATCTACGAG; 3': CCATCTCCTGCTCGAAGTC) (Chan et al., 1998) were also amplified. The conditions for PCR amplification were denaturation for 0.5 min at 96°C, annealing for 1 min at 55°C, and extension for 2 min at 72°C, followed by a single cycle at 72°C for 30 min. The cycle numbers for the amplification in TRAP, cathepsin K, IGF-1, MT, and β-actin cDNAs were determined by ensuring that PCR amplification was at submaximum and the intensity of the band

corresponded exactly to the amount of starting material. The PCR products were analyzed on a 2.5% NuSive GTG agarose gel (FMC BioProducts, ME, USA) and stained with ethidium bromide. The band densities were estimated using a computer program (NIH Image J). The mRNA levels of TRAP, cathepsin K, IGF-1 and MT were normalized to the mRNA level of β-actin.

Statistical analysis

The statistical significance was assessed by one-way ANOVA followed by Dunnett test. The significance level chosen was as $p < 0.05$.

Results

Effect of InHg on TRAP activity in the cultured scales of goldfish

InHg was significantly decreased the TRAP activities of the scales by 6 hrs of incubation ($p < 0.01$ for 10^{-5} M; $p < 0.001$ for 10^{-4} and 10^{-3} M) (Fig. 1). Thus, increased doses of InHg resulted in greater effects on decreasing TRAP activities dose-dependently.

By the long incubation time period (18 to 64 hrs), only at 10^{-4} M, significant difference ($p < 0.01$) between InHg-treated scales and control scales was obtained by 18 hrs of incubation (Fig. 2).

Effect of InHg on ALP activity in the cultured scales of goldfish

The ALP activity of the control scales by 6 hrs of incubation was 5.33 ± 0.41 (nmol produced pNP /mg scale /hr) which did not show any difference with InHg-treated groups (10^{-8} to 10^{-3} M) (Fig. 3). Thus, the ALP activity did not change during 6 hrs of incubation with InHg compared to the control.

However, the ALP activity in the InHg-treated scales decreased significantly by 36 hrs ($p < 0.05$ for 10^{-6} M, 10^{-5} M or 10^{-4} M) and 64 hrs ($p < 0.05$ for 10^{-6} M; $p < 0.01$ for 10^{-5} M and $p < 0.001$ for 10^{-4} M) of

incubation from the values of the control scales although it did not change at 18 hrs of incubation (Fig. 4).

Changes of TRAP, cathepsin K, IGF-1, and MT mRNA expression in InHg-treated scales

After 18 hrs of incubation, the mRNA expressions of TRAP, cathepsin K and IGF-1 in InHg-treated scales were significantly ($p < 0.001$ for TRAP; $p < 0.001$ for cathepsin K and $p < 0.001$ for IGF-1) lower than those in the control scales (Fig. 5). Conversely, the mRNA expression was significantly ($P<0.001$) increased for MT when treated with $HgCl_2$ (Fig. 5).

Discussion

The present study demonstrated that fish scale sensitively responded to InHg. A high co-relation of mercury between scales and muscles was reported in largemouth bass (Lake et al., 2006). This indicates that accumulation of mercury is occurred in the fish scale although mercury did not accumulate in the vertebral bone of fish (Camusso et al., 1995). It is also well-known that the scale is a more active tissue in fish calcium regulation than vertebral bone (Mugiya and Watabe, 1977; Yamada, 1961; Berg, 1968; Bereiter-Hahn and Zylberberg, 1993). Therefore, we strongly believe that the fish scale is capable to accumulate mercury and respond to mercury similarly like calcium.

In mammals, the influence of mercury on bone metabolism has been studied only by *in vivo* experiments and investigated in bone formation or osteoblastic activity (Yonaga et al., 1985; Jin et al., 2002). Mercury inhibited the growth of tibia in rats (Yonaga et al., 1985) and decreased serum levels of osteoblastic markers (ALP and osteocalcin) (Jin et al., 2002). In our knowledge, our study is the first to indicate direct effect of InHg on osteoclasts. The inhibitory action of InHg on osteoclasts after 6 hrs incubation was stronger than that of 18 to 64 hrs incubation. As for

organic mercury, similar results were obtained in our scale assay system (Suzuki et al., 2004b). Furthermore, we recently succeed to clone osteoclastic markers: TRAP and cathepsin K in fish for the first time (Azuma et al., 2007) and examined mRNA expressions of these markers in the $HgCl_2$-treated scales. In the present study, we confirmed that the both mRNA expressions of TRAP and cathepsin K decreased as TRAP enzyme activity did.

It was found that the mRNA expression of MT in InHg-treated scales increased in the present study. This result is similar to that in mammals because it has been demonstrated that MT plays a protective role in mercury-induced toxicity in bone (Jin et al., 2002). Fish are aquatic animals with scales that are always exposed to environmental water. In an in vitro experiment for 6 and 18 hrs of incubation, therefore, osteoblasts may be resistant to mercury as a result of MT production. On the other hand, IGF-1 mRNA expression decreased compared to the control. As IGF-1 participates in osteoblastic growth and differentiation, we speculated that mercury has toxic effect on osteoblasts under long-term exposure.

We previously demonstrated that the osteogenesis of regenerating scale is very similar to that of mammalian membrane bone and a good model of osteogenesis (Yoshikubo et al., 2005). Using this system,

furthermore, we first demonstrated that calcitonin, a hypocalcemic hormone, suppressed osteoclastic activity in teleosts as well as in mammals (Suzuki et al., 2000) and that melatonin, a major hormone secreted from the pineal gland, suppressed the functions in both osteoclasts and osteoblasts (Suzuki and Hattori, 2002). Osteoblasts in the scale responded to estrogen as they do in mammals (Yoshikubo et al., 2005). In addition, the effects of endocrine disrupters, such as bisphenol-A (Suzuki and Hattori, 2003) and tributyltin (Suzuki et al., 2006), and heavy metals, i.e., cadmium and organic mercury (Suzuki et al., 2004b), on osteoblasts and osteoclasts have been examined. Moreover, we indicated that cadmium (even at 10^{-13} M) responded to TRAP activity in the scale (Suzuki et al., 2004b). Considering these results together with present data, our scale assay system will be useful for analysis of environmental contaminant on bone metabolism.

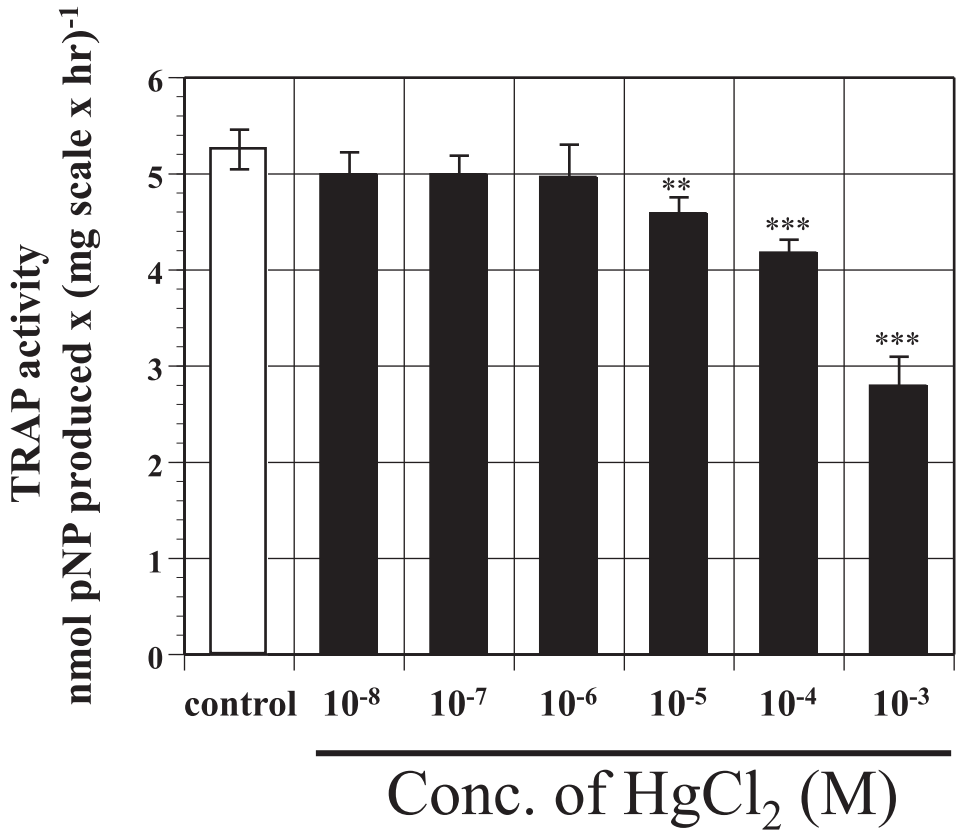

Fig. 1. Effect of $HgCl_2$ (10^{-8} to 10^{-3} M) on TRAP activity in the cultured scales incubated for 6 hrs. **, *** indicate statistically significant differences at $p < 0.01$ and $p < 0.001$, respectively, from the value in the control scales.

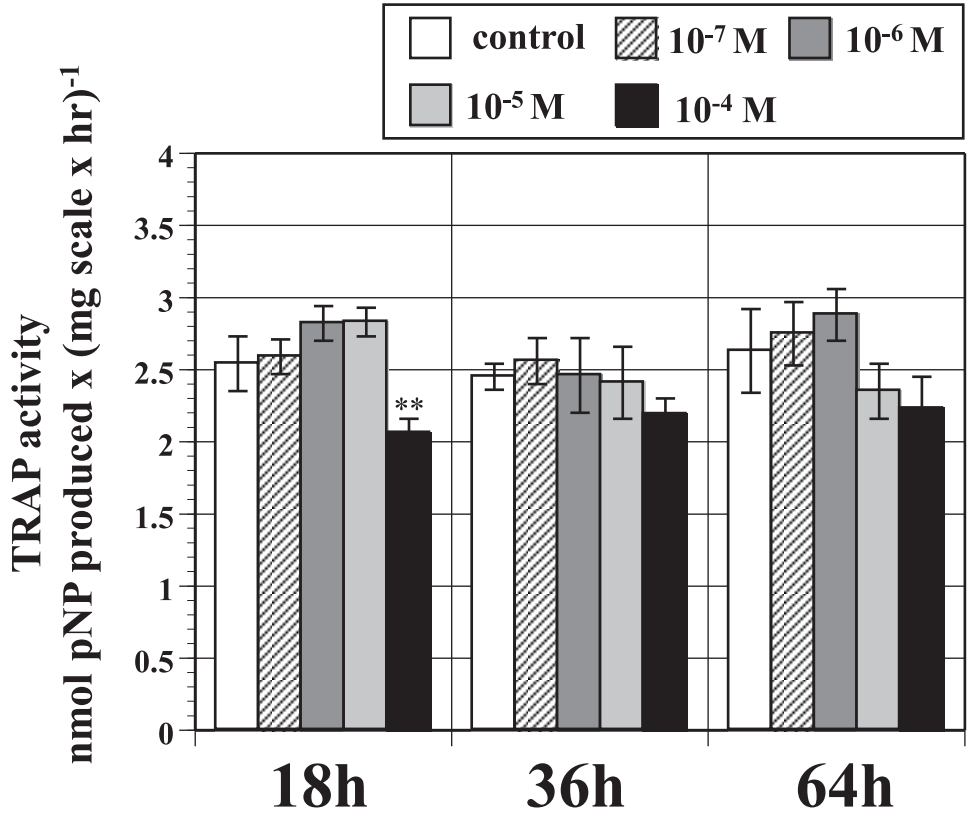

Fig. 2. Effect of $HgCl_2$ (10^{-7} to 10^{-4} M) on TRAP activity in the cultured scales incubated for 18, 36, and 64 hrs. ** indicates statistically significant difference at $p < 0.01$ from the values in the control scales.

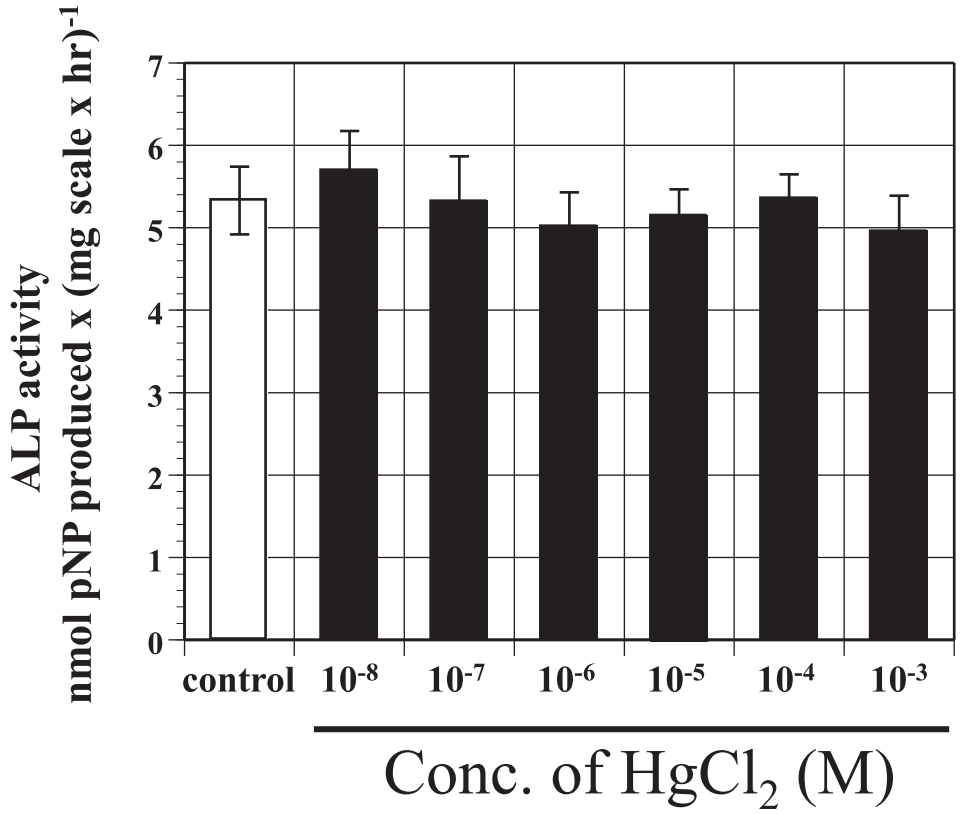

Fig. 3. Effect of $HgCl_2$ (10^{-8} to 10^{-3} M) on ALP activity in the cultured scales incubated for 6 hrs. There was no significant difference between $HgCl_2$-treated scales and control scales.

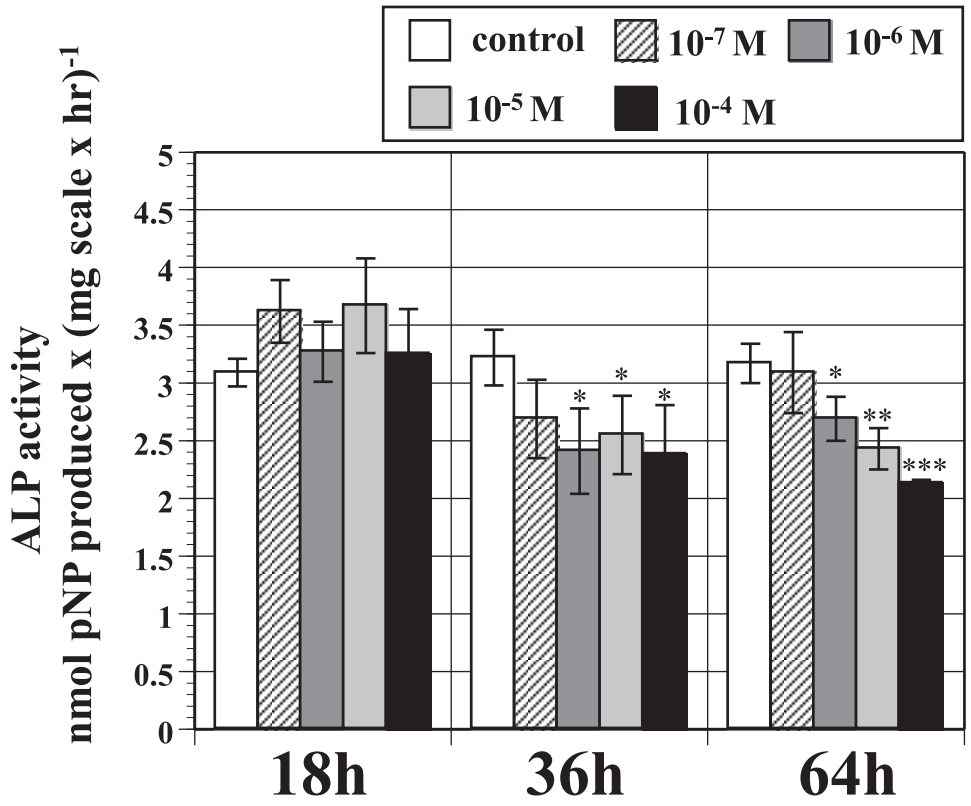

Fig. 4. Effect of $HgCl_2$ (10^{-7} to 10^{-4} M) on ALP activity in the cultured scales incubated for 18, 36, and 64 hrs. *, **, *** indicate statistically significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, from the values in the control scales.

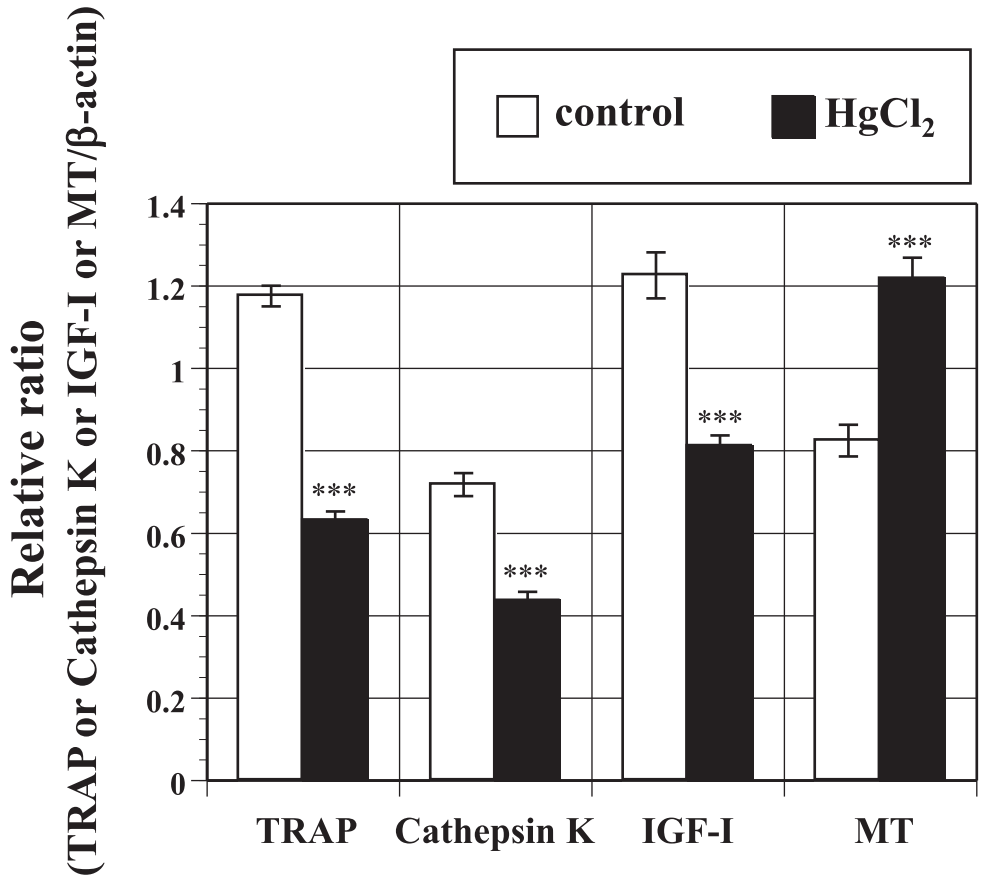

Fig. 5. Changes in the mRNA expression of TRAP, cathepsin K, IGF-1, and MT in $HgCl_2$ (10^{-4} M)-treated scales of goldfish incubated for 18 hrs of culture. *** indicates statistically significant difference at $p < 0.001$ from the values in the control scales.

Ⅲ. Effects of InHg and MeHg on osteoclasts and osteoblasts in the scales of marine teleosts as a model system of the bone

Introduction

Mercury is widely distributed in aquatic environments. Elemental mercury (Hg^0) appears to be emitted from fumaroles due to volcanic activity in the bottom of the sea (Sakamoto et al., 1995; Selin, 2009). Hg^0 has been shown to be oxidized to InHg in aquatic environments, and particularly easily in seawater (Yamamoto, 1996). InHg is convertible to MeHg in natural environments, and most of the mercury in fish tissue was reported to be MeHg (WHO, 2008; WHO, 2010; Depew et al., 2012). The toxicokinetics (absorption, distribution, metabolism, and excretion) of mercury are highly dependent on the form of mercury (WHO, 2008; WHO, 2010). MeHg is a highly toxic compound that bioaccumulates significantly in fish. In long-finned eels, MeHg was approximately 1,000,000 times higher than in environmental water (Redmayne et al., 2000). Humans are mainly exposed to MeHg through consumption of fish with bioaccumulations of mercury (WHO, 2008). In humans, the main target organs of MeHg are brain, kidney, and liver (WHO, 2008). Many studies have thus focused on these tissues. Little attention, however, has been paid to bone metabolism in this area of research, as mercury bioaccumulation in bone was shown to be extremely low compared to that in neural tissues

(Boyer et al., 1978; Doyle, 1979; Berglund et al., 2000).

As mentioned above, muscle in fish is the main edible part, and it accumulates MeHg (Depew et al., 2012). In largemouth bass, the total mercury concentration in muscle was highly correlated with that of scales (r = 0.89) (Lake et al., 2006), indicating the importance of examining the interaction between mercury exposure and bone metabolism in fish. Additionally, Lake et al. suggested that assessment of mercury in fish scales may be suitable for prediction of mercury levels in muscle tissue. Mercury accumulated in the scales of fish (herring and sockeye salmon) was also detected by a combination of laser ablation with relatively low-cost cold vapor atomic fluorescence spectrophotometry (Beaudin et al., 2010). Fish scales may thus be another mercury-sensitive organ.

Considering the above, we developed the assay system with nibbler fish to examine the effects mercury on bone metabolism in marine teleosts. First, we morphologically examined the colocalization of osteoclasts and osteoblasts in the scales of nibbler fish. We then determined the location of scales with similar cell activities to enhance accuracy and reproducibility, and developed an assay system, which we used to examine the effects of InHg and MeHg on scale osteoclasts and osteoblasts. To confirm the effects of InHg and MeHg on osteoclasts and osteoblasts, the

mRNA expressions of an osteoclast marker (tartrate-resistant acid phosphatase: TRAP) and an osteoblastic marker (collagen type I, α1: COL1A1) were partially sequenced and investigated using a quantitative PCR method. In addition, the mRNA expression of metallothionein (MT), which is a metal-binding protein that protects the organism from heavy metal (Hamer, 1986; Klaassen et al., 1999; Jin et al., 2002), was also examined.

In marine teleosts, this is the first study to indicate that both InHg and MeHg suppress marker enzyme activities of osteoclasts and osteoblasts in scales as a model system of bone.

Materials and Methods

Animals

Nibbler fish (*Girella punctata*) (both sexes, n = 80, 64.3 ± 1.3 g) were captured by fishing in Tsukumo Bay of the Noto Peninsula (Ishikawa Prefecture). After acclimation for around two weeks, these fish were used in the present experiments. All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of Kanazawa University.

Morphological study of osteoclasts and osteoblasts in the scales of nibbler fish

Scales were collected from nibbler fish anesthetized with 0.04% of a 2-phenoxyethanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan) solution and fixed using a 4% paraformaldehyde solution neutralized with a phosphate buffer solution (pH 7.2; Wako) for 5 min. Subsequently, osteoclasts were observed by TRAP staining using the methods of Cole and Walters (1987). Osteoblasts were detected by alkaline phosphatase (ALP) staining using a kit (NBT / BCIP Stock Solution, Roche Applied Science, Mannheim, Germany). After staining, the osteoclasts and

osteoblasts were observed under a microscope.

Development of an in vitro assay system with the scales of nibbler fish

Nibbler fish were anesthetized with a 0.04% 2-phenoxyethanol (Wako) solution, and body scales were then removed. The location of the scales used in the present study is indicated in Fig. 6. In nibbler fish, we chose a line of scales of approximately the same size. In each line, the removed scales were put into a 96-well microplate in turn, and divided into a control and an experimental group (See Fig. 6). To examine the variance in osteoclastic and osteoblastic activity in the two groups of each line, we measured the activity of osteoclasts and osteoblasts with TRAP and ALP as the respective markers, as these markers have been shown to be affected by a number of hormones and other factors in osteoclasts and osteoblasts in mammals (Vaes, 1988; Dimai et al., 1998; Suda et al., 1999).

The methods for measuring TRAP and ALP activities were as follows. An aliquot of 100 µl of an acid buffer (0.1 M sodium acetate, including 20 mM tartrate, pH 5.3) or an alkaline buffer (100 mM Tris-HCl, pH 9.5; 1 mM $MgCl_2$) was added to each well. Then, the scales were put into their own well in a 96-well microplate. This microplate was frozen at -80°C immediately and then kept at -20°C until analysis. An aliquot of 100

μl of 20 mM para-nitrophenyl phosphate in an acid or alkaline buffer was then added to each well of a melted solution in the microplate. This plate was incubated at 23°C for 60 min while being shaken. After incubation, the reaction was stopped by adding 50 μl of 3 N NaOH. One-hundred-fifty μl of a colored solution was transferred to a new plate, and the absorbance was measured at 405 nm. The absorbance was converted into the amount of produced para-nitrophenol (pNP) using a standard curve for pNP.

After measuring the activity of both TRAP and ALP , the size of the scales was measured with Image J. Afterward, TRAP and ALP activities were normalized to the surface area (mm^2) of each scale (Suzuki et al., 2009).

Effects of InHg and MeHg on TRAP and ALP activities in the cultured scales of nibbler fish

Scales collected from nibbler fish under anesthesia with a 0.04% 2-phenoxyethanol (Wako) solution were incubated for 6 hrs in L-15 medium (Life Technologies Corporation, Carlsbad, CA USA) with added InHg and MeHg (each 10^{-6} to 10^{-4} M; Wako) and compared with a mercury-free medium as a control. After incubation, TRAP and ALP activities were measured using the same method described above.

Furthermore, to evaluate the influence of InHg and MeHg on osteoblasts with longer exposure times, scales were incubated in mercury for 18 and 36 hrs.

Sequencing of TRAP, COL1A1, MT, and elongation factor 1α

Scales were collected from nibbler fish under anesthesia with a 0.04% 2-phenoxyethanol (Wako) solution. Total RNAs were prepared from scales of nibbler fish using a total RNA isolation kit (NucleoSpin RNA II, Takara Bio Inc., Otsu, Japan). Complementary DNA was synthesized using the PrimeScriptTM RT reagent kit (Takara Bio Inc.). Using cDNA from the scales of nibbler fish, partial fragments of TRAP and COL1A1 were amplified by degenerate PCR method. The degenerated primer sets for TRAP (sense: GCNGGNAAYCAYGAYCA; antisense 1: TCIADRAARTTICCNGCNC; antisense 2: ARRTTRTGRTCRTGICC RCA) and for COL1A1 (sense: TGYCAYCCNGAYTGGAA; antisense: YTCDATYTCRTTISWNCC) were designed from multiple amino acid sequence alignments of each vertebrate family member. To amplify the TRAP cDNA fragment, particularly, nested PCR was done.

The sequence of the open reading frame in MT was determined by use of the 3' RACE System for Rapid Amplification of cDNA Ends

(Life Technologies Corporation). The gene-specific primer for MT (ATGGATCCNTGCGAA) was also designed by the multiple amino acid sequence alignment of MT vertebrate family members. These sequences were analyzed by a direct sequence method using an ABI PRISM 3130xl Genetic Analyzer.

To normalize each mRNA expression, elongation factor 1α (EF-1α) was also cloned partially. The primers (sense: CCATGT CGACTCTGGGAAAT; antisense: TTCAGGAACTTGGGGTTGTC) were designed from the sequence of lamprey EF-1α (AB183717). Then, we also determined the partial sequence of EF-1α by direct sequencing.

Changes in TRAP, COL1A1, and MT mRNA expression of the scales by InHg and MeHg treatment

Scales were collected from nibbler fish under anesthesia with a 0.04% 2-phenoxyethanol (Wako) solution. To examine changes in TRAP, COL1A1, and MT mRNAs that responded to InHg or MeHg, these scales were incubated for 6 hrs in L-15 medium (Life Technologies Corporation) with added InHg (10^{-5} M) or MeHg (10^{-5} M) and compared with the control (without mercury). After incubation, the scales were frozen at -80°C for mRNA analysis.

Total RNAs were prepared from scales of nibbler fish, and cDNA was synthesized using a kit as described above. The PCR primer sequences—sense: TGGATGAGAAGCCCAGAGG; anti-sense: CCG CAGAGGTAAGCAGTGG—were designed from TRAP (AB874604). The primer sets for COL1A1 (AB874603) (sense: 5′-GTGAGGTCGCCAA GAAGAAC-3′; antisense: 5′-ATGAGACGCAGGAAGGTCAG-3′) and for MT (AB874602) (sense: 5′-TCAAAGAGTGGAAAATGCAACTG-3′; antisense: 5′-GATGGGCAGCATGAGCAG-3’) were used. EF-1α (AB874605) was amplified using the following primers: sense: 5′-GTATGGTCGTCACCTTTGCTC-3′; antisense: 5′- GTGGGTCGTTCTTG CTGTC-3′. The PCR amplification was performed using the real-time Mx3000p PCR apparatus (Agilent Technologies, Santa Clara, CA, US) (Suzuki et al., 2011a). The annealing temperature of TRAP, COL1A1, MT, and EF-1α was 60°C. The initial reaction condition was 10 s at 95°C, followed by 40 cycles of denaturation at 95°C for 10 s and annealing/extension at 60°C for 40 s. The TRAP, COL1A1, and MT mRNA levels were normalized to the EF-1α mRNA level.

Statistical analysis

Comparison between group 1 and 2 (each 8 scales) (See Fig. 6) was assessed by Student's *t*-test. The mean value from eight individual scales of one nibbler fish in the control group was compared with that in the experimental group. The data were assessed using the paired *t*-test, and the significance level chosen was $p < 0.05$. All results are expressed as means $\pm$ SEM (n = 6 or 8).

Results

Osteoclasts and osteoblasts in the scales of nibbler fish

We detected osteoclasts in the scales of nibbler fish. Arrows indicate multinucleated osteoclasts (Fig. 7B) in the grooves (asterisks in Figs. 7A, B) of the scales. ALP-stained osteoblasts (arrows in Fig. 7D) were detected around the grooves (asterisks in Figs. 7C, D). Osteoclastic and osteoblastic cells were colocalized in the grooves of the scales. In addition, ALP-stained osteoblasts (arrows in Fig. 8B) were detected around the ridges (arrowheads in Fig. 8B).

Comparison of TRAP and ALP activities among the alternate scales in each line

In each line, TRAP activity was not changed significantly among alternative scales (Fig. 9A). In the case of ALP activity as well as, there was no significant difference between the respective value of group 1 and group 2 (Fig. 9B).

Effects of InHg and MeHg on TRAP activity in the cultured scales of nibbler fish

The results are shown in Fig. 10. TRAP activity significantly decreased by InHg (10^{-5} and 10^{-4} M, $p < 0.05$) treatment at 6 hrs (Fig. 10A). Also, MeHg was significantly decreased in the TRAP activities of the scales at 6 hrs (10^{-6} and 10^{-5} M: $p < 0.05$, 10^{-4} M: $p < 0.01$) (Fig. 10B).

Effects of InHg and MeHg on ALP activity in the cultured scales of nibbler fish

During 6 hrs of incubation, ALP activity did not change from the values in the control group (Fig. 11A). However, ALP activity in the InHg-treated scales decreased significantly at 18 (10^{-5} and 10^{-4} M: $p < 0.05$) and 36 (10^{-5} M: $p < 0.05$; 10^{-4} M: $p < 0.01$) hrs of incubation from the value of the control scales (Figs. 11B and 11C).

Similar changes were induced by MeHg. ALP activity did not change at 6 hrs (Fig. 12A). In MeHg as well as InHg, ALP activity in the treated scales decreased significantly at 18 hrs (10^{-6} M, $p < 0.05$; 10^{-5} and 10^{-4} M, $p < 0.01$) and 36 hrs (10^{-6} M, $p < 0.05$; 10^{-5} M, $p < 0.01$; 10^{-4} M, $p < 0.001$) of incubation from the values of the control scales (Figs. 12B, C).

Sequencing of TRAP, COL1A1, MT, and EF-1α

The sequences of TRAP, COL1A1, and MT are shown in Fig. 13.

We determined the sequences of TRAP, COL1A1, and MT from the scales of nibbler fish. Deduced amino acid sequences of TRAP and MT showed high identity to salmon (84.6%) in TRAP and to goldfish (75.0%) in MT. In TRAP and MT, there was a relatively high sequence identity to other vertebrate counterparts (TRAP: 57.7% to human and 60.0% to mouse; MT: 65.6% to human and 69.9% to rat). However, the sequence of COL1A1 was well-conserved among vertebrates, at least in the obtained sequence. Sequence identity of nibbler fish COA1A1 to goldfish, human, and mouse COL1A1 was 88.7, 82.7, and 84.2%, respectively.

Changes in TRAP, COL1A1, and MT mRNA expression of the scales by InHg and MeHg treatment

The relative ratio of amplified TRAP, COL1A1, and MT cDNAs to EF-1α cDNA is shown in Fig. 14. After 6 hrs of incubation, the expression of TRAP mRNA in both InHg- and MeHg-treated scales was lower than that in the control scales (InHg: $p < 0.05$; MeHg: $p < 0.01$), although COL1A1 mRNA did not change significantly. Conversely, MT mRNA expression was increased by both InHg ($p < 0.05$) and MeHg treatment ($p < 0.01$).

Discussion

In this study, we found that the enzyme activity of TRAP (osteoclastic marker) was decreased at 6hrs following treatment by both InHg and MeHg. In osteoblasts, also, both InHg and MeHg inhibited the ALP enzyme activity at 18 and 36 hrs after incubation. It is known that the scales are calcified tissue that contains osteoclasts and osteoblasts (Bereiter-Hahn and Zylberberg, 1993; Yoshikubo et al., 2005; Suzuki et al., 2007; Suzuki et al., 2008; de Vrieze et al., 2010; Suzuki et al., 2011a; Thamamongood et al., 2012, Yano et al., 2013). Additionally, it has been reported that scales are a better potential internal calcium reservoir than body skeletons, jaws, and otolithes examined by the ^{45}Ca-labeling study of calcified tissues of goldfish and killifish (Mugiya and Watabe, 1977). In fish, thus, scales accumulate mercury and sensitively respond to mercury exposure in a short time.

In nibbler fish (a marine teleost), we have shown that both InHg and MeHg directly affect osteoclasts by both analyses of TRAP enzyme activity and TRAP mRNA expression in the present study. In goldfish (freshwater teleost), we previously reported that InHg (10^{-5} to 10^{-3} M) (Suzuki et al., 2011b) and MeHg (10^{-8} to 10^{-6} M) (Suzuki et al., 2004)

significantly suppressed TRAP enzyme activity. Particularly, the response of MeHg in nibbler fish is lower than that in goldfish. It is known that the exchange of calcium in the scales of freshwater teleost is faster than that in marine teleost because freshwater teleosts live in a low-calcium environment. In fact, the response of the calcemic hormone estrogen in the scale osteoclasts of freshwater teleosts was higher than that in those of marine teleosts (Persson et al., 1994; Persson et al., 1995; Guerreiro et al., 2002). We believe that osteoclasts in freshwater teleosts have some important roles in calcium metabolism as compared with those in marine teleosts.

In the case of osteoblasts, the ALP enzyme activity did not change from the values in the control group during 6 hrs of incubation with either InHg or MeHg. However, ALP activity in the InHg (10^{-5} and 10^{-4} M)- and MeHg (10^{-6} to 10^{-4} M)-treated scales decreased significantly at 18 and 36 hrs of incubation from the value of the control scales. From each marker mRNA expression analysis, at 6 hrs of incubation, the expression of TRAP mRNA in InHg- and MeHg-treated scales was significantly lower than that in the control scales, although the mRNA expression of COL1A1 (osteoblastic marker) did not change significantly. Conversely, MT mRNA expression significantly increased with both InHg and MeHg treatment.

This result is similar to those from studies in mammals, as it has been demonstrated that MT plays a protective role in mercury-induced toxicity in bone (Jin et al., 2002). As it has been reported that osteoblasts express MT and protect from heavy metal (Angle et al., 1990; Nagata and Lönnerdal, 2011), the activation of MT in osteoblasts may be involved in resistance to mercury.

In mammals, the influence of mercury on bone metabolism has been studied mainly by in vivo experiments and investigated in bone formation or osteoblastic activity (Yonaga et al., 1985; Jin et al., 2002). Both MeHg and InHg inhibited the growth of tibia in rats (Yonaga et al., 1985), and InHg decreased the serum levels of osteoblastic markers (ALP and osteocalcin) in rats (Jin et al., 2002). Mammalian bone has resorptive cells (osteoclasts) and formative cells (osteoblasts). These cells act on the regulation of calcium homeostasis in mammals as well as in fish. Simultaneous incubation of osteoclasts and osteoblasts is therefore necessary for the evaluation of the effects of mercury on bone metabolism. However, a co-culture of osteoclasts and osteoblasts is not particularly easy to obtain because of the difficulty in handling osteoclasts. Pre-osteoclasts are triggered by the receptor activator NF-κB—the receptor activator of the NF-κB ligand binding of osteoclasts and osteoblasts followed by

differentiation, activation, and multinucleation (Suda et al., 1999; Teitelbaum, 2000; Hofbauer et al., 2004). In the present study, therefore, we used scales from a marine teleost to develop an *in vitro* assay system. This system can be used to simultaneously detect both osteoclastic and osteoblastic activities with TRAP and ALP enzyme activities. Furthermore, we determined TRAP, COL1A1, and MT cDNAs from the scales of nibbler fish. Thus, we conclude that the in vitro assay system we developed may be a useful means for analyzing heavy metal in bone metabolism. In the future, we will further examine different toxic mechanisms between MeHg and InHg using fish scales as a model of bone.

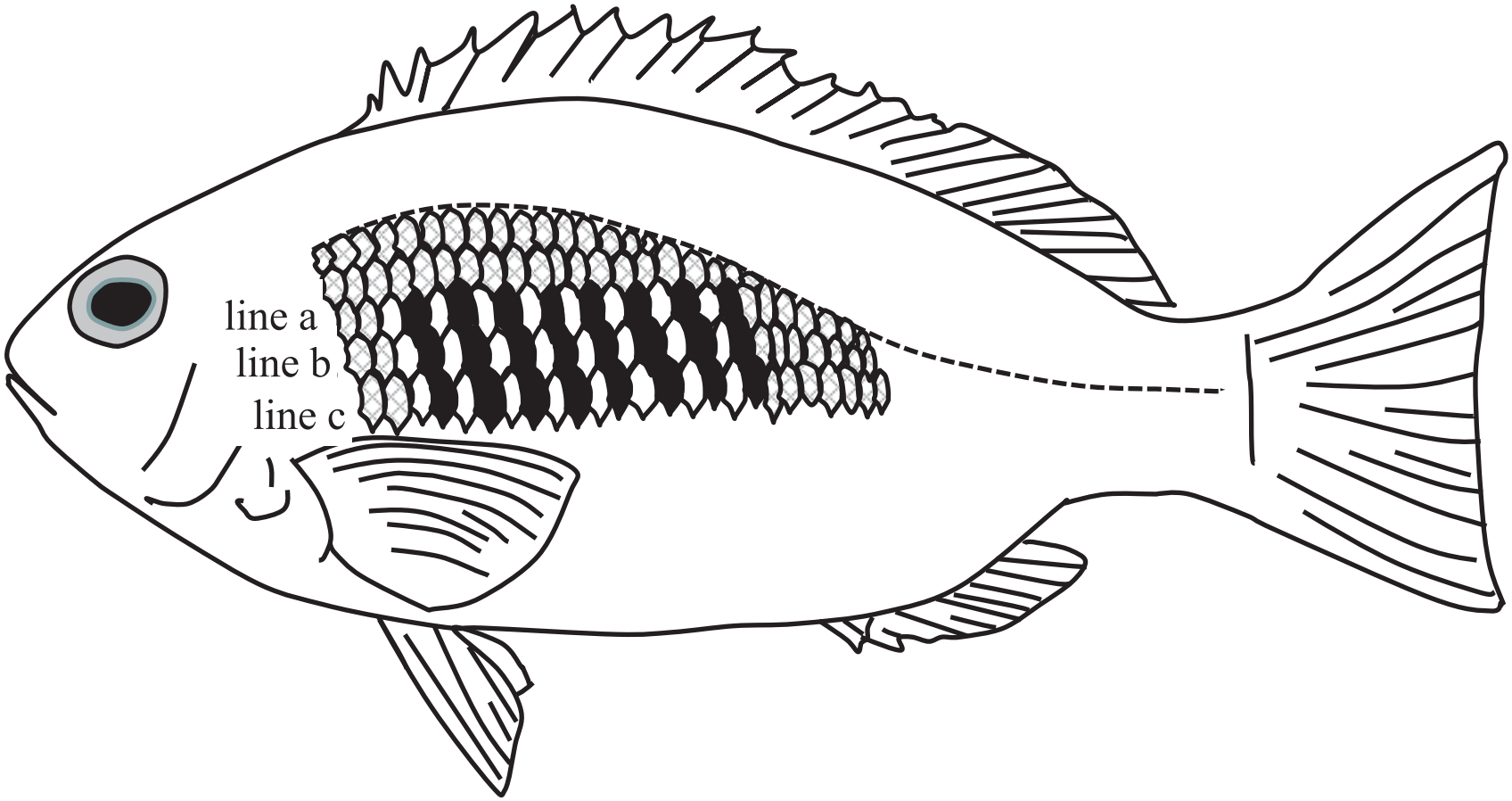

Fig. 6 Location of the scales used in the present study. In nibbler fish, we chose the line of scales of approximately the same size. In each line, the removed scales were separated in two group, a control group and an experimental group. The scales of group 1 are shown in white; the other scales (group 2) are indicated in black.

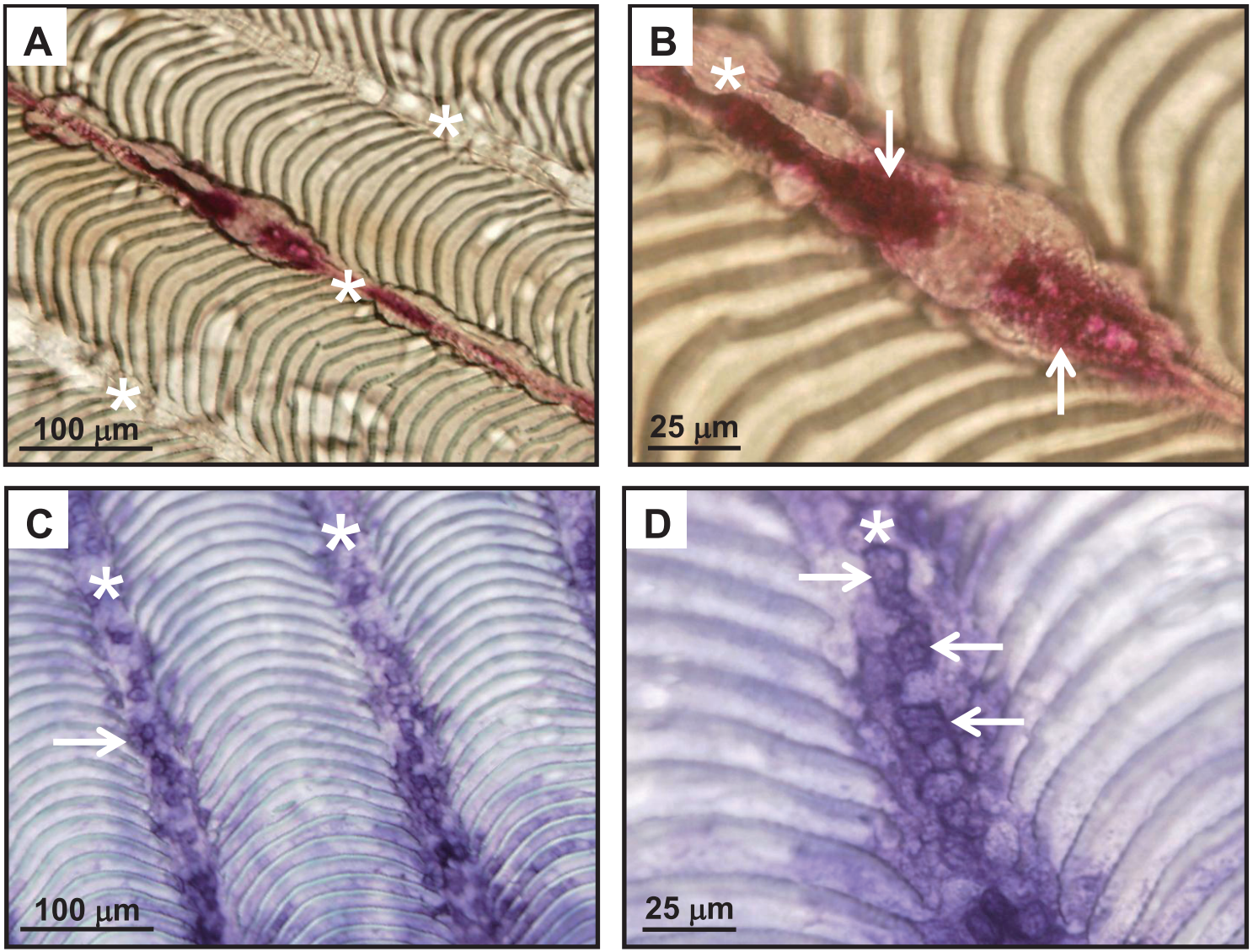

Fig. 7 Microscopic views of nibbler fish scales stained for osteoclasts (A, B) and osteoblasts (C, D). Panels (B) and (D) are enlarged view of (A) and (C), respectively. Arrows indicate multinucleated osteoclasts (B) and osteoblasts (D). Asterisks show grooves of the scales in nibbler fish.

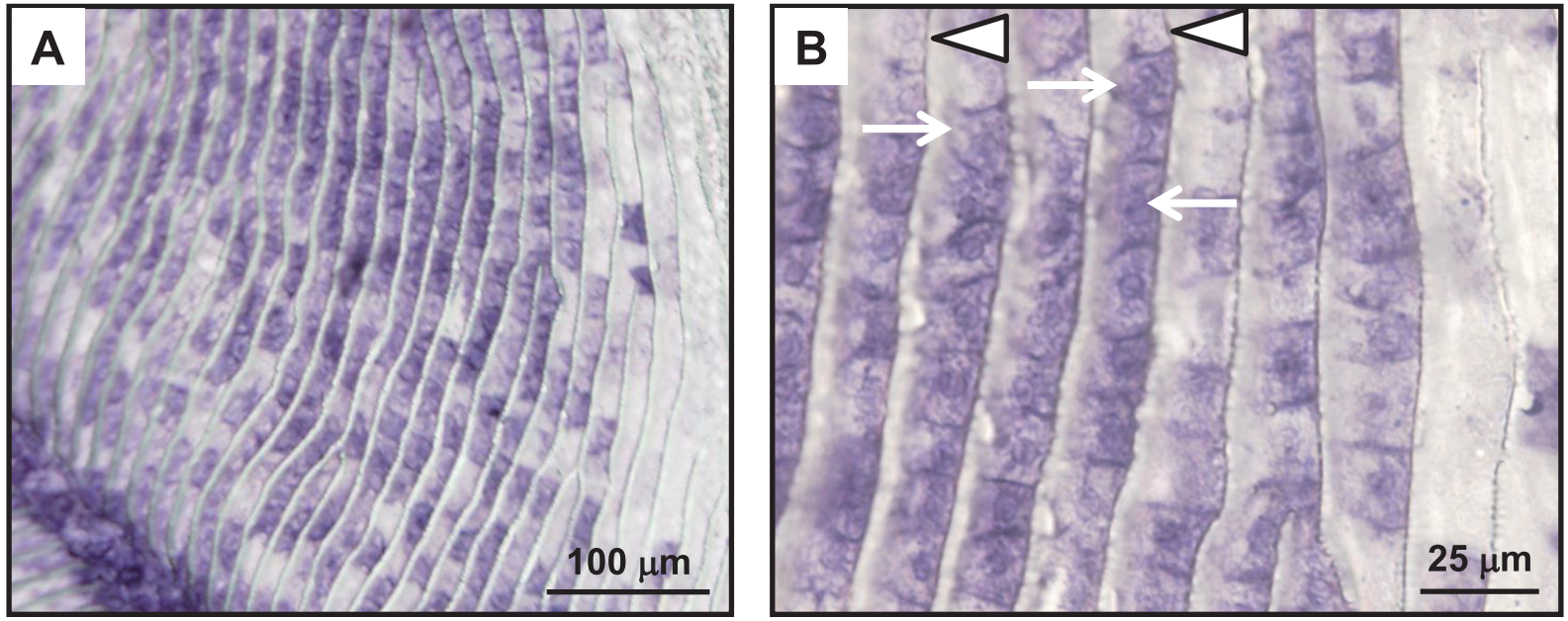

Fig. 8 Microscopic views of nibbler fish scales stained for osteoblasts. Panel (B) is an enlarged view of (A). Arrows indicate osteoblasts. Arrowheads show ridges of the scales in nibbler fish.

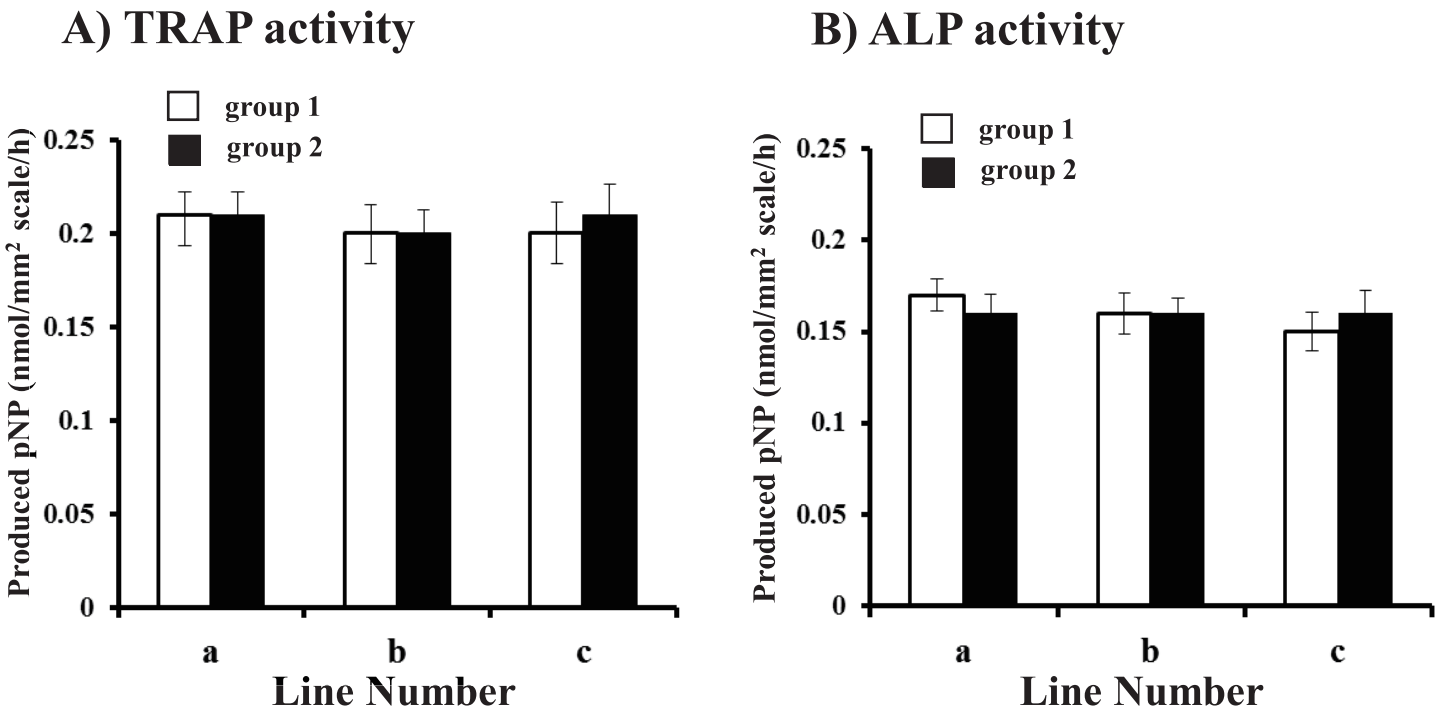

Fig. 9 TRAP (A) and ALP (B) activities of the scales in two groups in each line. There was no significant difference in TRAP and ALP activity values scales between group 1 and group 2 (n = 8 scales).

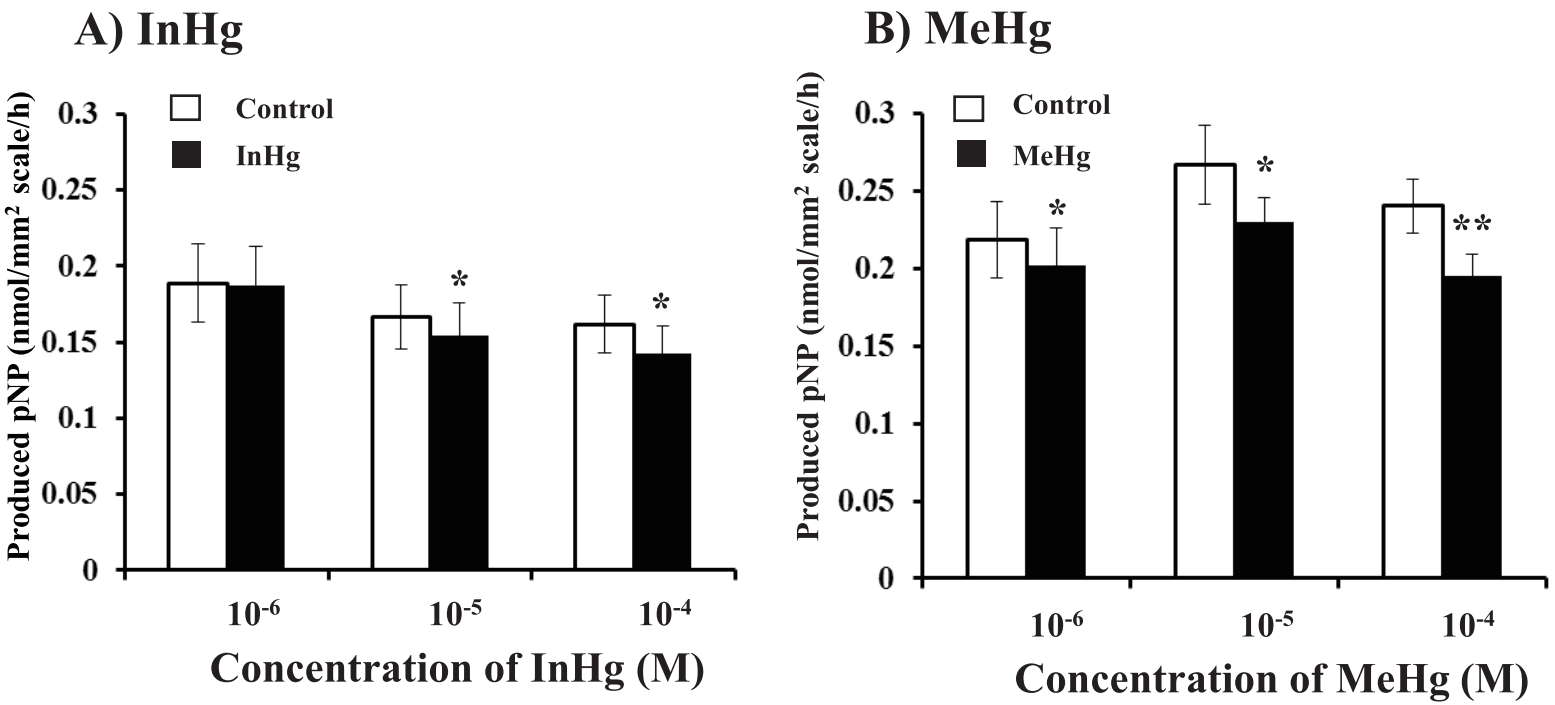

Fig. 10 Effect of InHg (10^{-6} to 10^{-4} M) (A) and MeHg (10^{-6} to 10^{-4} M) (B) on TRAP activity in the cultured scales at 6 hrs. * and ** indicate statistically significant difference at $p < 0.05$, $p < 0.01$, respectively, from the values of the control scales (n = 6 samples; one sample from one fish).

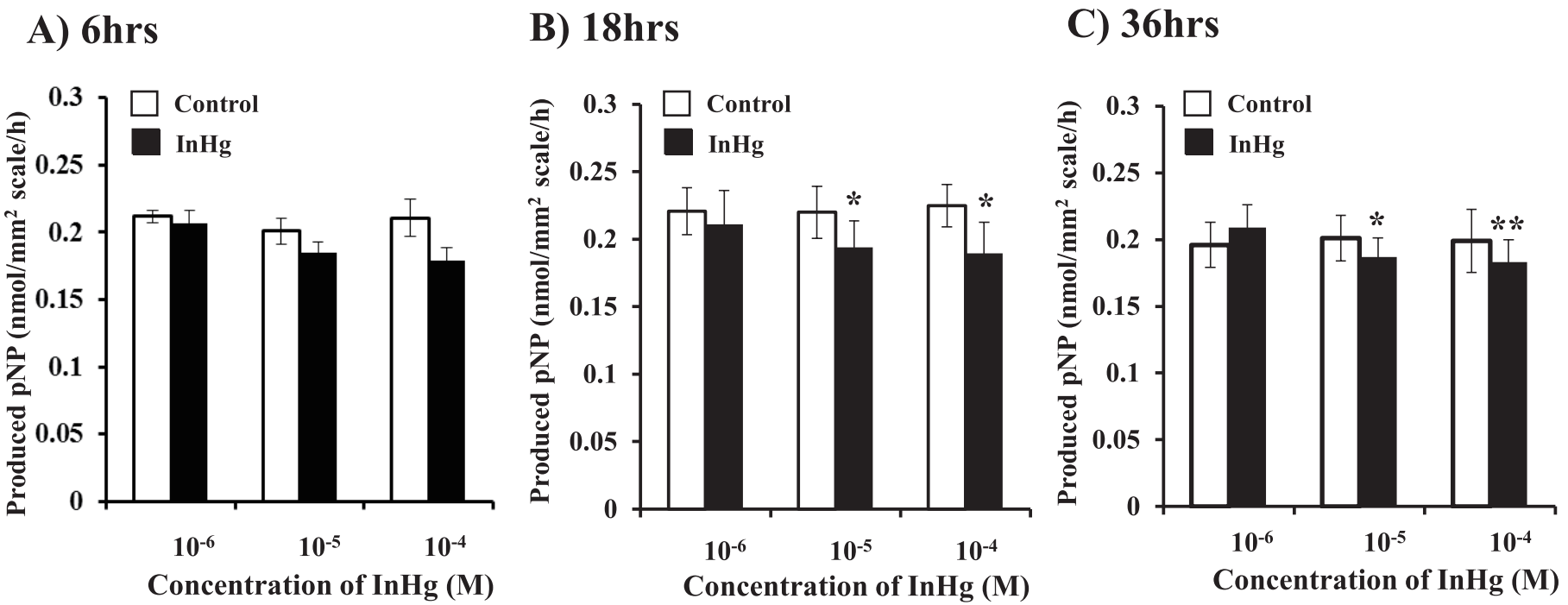

Fig. 11 Effect of InHg (10^{-6} to 10^{-4} M) on ALP activity in the cultured scales at 6 (A), 18 (B), and 36 hrs (C). * and ** indicate statistically significant differences at $p < 0.05$, $p < 0.01$, respectively, from the values of the control scales (n = 6 samples; one sample from one fish).

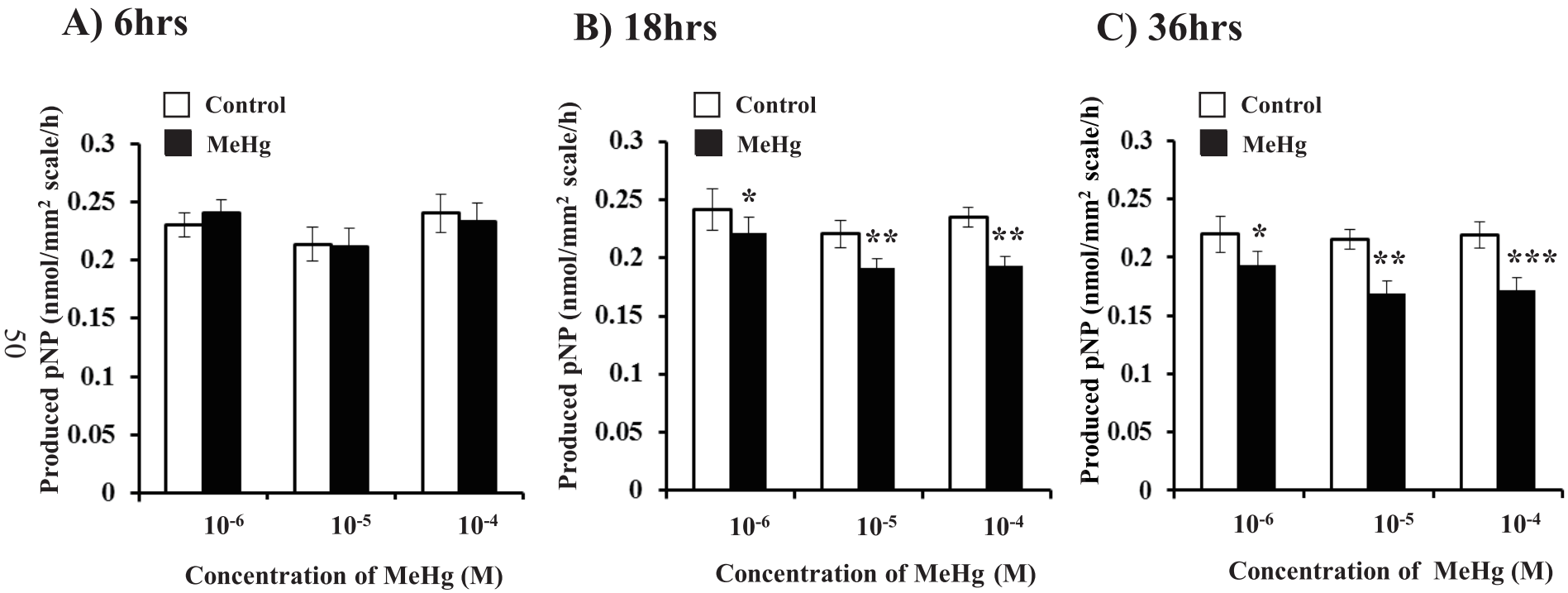

Fig. 12 Effect of MeHg (10^{-6} to 10^{-4} M) on ALP activity in the cultured scales at 6 (A), 18 (B), and 36 hrs (C). *, ** and *** indicate statistically significant difference at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, from the values of the control scales (n = 6 samples; one sample from one fish).

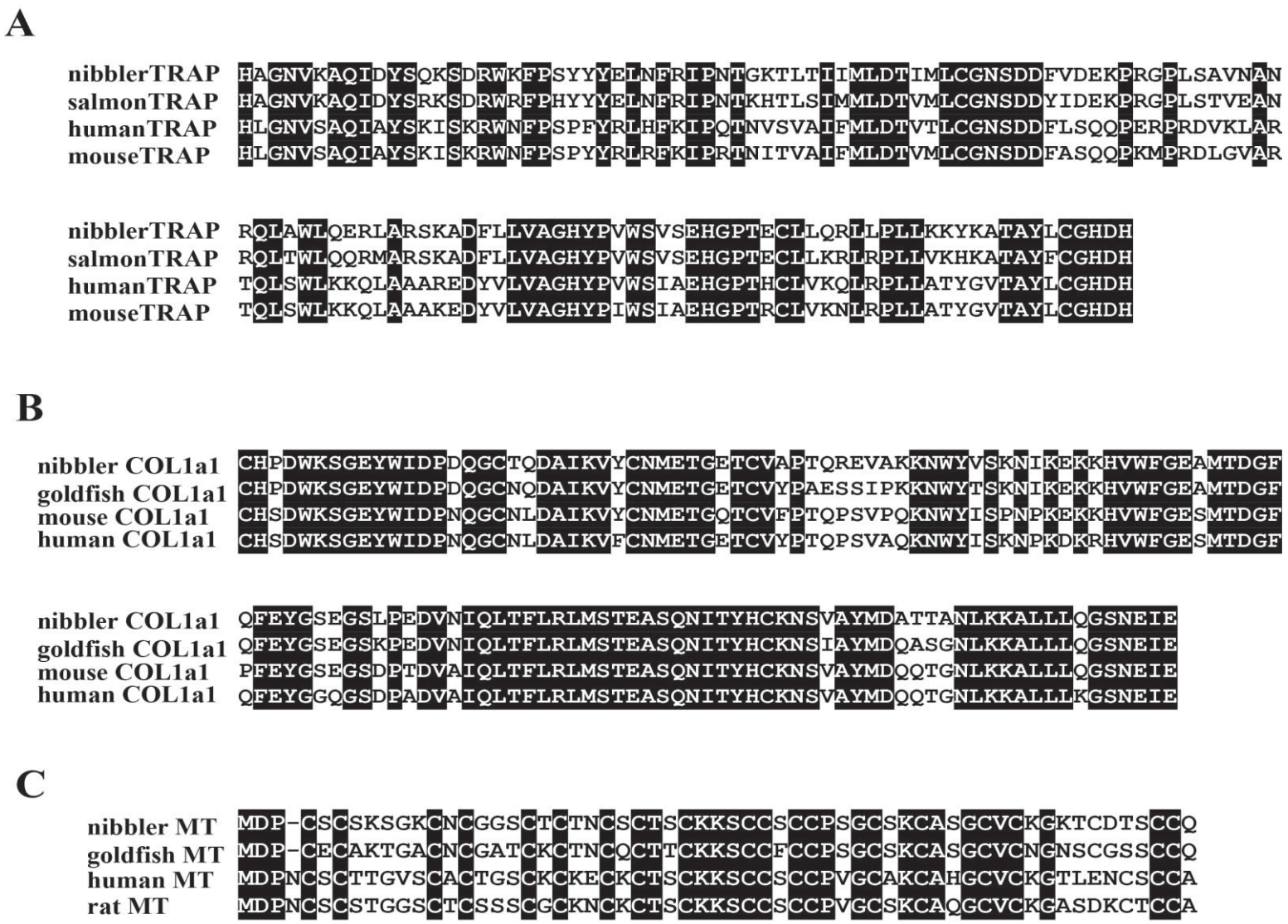

Fig. 13 Alignment of amino acid sequences of TRAP (A), COL1A1(B), and MT (C). Conserved amino acid sequences are described by black boxes. The accession numbers are as follows: nibbler TRAP, AB874604; salmon TRAP, NP_001134890.1; human TRAP, NP_001602.1; mouse TRAP: NP_031414.1; nibber COL1A1, AB874603 ; goldfish COL1A1, BAG72200.1; mouse COL1A1, AAA88912.1; human COL1A1, CAA98968.1; nibbler MT, AB874602; goldfish MT, AAB32777.1; human MT, NP_789846.1; rat MT, NP_620181.1.

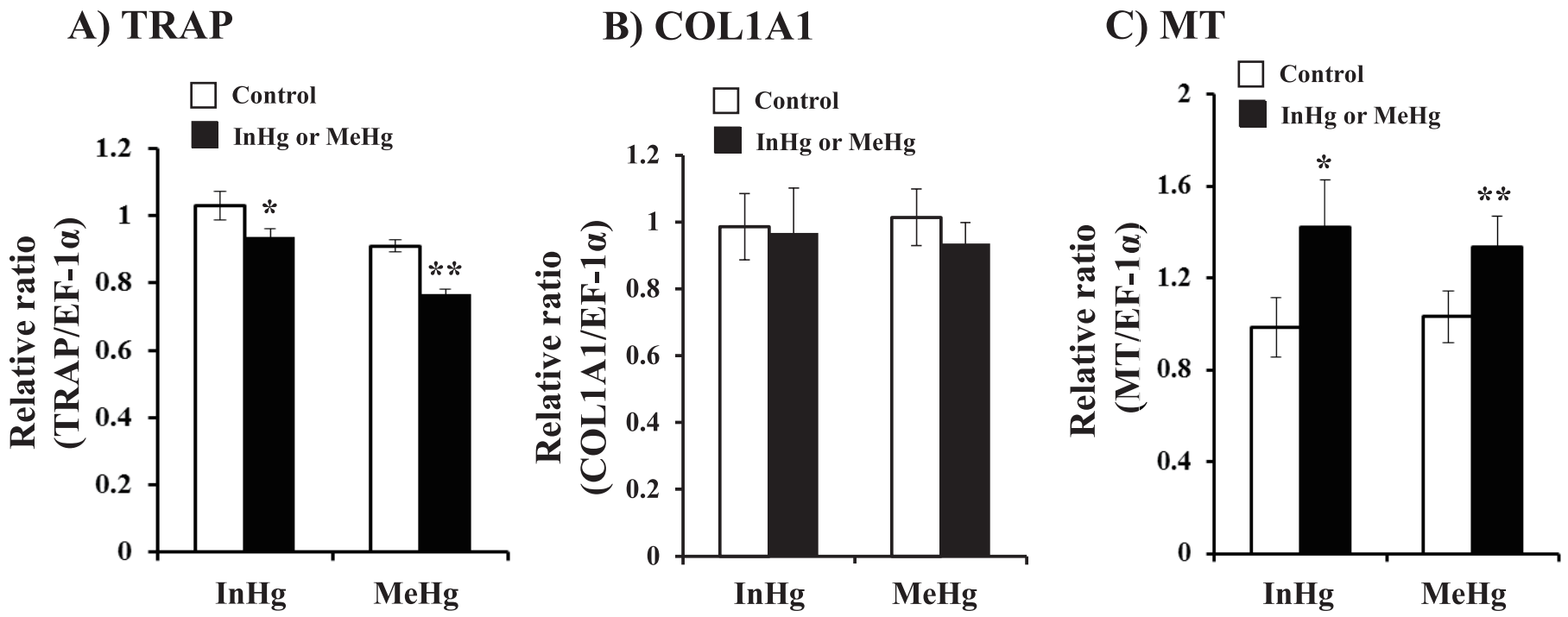

Fig. 14 Expression of TRAP (A), COL1A1 (B) and MT (C) mRNAs in the InHg- and MeHg-treated scales of goldfish at 6 hrs of incubation. TRAP, COL1A1, and MT mRNA levels were normalized to the EF-1α mRNA level. * and ** indicate statistically significant difference at $p < 0.05$, $p < 0.01$, respectively, from the values of the control scales (n = 8 samples; one sample from one fish).

Ⅳ. Polychlorinated biphenyl (118) activates osteoclasts and induces bone resorption in goldfish

Introduction

It has been reported that polychlorinated biphenyl (PCB) congeners act as endocrine-disrupting compounds (Lind et al. 2004a, b; Bovee et al. 2011; Nakayama et al. 2011; Ju et al. 2012). As bone formation and resorption are controlled by several hormones and vitamins (see a review, Peacock 2010), PCBs might disturb bone metabolism. In some animals, actually, the bone disruption caused by PCB has been reported (rat, Lind et al. 2004a; bear, Sonne et al. 2004; sheep, Gutleb et al. 2010; alligator, Lind et al. 2004b; turtle, Holliday and Holliday 2012; salmon, Olufsen and Arukwe 2011; zebrafish, Ju et al. 2012). In humans, changes in bone metabolism associated with exposure to PCBs have also been investigated (Hodgson et al. 2008). However, the direct effects of PCBs on osteoclasts and osteoblasts have not yet been elucidated in any animals.

The teleost scale is a calcified tissue that contains osteoblasts, osteoclasts, and the bone matrix of two layers (bony layer—a thin, well-calcified external layer; a fibrillary layer—a thick, partially calcified layer) (Bereiter-Hahn and Zylberberg 1993; Suzuki et al. 2000, 2007; Yoshikubo et al. 2005; Ohira et al. 2007). The bone matrix, which includes type I collagen (Zylberberg et al. 1992), osteocalcin (Nishimoto et al.,

1992), and hydroxyapatite (Onozato and Watabe 1979), is present in the scale as well as in mammalian bone. Recently, we detected both cathepsin K and TRAP mRNA expression in scale osteoclasts (Azuma et al. 2007). In osteoblasts, we detected osteoblast-specific markers, such as ALP, runt-related transcription factor 2, osterix, osteocalcin, type I collagen, and RANKL (Thamamongood et al. 2012). Therefore, the features of osteoclasts and osteoblasts in scales are similar to those in mammals.

In fish as well as mammals, plasma calcium level was regulated by hormones such as parathyroid hormone (Suzuki et al. 2011a) and calcitonin (Suzuki et al. 2000, 2004a). In an in vivo experiment, fugu parathyroid hormone I induced hypercalcemia resulted from the increase of both osteoblastic and osteoclastic activities in the scale and caused to decrease scale calciumcontents (Suzuki et al. 2011a). Scale osteoclastic activation was also observed in the prostaglandin E2-injected goldfish (Omori et al. 2012). It is reported that the scales are a better potential internal calcium reservoir than the body skeletons, jaws, and otoliths, examined by the ^{45}Ca-labeling study for the calcified tissues of goldfish and killifish (Mugiya and Watabe 1977). Thus, we conclude that teleost scale is an active and functional calcium reservoir.

In fish, PCB (118) is the highest congener compared with

PCB-105, -156, -167, -123, -157, -114, -189, -77, -126, -81, or -169 (Bhavsar et al. 2007). Furthermore, it has been reported that trabecular bone mineral content was almost 30% lower in the PCB (118) (49 g/kg body weight/day) at the metaphysis in sheep (Gutleb et al. 2010), although the detail mechanism has not yet been elucidated. We therefore analyzed the effect of PCB (118) on scale osteoclastic and osteoblastic activities, as well as plasma calcium levels, in the goldfish scales. In addition, effect of PCB (118) on osteoclasts and osteoblasts was investigated in vitro. This is the first to demonstrate that PCB (118) activates osteoclasts and activates osteoclasts and induced bone resorption in fish.

Materials and methods

Animals

To examine the effect of PCB (118) on the bone metabolism, immature goldfish (4-6 g), in which the endogenous effects of sex steroids are negligible, were used for the *in vivo* study. A previous study (Suzuki et al. 2000) indicated that the sensitivity for calcemic hormones was higher in mature female than in mature male teleosts. Therefore, female goldfish (*Carassius auratus*) (30-40 g) were purchased from a commercial source (Higashikawa Fish Farm, Yamatokoriyama, Japan) and used for the in vitro experiments.

All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Kanazawa University.

Effects of PCB (118) on scale osteoclastic and osteoblastic activities and the plasma calcium in goldfish at day 1 and 2 after PCB (118) injection (in vivo experiment)

PCB (118) was solubilized in dimethyl sulfoxide (DMSO) at a concentration of 10 ppm. Goldfish (body weight 4-6 g) were anesthetized

with ethyl 3-aminobenzoate and methanesulfonic acid salt (Sigma-Aldrich, Inc., MO, USA) and taken the blood (about 100 μl) from caudal vessels of each individual into heparinized syringes just before PCB (118) injection. After centrifugation at 15,000 rpm for 3 min, the plasma was immediately frozen and kept at -80 °C until use. In the experimental group (n = 10), In the experimental group (n = 10), thereafter, PCB (118) was intraperitoneally injected (100 ng/g body weight). The goldfish in the control group (n = 10) were injected with DMSO in the same manner. These goldfish were kept in the aquarium for 1 and 2 days. During the experimental periods, these goldfish were not given any food to exclude intestinal calcium uptake from diets. Each day after injection, the scales were collected from each goldfish. At day 2 after injection, blood samples (about 100 μl) were collected from the gill using a heparinized capillary from individual, anesthetized goldfish. After centrifugation at 15,000 rpm for 3 min, the plasma was also immediately frozen and kept at -80 °C until use. The plasma total calcium level (mg/100 ml) was determined using an assay kit (Calcium C; Wako Pure Chemical Industries, Ltd., Osaka, Japan). Then, we measured the activities of ALP and TRAP activities as respective indicators of each activity in osteoclasts and osteoblasts (Suzuki et al. 2000, 2009; Suzuki and Hattori 2002). The measurement methods (Suzuki et al.

2009) of ALP and TRAP activities were as follows. The incubated scale was transferred to its own well in a 96-well microplate after washing with saline. An aliquot of 100 μl of an alkaline buffer (100 mM Tris-HCl, pH 9.5; 1 mM $MgCl_2$; 0.1 mM $ZnCl_2$) for ALP activity or an acid buffer (0.1 M sodium acetate including 20 mM tartrate, pH 5.3) for TRAP activity was added to each well. This microplate was frozen at -80°C immediately and then kept at -20°C until analysis. After thawing, an aliquot of 100 μl of 20 mM *para*-nitrophenyl-phosphate in an alkaline buffer or an acid buffer was added to each well. This plate was then incubated at 20°C for 30 min with shaking. After incubation, the reaction was stopped by adding 50 μl of a 3 N NaOH-20 mM EDTA solution. Aliquots of 150 μl of a colored solution were transferred to a new plate, and the absorbance was measured at 405 nm. The absorbance was converted into the amount of produced pNP using a standard curve for pNP. After measurement of the absorbance, the ALP and TRAP activities were normalized by the surface area (mm^2) of each goldfish scale. The results are shown as the means ± SE of eight scales.

PCB (118) contents in the scales of goldfish (in vivo experiment)

At day 1 and 2 after PCB (118) injection, the scales were collected from goldfish and then immediately frozen and kept at -80°C

until use. The PCB (118) contents were analyzed by the methods of Hirai et al. (2005). Because a single sample volume was very small, we conducted three measurements to obtain a pulled sample. Thus, the mean of three measurements was described in the results.

Effects of PCB (118) on osteoclastic and osteoblastic activities in the cultured scales of goldfish (in vitro experiment)

Scales collected from goldfish (n = 10) after anesthesia with ethyl 3-aminobenzoate and methanesulfonic acid salt (Sigma-Aldrich) and incubated for 6 and 18 h in Leibovitz's L-15 medium (Invitrogen, Grand Island, NY, USA) containing a 1% penicillin-streptomycin mixture (ICN Biomedicals, Inc., OH, USA) supplemented with PCB (118) (0.025, 0.25, and 2.5 ppm). In an *in vivo* experiment, around 0.05 to 0.1 ppm PCB was detected in the PCB-injected scales. Based on these PCB contents in the scales, we decided the administration doses of PCB in an in vitro experiment. The PCB concentration in one goldfish was performed using 48 scales from each left or right side. The 48 scales used in the present study were considered to use as follows: (1) eight scales for TRAP analysis by 0.025 ppm, (2) eight scales for TRAP analysis by 0.25 ppm, (3) eight scales for TRAP analysis by 2.5 ppm, (4) eight scales for ALP analysis by

0.025 ppm, (5) eight scales for ALP analysis by 0.25 ppm, and (6) eight scales for ALP analysis by 2.5 ppm. The respective mean for TRAP (obtained from eight individual scales of one goldfish) and ALP (obtained from eight individual scales of one goldfish) activities from the left side (experimental group) was compared with those of the right side (control group). Using 10 individual goldfish, the same experiment was done repeatedly. The experiments for 0.25 and 2.5 ppm PCB (118) were carried out in the same manner. After incubation, TRAP and ALP activities were measured using the same methods described above (Suzuki et al. 2009). The results are shown as means ± SEM (n = 10).

Changes in TRAP, cathepsin K, and RANKL mRNA expressions in PCB (118)-treated goldfish scales (in vitro experiment)

Scales were collected from goldfish under anesthesia with ethyl3-aminobenzoate and methanesulfonic acid salt (Sigma-Aldrich). To examine changes in TRAP, cathepsin K, and RANKL mRNAs that responded to PCB (118), these scales were incubated for 18 h in Leibovitz's L-15 medium (Invitrogen) containing a 1% penicillin-streptomycin mixture (ICN Biomedicals). In the prostaglandin E2-treated scales of goldfish, we previously reported that TRAP, cathepsin K, and RANKL mRNA

expression increased at 18 h of incubation (Omori et al. 2012). Therefore, this incubation period was adopted. After incubation, the scales were frozen at-80°C for mRNA analysis.

Total RNAs were prepared from goldfish scales using a total RNA isolation kit for fibrous tissue (Qiagen GmbH, Hilden, Germany). Complementary DNA synthesis was performed using a kit (Qiagen GmbH). Gene-specific primers for TRAP (sense, 5′ -AACTTCCGCAT TCCTCGAACAG-3′ ; antisense, 5′ -GGCCAGCCACCAGGAGA TAA-3′) (Azuma et al. 2007), cathepsin K (sense, 5′ -GCTATGGAGC CACACCAAAAGG- 3′ ; antisense, 5′ -CTGCGCTTCCAGCTCTC ACAT-3′) (Azuma et al. 2007), and RANKL (sense, 5-GCGCTTA CCTGCGGAATCATATC-3′ ; antisense, 5′ -AAGTGCAACAGAA TCGCCACAC-3′) (Suzuki et al. 2011a) were used. The amplification of β-actin cDNA using a primer set (5′ ,CGAGCGTGGCTACAGCTTCA; 3′ , GCCCGTCAGGGAGCTCATAG) (Azuma et al. 2007) was performed. The PCR amplification was analyzed by real-time PCR apparatus (Mx3000p; Agilent Technologies, CA, USA) (Suzuki et al. 2011a). The annealing temperature of TRAP, cathepsin K, RANKL, and β-actin was 60°C. The TRAP, cathepsin K, and RANKL mRNA levels were normalized to the β-actin mRNA level.

Statistical analysis

All results are expressed as the means ± SE (n = 10). The statistical significance between control and experimental group was assessed by Student's *t* test (*in vivo* experiment) or paired *t* test (*in vitro* experiment). In all cases, the selected significance level was $P < 0.05$.

Results

Effects of PCB (118) on scale osteoclastic and osteoblastic activities and the plasma calcium in goldfish at 1 and 2 days after PCB (118) injection in vivo

We measured the activities of ALP and TRAP activities as respective indicators of each activity in osteoclasts and osteoblasts. At day 2, scale TRAP activity in PCB-injected goldfish increased significantly (Fig. 15a), while ALP activity did not change significantly at day 1 and 2 (Fig. 15b). Corresponding to the elevation of osteoclastic activity, plasma calcium levels increased significantly at day 2 after PCB administration (Fig. 16).

PCB (118) contents in the scales of goldfish in vivo

At day 1 and 2 after PCB (118) injection, PCB (118) was detected in the scales. At day 1, PCB contents in the control and PCB-injected scales were determined as 0.39 and 79 (ng/g-wet), respectively. At day 2, PCB (ng/g-wet) of 0.38 and 55 was detected in the control and PCB injected scales, respectively.

Effect of PCB (118) on osteoclastic and osteoblastic activities in the cultured scales of goldfish in vitro

PCB (118) significantly increased the TRAP activities of the scales by 6 h of incubation ($p < 0.05$ for 0.25 ppm) (Fig. 17a). At 18 h of incubation, the TRAP activities in the PCB (118)-treated scales also significantly increased ($p < 0.05$ for 0.025 and 2.5 ppm; $p < 0.001$ for 0.25 ppm) (Fig. 18a).

In case of the ALP activities, it significantly increased ($p < 0.05$) only by the concentration of 2.5 ppm at the 6 and 18 h incubation (Figs. 17b and 18b).

Changes in TRAP, cathepsin K, and RANKL mRNA expressions in PCB (118)-treated goldfish scales in vitro

The mRNA expression of osteoclastic markers (TRAP and cathepsin K) increased significantly by PCB (118) (0.25 ppm) treatment (Fig. 19a, b).

Similar results were obtained in RANKL. The mRNA expression of RANKL, an activating factor of osteoclasts, increased significantly in the osteoblasts in the PCB (118)-treated scales (Fig. 19c).

Discussion

In the present study, we are the first to demonstrate that PCB(118) induced hypercalcemia resulting from increasing osteoclastic activity *in vivo*. In an *in vitro* experiment, the data were reproduced and osteoclastic marker mRNA expression as well as enzyme activity increased. In fish, PCB (118) is the highest congener compared with PCB-105, -156, -167, -123, -157, -114, -189, -77, -126, -81, or -169 (Bhavsar et al. 2007). In aquatic environment, PCB (118) was detected (Hope 2008; Aksoy et al. 2011). Therefore, we paid attention to bone metabolism by PCB (118) pollution.

At day 1 and 2 after PCB (118) injection intraperitoneally, we detected PCB (118) in the scale. As described in the "Introduction", the scales are potential internal calcium reservoir than the body skeletons, jaws, and otoliths. Lake et al. (2006) reported that the correlation between the total mercury concentration of the scales and that of the muscles was high ($r = 0.89$). In sheep, PCB was accumulated and detected in bone at 2 months after administration (Jan et al. 2006). We therefore suggest that scale PCB content can be used as an environmental PCB monitor to estimate the environmental pollution of PCB.

In the present study, we measured hydroxy-PCB which is a kind of metabolites from PCB because hydroxy-PCB possessed specific and competitive interactions with the plasma thyroid hormone transport protein, transthyretin (Lans et al. 1993). In PCB-treated scales, however, hydroxyl-PCB was not detected. Therefore, this phenomenon of osteogenesis seems to be direct action of PCB (118)

In an *in vivo* experiment, osteoblastic activity increased by the high concentration of PCB (118) (2.5 ppm). This indicates that PCB (118) is affected on osteoblasts. Osteogenesis is regulated by osteoblasts (Suda et al. 1999; Teitelbaum 2000; Lacey et al. 2012). RANKL produced by cells in the osteoblast lineage binds to RANK in mononuclear hemopoietic precursors and promotes the formation and activity of multinucleated osteoclasts (Suda et al. 1999; Teitelbaum 2000; Lacey et al. 2012). Our present study indicated that RANKL mRNA expression was promoted by PCB (118) treatment. In addition, osteoclastic

Marker (TRAP and cathepsin K) mRNA expression also increased significantly. Therefore, we strongly suggest that PCB (118) promotes osteoclastogenesis by the RANK–RANKL pathway.

In the present study, we succeeded to analysis the PCB (118) on osteoclasts and osteoblasts. Our results suggest that scale is a good model

for analysis of bone metabolism. We previously demonstrated that the osteogenesis of regenerating scale is very similar to that of mammalian membrane bone and a good model of osteogenesis (Yoshikubo et al. 2005). Using this system, furthermore, we first demonstrated that calcitonin, a hypocalcemic hormone, suppressed osteoclastic activity in teleosts as well as in mammals (Suzuki et al. 2000) and that melatonin, a major hormone secreted from the pineal gland, suppressed the functions in both osteoclasts and osteoblasts (Suzuki and Hattori 2002). Osteoblasts in the scale responded to estrogen as they do in mammalian bone (Yoshikubo et al. 2005). In addition, the effects of endocrine disrupters, such as bisphenol-A (Suzuki and Hattori 2003) and tributyltin (Suzuki et al. 2006), and heavy metals, i.e., cadmium and mercury (Suzuki et al. 2004b, 2011b), on osteoblasts and osteoclasts have been examined. Moreover, we indicated that cadmium (even at 10^{-13} M) responded to TRAP activity in the scale (Suzuki et al. 2004b).

In conclusion, PCB (118) disrupts bone metabolism in goldfish both *in vivo* and *in vitro* experiments. Our results suggest that PCB (118) promotes osteoclastogenesis by the RANK–RANKL pathway. Furthermore, our previous and present results indicate that the scale assay system will be useful for the analysis of environmental contaminant on bone metabolism,

and findings of PCB (118) on bone in fish may be tied in to an overall health issue for mammals in general.

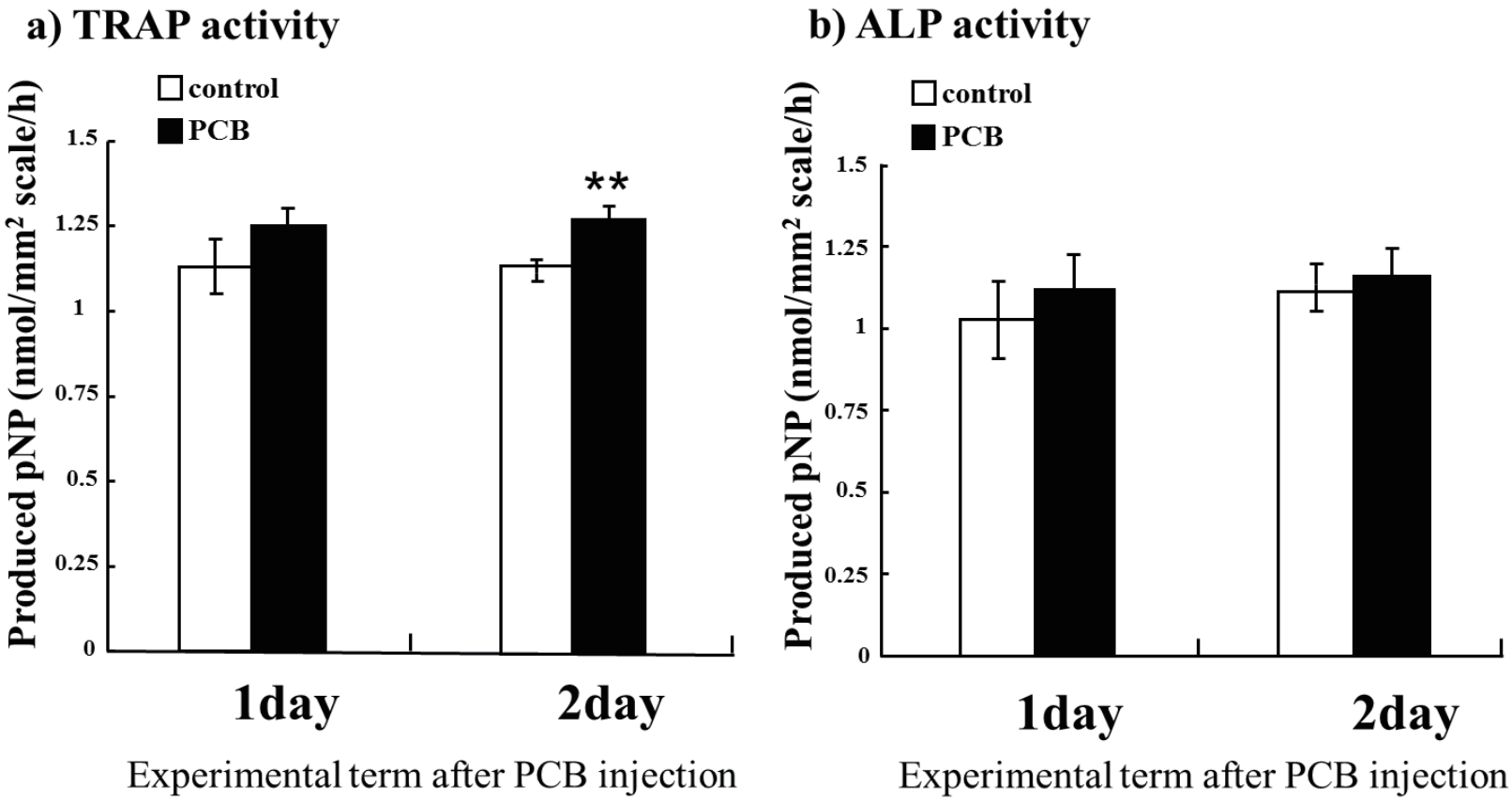

Fig. 15 Effects of PCB (118) injection on scale TRAP (a) and ALP (b) activities in goldfish. Each column and the vertical line represent the mean ± SEM (n = 10 samples; one sample from one fish). ** indicates statistically significant difference at $p < 0.01$ from the values in the control

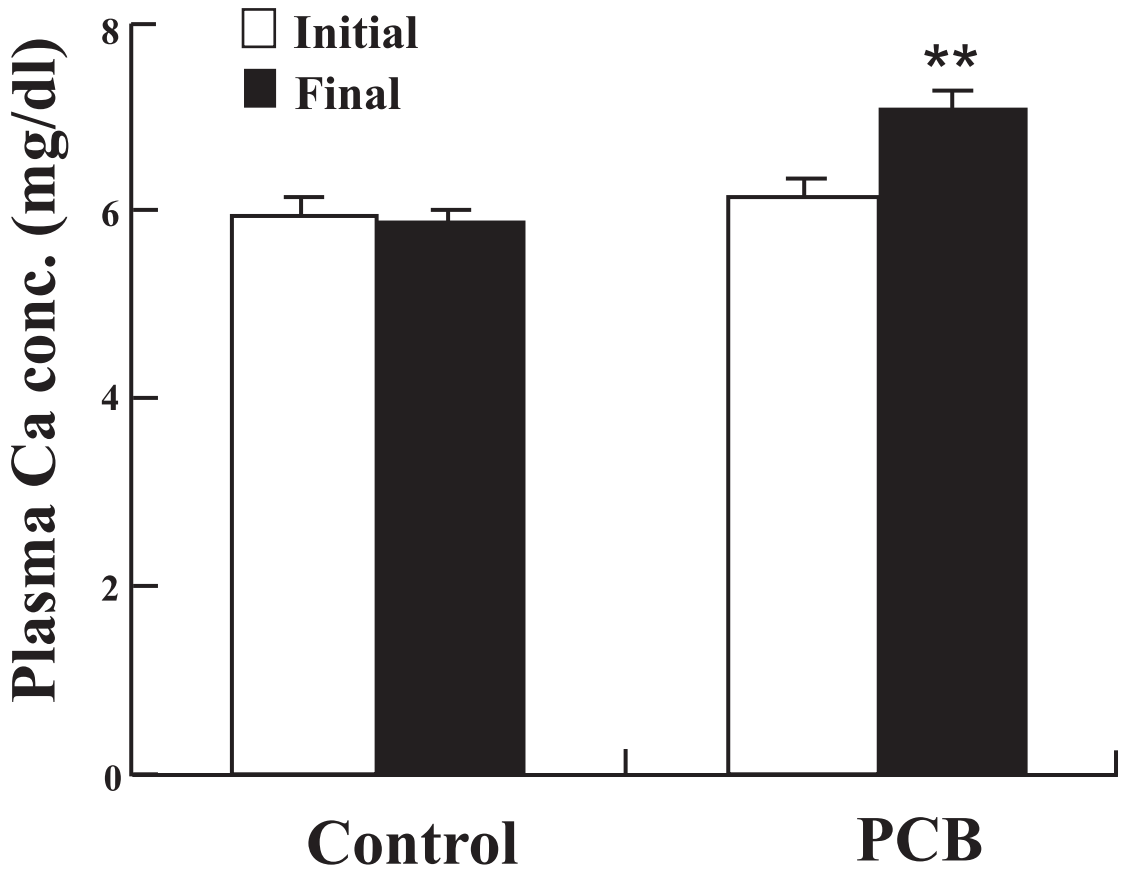

Fig. 16 Effects of PCB (118) injection on plasma calcium level (mg/100 ml) in goldfish. Each column and the vertical line represent the mean± SEM (n = 10 samples; one sample from one fish). ** indicates statistically significant difference at $P < 0.01$ from the values in the control

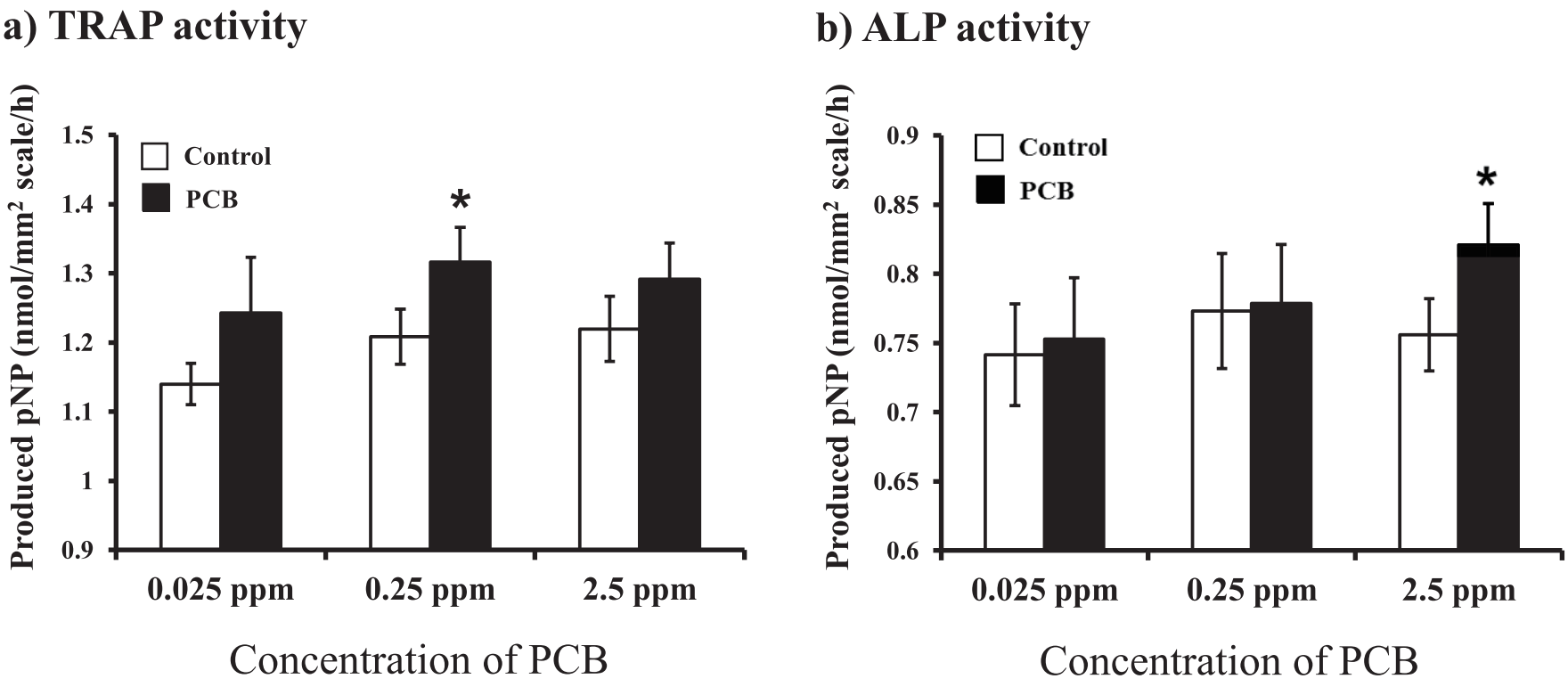

Fig. 17 Effects of PCB (118) administration on TRAP (a) and ALP (b) activities in the scales of goldfish at the 6 h of incubation. Each column and the vertical line represent the mean ± SEM (n = 10 samples; one sample from one fish). * indicates statistically significant difference at $p < 0.05$ from the values in the control

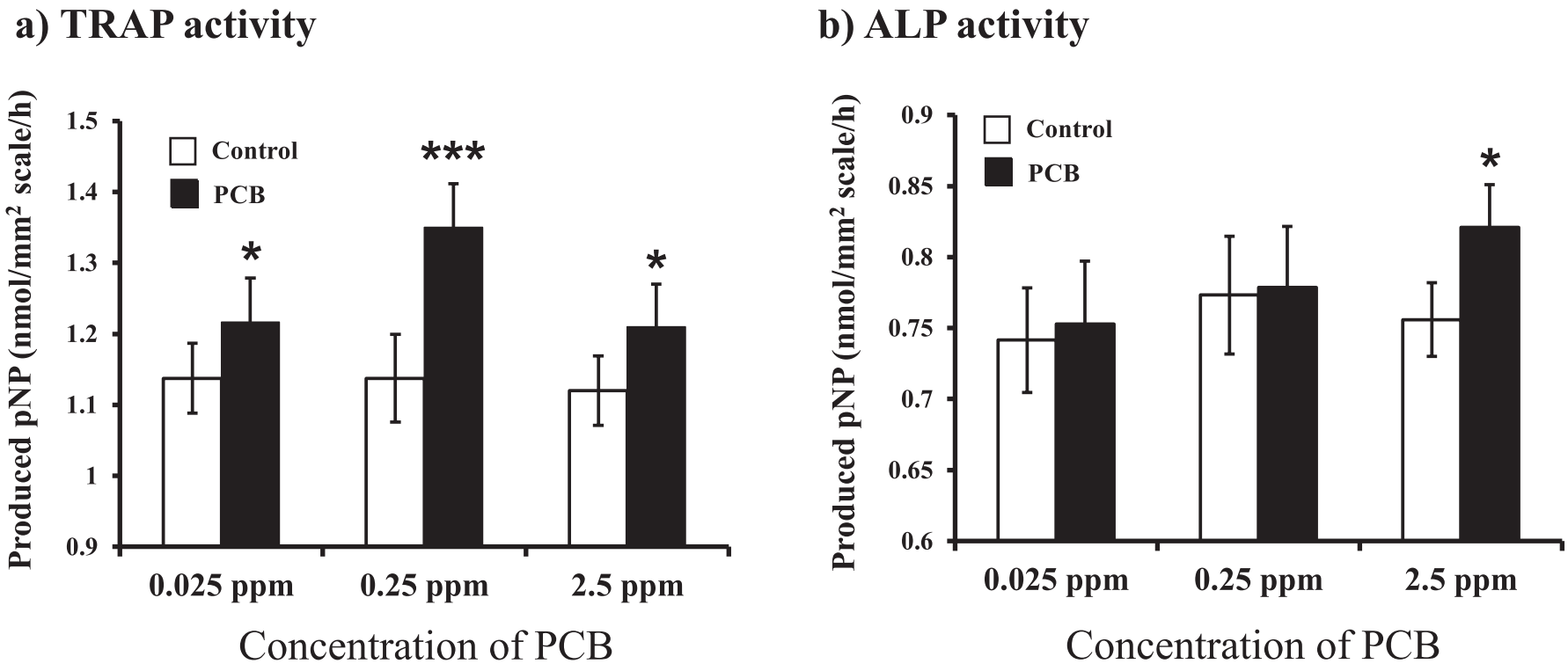

Fig. 18 Effects of PCB (118) administration on TRAP (a) and ALP (b) activities in the scales of goldfish at the 18 h of incubation. Each column and the vertical line represent the mean ± SEM (n = 10 samples; one sample from one fish). * and *** indicate statistically significant differences at $p < 0.05$ and $p < 0.001$, respectively, from the values in the control

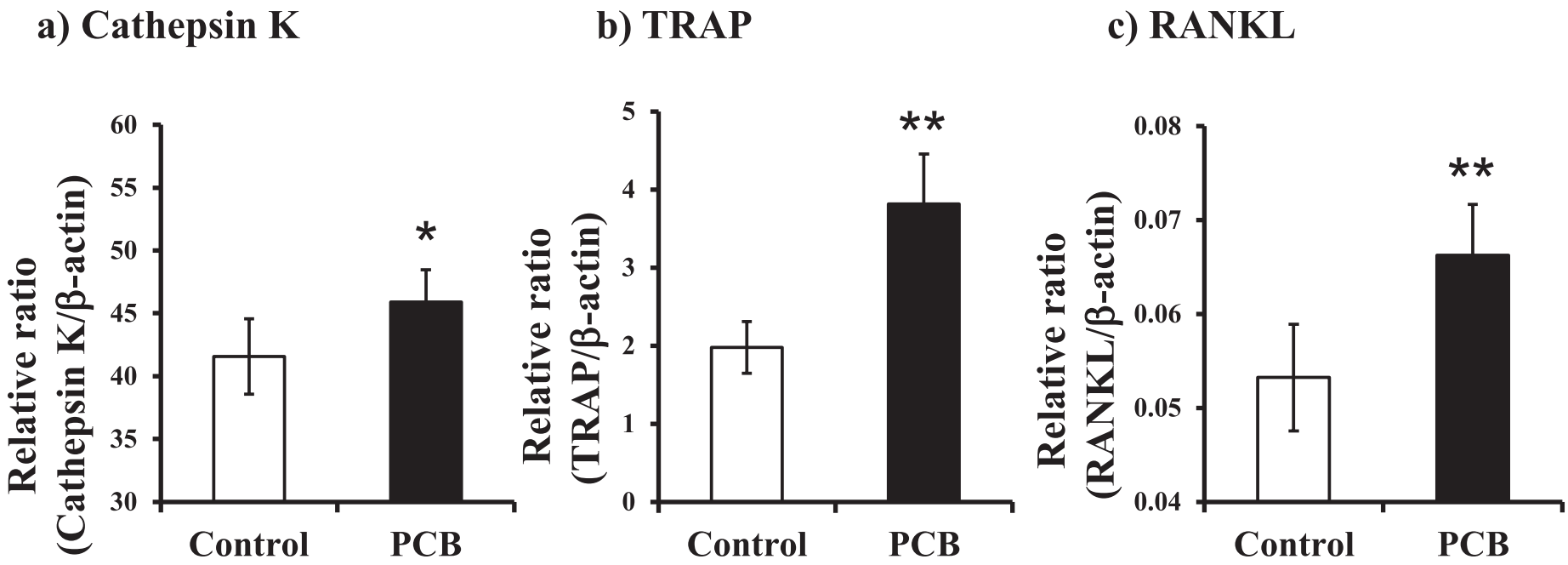

Fig. 19 Effect of PCB (118) (0.25 ppm) in the expression of osteoclastic markers: cathepsin K (a), TRAP (b), and RANKL (c) mRNAs in the scale. The cathepsin K, TRAP, and RANKL mRNA levels were normalized by the β-actin mRNA level. The values of ordinate indicate relative ratio of cathepsin K/β-actin (a), TRAP/β-actin (b), and RANKL/β-actin (c), respectively. Each column and the vertical line represent the mean ± SEM (n = 10 samples; one sample from one fish). * and ** indicate statistically significant differences at $p < 0.05$ and $p < 0.01$, respectively, from the values in the control

Ⅴ. General Discussion

Effects of InHg on osteoclasts and osteoblasts as analyzed by an in vitro *assay system with goldfish (fresh water teleosts)*

The present study demonstrated that fish scale was sensitive to InHg. A high correlation of mercury between scales and muscles was reported in largemouth bass (Lake et al., 2006). This indicates that accumulation of mercury has occurred in fish scales, although mercury did not accumulate in the vertebral bone of fish (Camusso et al., 1995). It is also well known that the scale is a more active tissue in fish calcium regulation than is the vertebral bone (Mugiya and Watabe, 1977; Yamada, 1961; Berg, 1968; Bereiter–Hahn and Zylberberg, 1993). Therefore, we strongly believe that fish scale is capable of accumulating mercury and responding to mercury similarly to calcium.

In the present study, TRAP activity in the scales significantly decreased with treatment of InHg (10^{-5} to 10^{-3} M) during 6 hrs of incubation. In addition, mRNA expressions of osteoclastic markers TRAP and cathepsin K significantly decreased as compared with the control with 18 hrs of incubation. On the other hand, ALP activity decreased after exposure to InHg at concentrations of 10^{-6} to 10^{-4} M for 36 and 64 hrs, although its activity did not change after 6 and 18 hrs. It was found that the mRNA expression of MT in InHg-treated scales increased with 18 hrs of

incubation. In an *in vitro* experiment for 6 and 18 hrs of incubation, therefore, osteoblasts may be resistant to mercury as a result of MT production. On the other hand, IGF–I mRNA expression decreased as compared to the control with 18 hrs of incubation. As IGF–I participates in osteoblastic growth and differentiation, we speculate that mercury has a toxic effect on osteoblasts under long-term exposure.

Effects of InHg and MeHg on osteoclasts and osteoblasts as analyzed by an in vitro *assay system with nibbler fish (marine teleosts)*

In nibbler fish (marine teleosts), we found that the enzyme activity of TRAP (osteoclastic marker) was decreased at 6 hrs following treatment by both InHg and MeHg. In osteoblasts, also, both InHg and MeHg inhibited ALP enzyme activity with 18 and 36 hrs of incubation. In goldfish (freshwater teleosts), we previously reported that InHg (10^{-5} to 10^{-3} M) (Suzuki et al., 2011b) and MeHg (10^{-8} to 10^{-6} M) (Suzuki et al., 2004) significantly suppressed TRAP enzyme activity. Particularly, the response of MeHg in nibbler fish is lower than that in goldfish. In fact, the response of the calcemic hormone estrogen in the scale osteoclasts of freshwater teleosts was higher than that in those of marine teleosts (Persson et al., 1994; Persson et al., 1995; Guerreiro et al., 2002). It is known that the

exchange of calcium in the scales of freshwater teleosts is faster than that in marine teleosts because freshwater teleosts live in a low-calcium environment.

From each marker mRNA expression analysis at 6 hrs of incubation, the expression of TRAP mRNA in InHg- and MeHg-treated scales was significantly lower than that in the control scales, although the mRNA expression of COL1A1 (osteoblastic marker) did not change significantly. Conversely, MT mRNA expression significantly increased with both InHg and MeHg treatments. This result is similar to that in goldfish, as it has been demonstrated that MT plays a protective role against mercury-induced toxicity in bone (Jin et al., 2002).

In the present study, using the scales of marine teleosts, we developed an *in vitro* assay system. This system can be used to detect both osteoclastic and osteoblastic activities simultaneously with TRAP and ALP enzyme activities. Furthermore, we sequenced TRAP, COL1A1, and MT cDNAs from the scales of nibbler fish. Thus, we conclude that the in vitro assay system we developed may be a useful means for analyzing heavy metal in bone metabolism. In the future, we will further examine the difference in toxic mechanism between MeHg and InHg using fish scales as a model of bone.

Influence of PCB on bone metabolism as analyzed by both in vivo *and* in vitro *experiments with goldfish*

The present study is the first to demonstrate that PCB (118)-induced hypercalcemia results from increasing osteoclastic activity *in vivo*. In an *in vitro* experiment, the data were reproduced, and osteoclastic marker mRNA expression as well as enzyme activity increased. In addition, RANKL mRNA expression was promoted by PCB (118) treatment. Therefore, we strongly suggest that PCB (118) promotes osteoclastogenesis by the RANK–RANKL pathway.

We succeeded in analyzing the effect of PCB (118) on osteoclasts and osteoblasts. Our results suggest that scale provides a good model for analyzing bone metabolism. We previously demonstrated that the osteogenesis of regenerating scale is very similar to that of mammalian membrane bone and a good model of osteogenesis (Yoshikubo et al., 2005). Using this system, furthermore, we first demonstrated that calcitonin, a hypocalcemic hormone, suppressed osteoclastic activity in teleosts as well as in mammals (Suzuki et al., 2000), and that melatonin, a major hormone secreted from the pineal gland, suppressed the functions in both osteoclasts and osteoblasts (Suzuki and Hattori, 2002). Osteoblasts in the scale responded to estrogen as they do in mammalian bone (Yoshikubo et al.,

2005). In addition, the effects of endocrine disrupters, such as bisphenol-A (Suzuki and Hattori, 2003) and tributyltin (Suzuki et al., 2006), and heavy metals, i.e., cadmium and mercury (Suzuki et al., 2004b; Suzuki et al., 2011b), on osteoblasts and osteoclasts have been examined. Moreover, we indicated that cadmium (even at 10^{-13} M) responded to TRAP activity in the scale (Suzuki et al., 2004b). Considering the results above, we concluded that fish scale is a suitable model for analyzing bone metabolism.

Ⅵ. General Summary

1) Effects of InHg on osteoclasts and osteoblasts as analyzed by an *in vitro* assay system with goldfish (fresh water teleosts)

This study is the first to indicate a direct effect of InHg on osteoclasts. In addition, both mRNA expressions of TRAP and cathepsin K decreased as TRAP enzyme activity decreased. TRAP activity in scale decreased significantly with treatment of InHg in a shorter time than did ALP activity. Osteoclasts decreased in a shorter time than did osteoblasts. We strongly believe that osteoblasts may be resistant to mercury as a result of MT production.

2) Effects of InHg and MeHg on osteoclasts and osteoblasts as analyzed by an *in vitro* assay system with nibbler fish (marine teleosts)

We successfully developed an *in vitro* assay system by using nibbler fish (marine teleosts) in the present study. This system can be used to detect both osteoclastic and osteoblastic activities simultaneously with TRAP and ALP enzyme activities. This is the first report concerning the effects of mercury on osteoclasts and osteoblasts in the marine teleost scale as a model system of bone. Furthermore, we sequenced TRAP, COL1A1, and MT cDNAs from the scales of nibbler fish. Thus, we conclude that our

developed *in vitro* assay system may be a useful means for analyzing the effects of heavy metal on bone metabolism.

3) Influence of PCB on bone metabolism as analyzed by both *in vivo* and *in vitro* experiments with goldfish

We successfully analyzed the effect of PCB (118) on osteoclasts and osteoblasts in both *in vivo* and *in vitro* experiments. PCB (118) induced hypercalcemia, resulting from increasing osteoclastic activity *in vivo*. Furthermore, we found that PCB (118) promotes osteoclastogenesis by the RANK–RANKL pathway, judging from *in vitro* experiments. Thus, our previous and present results indicate that the scale assay system will be useful for analyzing the effects of environmental contaminants on bone metabolism, and that finding PCB (118) in fish bone may be tied to an overall health issue for mammals in general.

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Abbreviation

ANOVA : analysis of variance

ALP: alkarine phosphatase

COL1A1: collagen type I α1

DDT: 2,2-bis-(*p*-chlorophenyl)-1,1,1-trichloroethane

DMSO: dimethyl sulfoxide

EF-1α: elongation factor 1α

HCHs: hexachlorocyclohexanes

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IGF-1: insulin-like growth factor-1

InHg : inorganic mercury ($HgCl_2$)

MeHg : methylmercury (CH_3HgCl)

MEM: Eagle's minimal essential medium

MS-222 : ethyl 3-aminobenzoate, methanesulfonic acid

MT: metallothionein

NIH Image J: a computer program by the National Institute of Mental Health (USA)

PCB : polychlorinated biphenyl

PCDD/Fs : polychlorinated dibenzo-*p*-dioxins and furans

pNP : *para*-nitrophenol

RANK: receptor activator of the NF- κ B

RANKL: receptor activator of the NF- κ B ligand

RT-PCR: reverse-transcription polymerase chain reaction

TRAP: tartrate-resistant acid phosphatase