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journal or publication title	Fisheries Science
volume	83
number	4
page range	543-550
year	2017-07-01
URL	http://doi.org/10.24517/00029370

doi: 10.1007/s12562-017-1086-0

Aquaculture

Sodium fluoride influences calcium metabolism resulting from the suppression of osteoclasts in the scales of nibbler fish *Girella punctata*

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ABSTRACT The influence of sodium fluoride (NaF) on calcium metabolism was examined in nibbler fish (marine teleosts). Two days after the administration of NaF (5 μg/g of body weight)(around 10^{-4} M in fish), we showed that plasma calcium levels significantly decreased in NaF-treated nibbler fish. In addition, we detected fluoride in the treated scales by scanning electron microscope with an energy-dispersive X-ray microanalysis, indicating that NaF directly affects their scales. Therefore, the influence of NaF on osteoblasts and osteoclasts in the scales was examined. In the scales of NaF-injected nibbler fish, tartrate-resistant acid phosphatase (TRAP) (osteoclastic marker enzyme) decreased, although alkaline phosphatase (osteoblastic marker enzyme) was activated. To confirm the effect of NaF on osteoclasts, furthermore, the mRNA expressions of osteoclastic markers (matrix metalloproteinase-9 and TRAP) were decreased significantly 2 days after incubation. In barred knifejaws, plasma calcium levels decreased as they did in nibbler fish. Therefore, NaF functions in both osteoblasts and osteoclasts and then influences calcium metabolism in marine fish. In the marine environment, high levels of fluoride (1.2 to 1.5 mg F^-/l) (around 10^{-5} to 10^{-4} M) are present in seawater. It is probable that teleosts living in seawater efficiently use fluoride to regulate their blood calcium levels.

Keywords Sodium fluoride · Osteoblasts · Osteoclasts · Scales · Calcium metabolism · Element analysis · Nibbler fish

Introduction

Fluoride is widely present in environmental waters and it is generally derived from geological sources [1, 2]. It is also known that fluoride levels in seawater (1.2 to 1.5 mg F^-/l) were higher than those in fresh water (0.01 to 0.3 mg F^-/l) (see a review [3]). In aquatic animals (particularly marine animals), fluoride tends to accumulate in the exoskeleton of invertebrates and hard tissue, including the bone and scales of fish. For example, krill, which has been used as a feed source for cultured fish, accumulates fluoride in pleon cuticle (2600 ppm/d.w. in *Euphausia superba* and 3300 ppm/d.w. in *Meganyctiphanes norvegica*) [4]. Furthermore, dietary fluoride derived from krill exoskeletons accumulates in the vertebral bones of yellowtail [5]. This suggests that fluoride influences bone metabolism in marine teleosts.

Teleost scale is calcified tissue that contains osteoblasts (bone-forming cells), osteoclasts (bone resorption cells), and the bone matrix of two layers [6-11]. Teleost scales play an important role in regulating the blood calcium level because teleost scales are known to function as internal calcium reservoirs, particularly during times of increased calcium demand, such as sexual maturation or starvation [6, 12-14]. Using fish scales, we have developed a fish scale assay system [7, 15]. This fish scale assay has been utilized to analyze hormonal functions [7, 11, 15, 16]. Using the scale assay system, we recently indicated that in goldfish *Carassius auratus* (freshwater teleosts), sodium fluoride (NaF) affects both osteoblasts and osteoclasts [17]. In marine teleosts, however, the direct effect of fluoride on osteoblasts and osteoclasts has not yet been reported.

In the present study, we examined the influence of NaF on calcium metabolism in a marine teleost species (nibbler fish *Girella punctata*). To confirm the results in nibbler fish,

we injected NaF into another marine teleost species (barred knifejaw *Oplegnathus fasciatus*) and examined the influence of NaF on plasma calcium levels. Furthermore, to investigate the mechanism of NaF on bone metabolism in marine teleost, we performed both *in vivo* and *in vitro* experiments with the scales of nibbler fish.

Thus, we concluded that NaF functions in both osteoblasts and osteoclasts and then influences calcium metabolism in marine teleosts as well as in freshwater teleosts.

Materials and methods

Animals

Nibbler fish *Girella punctata* (both sexes, n = 17, 38.5 ± 1.1 g) were captured by fishing in Tsukumo Bay of the Noto Peninsula (Ishikawa Prefecture). After acclimation for approximately 2 weeks, these fish were used in the present experiments. To confirm the results in nibbler fish, barred knifejaws *Oplegnathus fasciatus* (both sexes, n = 14, 124.2 ± 20.4 g) were used, and the effect of NaF on calcium metabolism was examined. Some of the barred knifejaws were gifts from both Notojima and Uozu aquariums.

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

Treatment and blood collection in nibbler fish (*in vivo* experiment)

Nibbler fish were anesthetized with 0.04% of a 2-phenoxyethanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and blood samples were then collected from the caudal vessel using a heparinized syringe from individual anesthetized nibbler fish (n = 6). Each collected

sample was put into a 1.5-ml tube. Thereafter, the tube was centrifuged at 15,000 rpm for 3 min. Then, the separated plasma was immediately frozen and kept at –80 °C until use. After blood sampling, NaF (purity: 99.0%, Wako Pure Chemical Industries, Ltd.) (5 μg/g of body weight) was injected intraperitoneally. The dose of NaF was determined in accordance with our previous report [17].

Nibbler fish in the control group (n = 6) were injected with saline (0.9% NaCl) in the same manner as experimental nibbler fish. These nibbler fish were kept in the aquarium for 2 days. These experimental periods were adopted because hormonal and toxicological effects appeared in goldfish in 2 days [16-21]. In addition, our preliminary experiment with nibbler fish indicated that the changes in plasma calcium levels were highest 2 days after injection. During the experimental periods, these nibbler fish were fasted to exclude intestinal calcium uptake from their diet. Two days after injection, blood samples were collected as described above. The separated plasma was immediately frozen and kept at –80 °C until use. The total calcium level (mg/100 ml) of the separated plasma was determined using an assay kit (Aqua-auto Kainos Calcium Reagent kit, KAINOS Laboratories, Inc., Tokyo, Japan). Calcium in the plasma samples binds to a color coupler (arsenazo III) and forms a blue complex under neutral pH. The depth of the blue color is proportional to the calcium concentration. We measured plasma calcium levels by colorimetry during this color development.

Treatment and blood collection in barred knifejaws (*in vivo* experiment)

To confirm the obtained results in nibbler fish, we examined the influence of NaF on plasma calcium levels using NaF-injected barred knifejaws.

These fish were anesthetized with 0.04% of 2-phenoxyethanol (Wako Pure Chemical Industries, Ltd.). Thereafter, blood sampling was done as described above. NaF (Wako Pure

Chemical Industries, Ltd.) (5 μg/g of body weight) was then injected intraperitoneally. Barred knifejaws in the control group were injected with saline (0.9% NaCl) in the same manner as experimental fish. These fish were kept in the aquarium for 2 days. Two days after injection, a blood sample was collected from the caudal vessel using a heparinized syringe from individual anesthetized fish. Thereafter, the total plasma calcium level (mg/100 ml) was determined using an Aqua-auto Kainos Calcium Reagent kit (KAINOS Laboratories, Inc.).

Detection of fluoride in the scales of nibbler fish after NaF injection (*in vivo* experiment)

To measure the deposition of fluoride in the scales of nibbler fish, epidermal tissues, including scales, from the left side of the anesthetized fish were dissected and lyophilized. Thereafter, scales were taken from the lyophilized tissue and coated with platinum palladium. Samples were observed using a scanning electron microscope (SEM, SU1510, Hitachi High-Technologies Corporation, Tokyo, Japan) with energy-dispersive X-ray microanalysis (EDS, EX-250X-ac, Horiba Ltd., Kyoto, Japan), as described in Mishima et al. (1995) [22]. SEM-EDS analytical conditions were as follows: the accelerating voltage was 15kV, and the measuring time was about 3600 seconds. The quantitative analysis was a standard method. SiO_2, MgF_2, MgO, GaP, MADD-10 feldspar (K), and wollastonite (Ca) were used as standard specimens. Using EDS mapping, whole scales of nibbler fish were analyzed. Quantitative analysis of the scale was performed in the range of 400 μm × 300 μm. Analysis was performed several times, and F represents the average value of the concentration.

Effects of NaF on scale osteoblastic and osteoclastic activities (*in vivo* experiment)

Before NaF or saline (0.9% NaCl) injection, the scales on the right side were removed under anesthesia with 0.04% of 2-phenoxyethanol (Wako Pure Chemical Industries, Ltd.). Two days after NaF or saline injection, scales on the right side were extracted from anesthetized nibbler fish to examine the influences of NaF on the osteoblasts and osteoclasts with alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) as markers because, in mammals, the effects of hormones and some bioactive substances on osteoblasts and osteoclasts have been investigated using ALP and TRAP as respective markers [23–25]. The mean for ALP or TRAP (8 scales from one fish) was compared with that of the right side (control group).

The methods for measuring ALP and TRAP activities were as follows. An aliquot of 100 µl of an alkaline buffer (100 mM Tris-HCl, pH 9.5; 1 mM $MgCl_2$) or an acid buffer (0.1 M sodium acetate, including 20 mM tartrate, pH 5.3) was added to each well. Then, each scale was put into its own well in a 96-well microplate. This microplate was immediately frozen at -80 ℃ and then kept at -20 ℃ until analysis. An aliquot of 100 µl of 20 mM para-nitrophenyl phosphate in an alkaline or acid buffer was then put into each well with the content of the defrosted plate, which had been stored previously at -20 ℃. This plate was incubated at 23 ℃ 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 reacted 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 both ALP and TRAP activities, the size of each scale was measured with ImageJ. Thereafter, ALP and TRAP activities were normalized to the surface area (mm^2) of each scale [26].

Osteoclastic marker mRNA analysis in NaF-treated nibbler fish scales (*in vitro* experiment)

To investigate the effect of NaF on osteoclasts, osteoclastic marker mRNA expression was measured in addition to marker enzyme activity *in vivo*. The nibbler fish (n = 5) were anesthetized with 0.04% of 2-phenoxyethanol (Wako Pure Chemical Industries, Ltd.). Then, the scales (n = 8) on the right side were collected from anesthetized nibbler fish. To examine changes of genes expressed in osteoclasts that responded to NaF (10^{-5} M), these scales were incubated for 1 and 2 days in Leibovitz's L-15 Medium (Thermo Fisher Scientific Inc., Grand Island, NY, USA) containing a 1% penicillin-streptomycin mixture (Thermo Fisher Scientific Inc.). This concentration of NaF was determined in accordance with experiments regarding *in vitro* cultures (effective doses: 10^{-7} to 10^{-4} M) of osteoblasts in rats [27] and the concentration of sea water (around 10^{-5} to 10^{-4} M)[3]. After incubation, the scales were frozen at -80 °C for mRNA analysis.

Total RNAs were prepared from nibbler fish scales using a total RNA isolation kit (Isogen, Nippon Gene, Tokyo, Japan). Complementary DNA synthesis was performed using a kit (PrimeScript™ II 1st strand cDNA Synthesis Kit, Takara Bio Inc., Shiga, Japan). Using the cDNA of control scales, partial fragments of matrix metalloproteinase-9 (MMP-9) were amplified using the degenerate PCR method. The degenerated primer sets for MMP-9 (sense 1: TACCCNTTTGATGGNAARG; antisense 1: GGAGTGATCCAANGGNAG; sense 2: CAGGGNGAYGCNCAYTTTG; antisense 2: TCCAAAYTCRTGNGCNGC) were designed from multiple amino acid sequence alignments of each vertebrate family member. MMP-9 cDNA fragments were amplified by nested PCR (annealing temperature:

40 ℃). The obtained PCR fragments were sequenced. Partial sequences of MMP-9 in nibbler fish were determined.

Gene-specific primers for MMP-9 and TRAP [28] are indicated in Table 1. Elongation factor-1α (EF-1α) cDNA was amplified using a primer set [28] (Table 1). The PCR amplification was analyzed using real-time PCR apparatus (Mx3000p, Agilent Technologies, Santa Clara, CA, USA) [16, 29]. In accordance with the manufacturer's protocol, relative quantification was performed by the standard curve method. The stocked cDNA was accurately diluted. We prepared standard curves using the diluted cDNA. The relative quantities determined can be compared across the plates. MMP-9, TRAP, and EF-1α were annealed at 60 °C. The MMP-9 and TRAP mRNA levels were normalized to the EF-1α mRNA level.

Statistical analysis

All results are expressed as the means ± SE. The statistical significance between the control and experimental groups was assessed using a student's *t*-test or paired *t*-test. In all cases, the selected significance level was $p < 0.05$.

Results

Effects of NaF on the plasma calcium level in nibbler fish and barred knifejaws (*in vivo* experiment)

The influence of NaF on calcium metabolism was examined in an *in vivo* experiment with nibbler fish (marine teleosts). Two days after administration, plasma calcium levels had

decreased significantly in the NaF-injected nibbler fish, although plasma calcium levels did not change in the control nibbler fish (Fig. 1).

To confirm the results obtained in nibbler fish, we examined the influence of NaF on plasma calcium levels using NaF-injected barred knifejaws. NaF also induced hypocalcemia in barred knifejaws, although plasma calcium levels did not change in the control barred knifejaws (Fig. 2).

Detection of fluoride in the scales of nibbler fish after NaF injection (*in vivo* experiment)

Fluoride was deposited in the scales of nibbler fish using SEM-EDS. We can detect fluoride in the scales of NaF-treated nibbler fish using SEM-EDS. As a result of having measured twice with different scales, the mean value of fluoride in the treated scales (1.57 weight %) was higher than that in the control scales (0.21 weight %).

Effects of NaF on osteoblastic and osteoclastic activities in the scales of nibbler fish (*in vivo* experiment)

We examined the influences of NaF on osteoblasts and osteoclasts with ALP and TRAP as respective markers. As a result, NaF treatment induced the upregulation of ALP activity (Fig. 3a). However, NaF injection significantly decreased TRAP activity (Fig. 3b).

The mRNA expression of osteoclastic markers with NaF treatments (*in vitro* experiment)

We partially determined MMP-9 (GenBank accession no. LC198841) sequences in nibbler fish (Fig. 4). Several amino acids were conserved among nibbler fish, medaka, mummichog, and zebrafish. Furthermore, 9 cysteine residues were well conserved in these fish.

Using the determined sequences, a gene-specific primer was designed (Table 1). MMP-9 mRNA expression in the NaF-treated and control scales was examined. After 1 day of incubation, MMP-9 mRNA expression tended to decrease with NaF treatment (Fig. 5a). Thereafter, the mRNA expression of MMP-9 in the NaF-treated scales decreased significantly as compared with that in control scales after 2 days of incubation (Fig. 5b).

TRAP mRNA expression was downregulated with NaF treatment, as was MMP-9. After 1 day of incubation, TRAP mRNA expression in the NaF-treated scales was slightly lower than that in the control scales (Fig. 5c). After 2 days of incubation, TRAP mRNA expression decreased significantly with NaF treatment, as did the TRAP enzyme (Fig. 5d).

Discussion

In the present study, we examined the effects of intraperitoneally injected NaF (5 μg/g of body weight) in marine teleosts, nibbler fish and barred knifejaws. In the marine environment, fluoride is present in a range from 1.2 to 1.5 mg F^-/l [3]. Therefore, the dose of fluoride in this study, corresponding to approximately 1.19 x 10^{-4} M in fish, is close to the environmental fluoride level (0.63 x 10^{-4} to 0.78 x 10^{-4} M) and is not an extreme dose. In the case of krill, fluoride was re-accumulated from environmental seawater at each molt stage because no internal deposition took place [4]. Krill maintain a high fluoride concentration in their cuticle during the molt cycle, indicating that fluoride is a mineral necessary for making cuticle in krill. In the two marine teleost species examined in this study, fluoride induced hypocalcemia. As seawater contains high calcium, fluoride may be involved in the suppression of calcium levels in marine teleosts. Thus, marine organisms may utilize fluoride to regulate minerals.

Considering that NaF injection induced osteoblastic marker enzyme activation, it is clear that fluoride has an important role in the regulation of bone metabolism in marine teleosts.

We previously reported that NaF induces hypercalcemia in goldfish (freshwater teleosts) [17]. We believe that the response to NaF in marine teleosts is different from that in freshwater teleosts. Receptor activators of nuclear factor-κB (RANK)/receptor activators of nuclear factor-κB ligand (RANKL)/osteoprotegerin (OPG) signaling may be involved in this phenomenon.

RANK/RANKL/OPG signaling is an important function in osteoclastic activation (see a review, 30). In osteoclasts, RANK binds the ligand (RANKL), resulting in the activation of osteoclasts, whereby multinucleated osteoclasts (an active type of osteoclasts) are induced. In addition, OPG that is a decoy receptor of RANKL inhibits osteoclastogenesis by binding to RANKL. Therefore, the RANKL/OPG ratio has been used as an important indicator of osteoclast activation [31]. Wnt/β-catenin signaling promotes new bone formation by upregulating OPG synthesis (see a review, 32). Furthermore, in rat osteoblasts, fluoride activated Wnt/β-catenin signaling [27]. In goldfish, we recently reported that OPG mRNA expression did not change significantly between NaF-treated and control scales, although NaF significantly increased RANKL mRNA expression [17]. In goldfish, therefore, the ratio of RANKL/OPG in NaF-treated scales was significantly higher than that in control scales, and then NaF treatment induced the activation of osteoclasts [17]. In the present study, NaF activated scale osteoblasts but suppressed scale osteoclasts in nibbler fish. We strongly believe that NaF acts on osteoblasts and then decreases the osteoclastic activity resulting from increased OPG production in nibbler fish. The suppression of osteoclasts with NaF treatment

was supported by an *in vitro* experiment for the mRNA expression of osteoclastic markers (MMP-9 and TRAP).

In the present study, we detected fluoride in the scales of nibbler fish. Fluoride can directly interact with the bone mineral matrix. The *in vitro* fluoridation of bone with fluoride can lead to the conversion of carbonated hydroxyapatite to carbonated fluorapatite, which is more stable and resistant to acid dissolution than is hydroxyapatite (see a review, 33). In the scales of nibbler fish, as well as in the bones and teeth of humans, fluoride may be converted into carbonated fluorapatite, as osteoblasts exist in the scales of nibbler fish [28]. In the control scales, we detected fluoride by using SEM-EDS. In marine teleosts, we believe that the deposition of fluoride occurs under natural conditions. To further understand the function of fluoride on bone metabolism, we will examine the existent form of fluoride that was deposited in the scales.

In the present study, we performed both *in vivo* and *in vitro* experiments. Considering both experiments, we concluded that NaF functions in both osteoblasts and osteoclasts and then influences calcium metabolism in marine teleosts. In the marine environment, however, fluoride levels are high (1.2 to 1.5 mg F^-/l) in seawater. Therefore, we believe that teleosts living in seawater efficiently use fluoride to regulate blood calcium levels.

Acknowledgments

This study was supported in part by grants to N.S. (Grant-in-Aid for Scientific Research [C] No. 16K07871 by JSPS), to A.H. (Grant-in-Aid for Scientific Research [C] No. 24570068 by JSPS), to T.S. (Scientific Research [C] No. 15K07126 by JSPS), and to H.M. (Scientific Research [C] No. 15K11034 by JSPS). This work was performed under the cooperative

research program of the Institute of Nature and Environmental Technology, Kanazawa University, Acceptance No. 17022, in 2017. We greatly appreciate Notojima and Uozu aquariums for providing us with barred knifejaw.

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Figure legends

Fig. 1. Effects of NaF on plasma calcium levels (mg/100 ml) in nibbler fish (a: control group; b: NaF injected group). NaF (5 μg/g of body weight) was injected intraperitoneally after initial blood sampling. Nibbler fish in the control group were injected with saline (0.9% NaCl) in the same manner as experimental nibbler fish. Blood sampling was then performed at 2 days. * indicates a statistically significant difference, at $p < 0.05$, from the initial values in the control or experimental group. $n = 6$ samples; one sample from one fish.

Fig. 2. Effects of NaF on plasma calcium levels (mg/100 ml) in barred knifejaws (a: control group; b: NaF injected group). NaF (5 μg/g of body weight) was injected intraperitoneally after initial blood sampling. Barred knifejaws in the control group were injected with saline (0.9% NaCl) in the same manner as experimental barred knifejaws. Blood sampling was then performed at 2 days. * indicates a statistically significant difference, at $p < 0.05$, from the initial values in the control or experimental group. $n = 7$ samples; one sample from one fish.

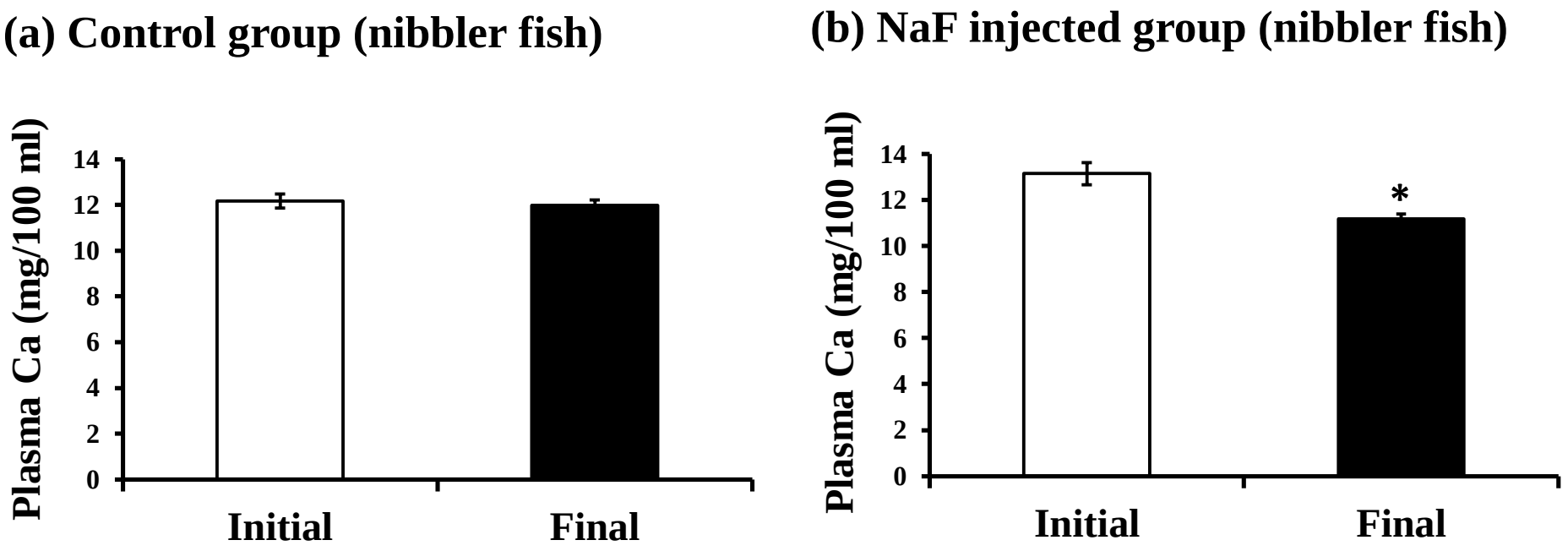
Fig. 3. Effects of NaF on alkaline phosphatase (ALP) (a) and tartrate-resistant acid phosphatase (TRAP) (b) activities (pNP nmol/mm^2 scale/h) in the scales of nibbler fish 2 days after NaF injection. Before NaF or saline (0.9% NaCT) injection, the scales on the right side were removed under anesthesia. Two days after NaF or saline injection, the scales on the right side were extracted from anesthetized nibbler fish. Each data indicates the ratio of final value/ initial value. ** and *** indicate statistically significant differences, at $p < 0.01$ and $p < 0.001$, respectively, from the values in the control scales. $n = 6$ samples; one sample from one fish.

Fig. 4. Alignment of amino acid sequences of matrix metalloproteinase-9 (MMP-9). Conserved amino acid sequences are indicated by black boxes. The accession numbers are as follows: nibbler fish, LC198841; medaka, NP_001098350.1; mummichog, JAR71850.1; zebrafish, AAI60656.1.

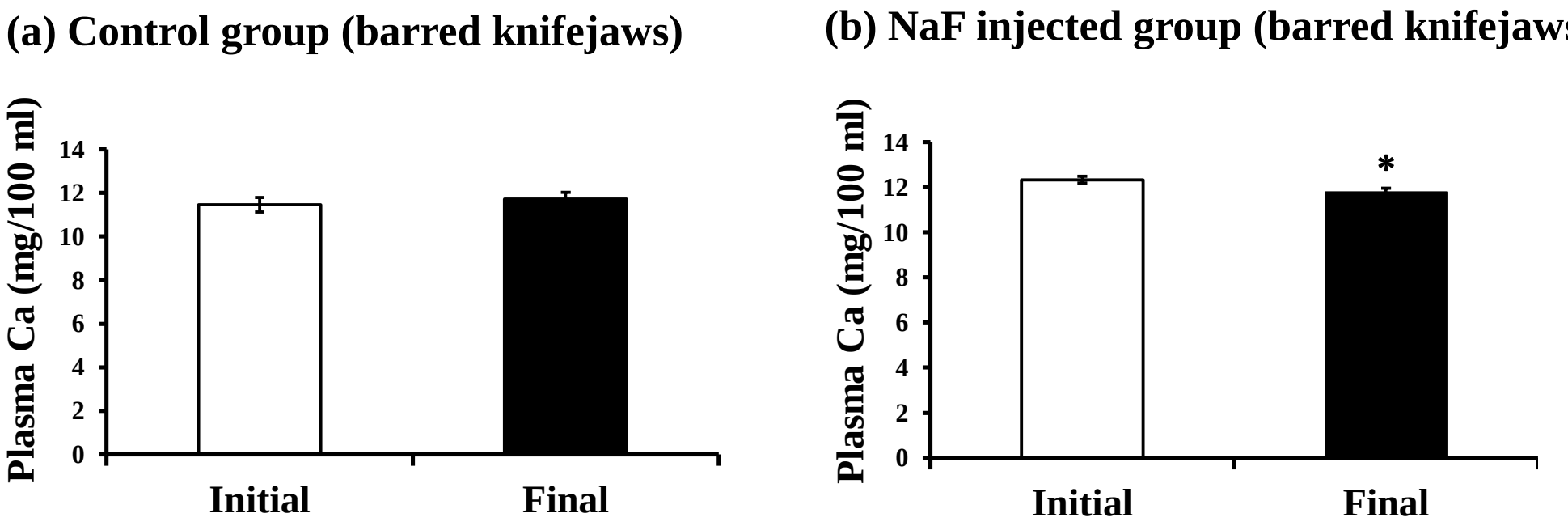
Fig. 5. Expression analysis of osteoclastic markers: matrix metalloproteinase-9 (MMP-9) and tartrate-resistant acid phosphatase (TRAP) mRNAs in scales treated with NaF (10^{-5} M) incubated for 1 and 2 days. a: MMP-9 mRNA expression after 1 day of incubation; b: MMP-9 mRNA expression after 2 days of incubation; c: TRAP mRNA expression after 1 day of incubation; d: TRAP mRNA expression after 2 days of incubation. MMP-9 and TRAP mRNA levels were normalized to the elongation factor-1α (EF-1α) mRNA level. The values of the ordinates indicate the relative ratios of MMP-9/ EF-1α (a and b) and TRAP/ EF-1α (c and d). * indicates statistically significant differences, at $p < 0.05$, from the values in the control scales. $n = 5$ samples; one sample from one fish.

Table legend

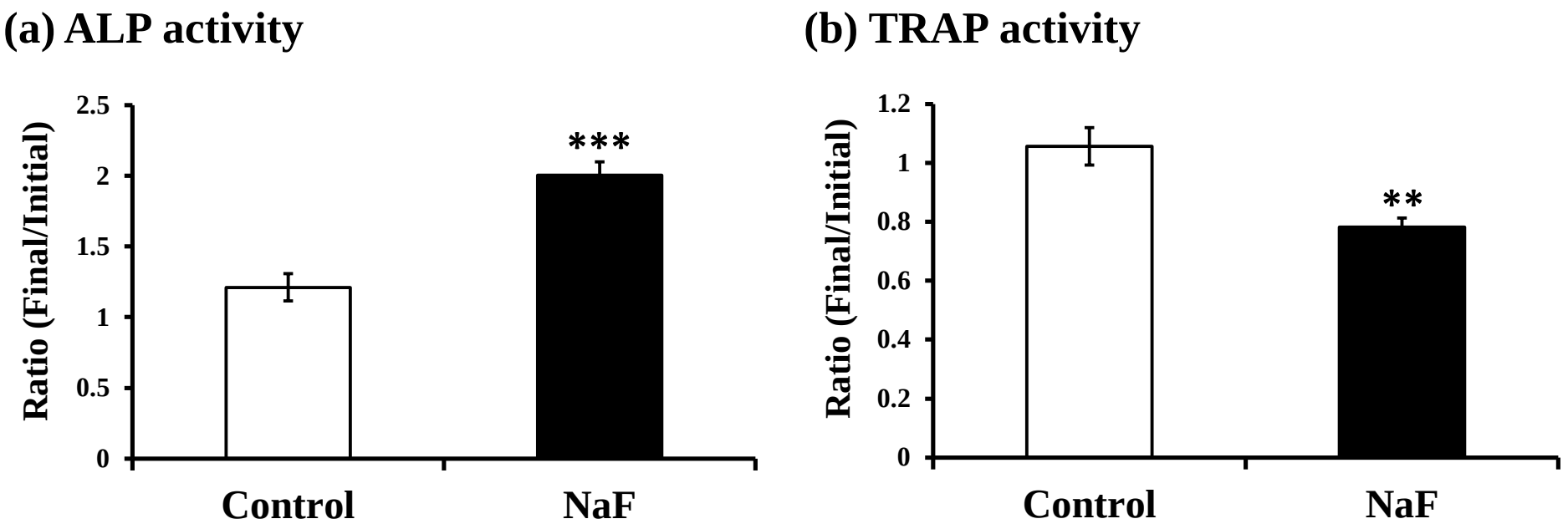
Table 1. Primer sequences for real-time quantitative PCR



Sato et al., Figure 1

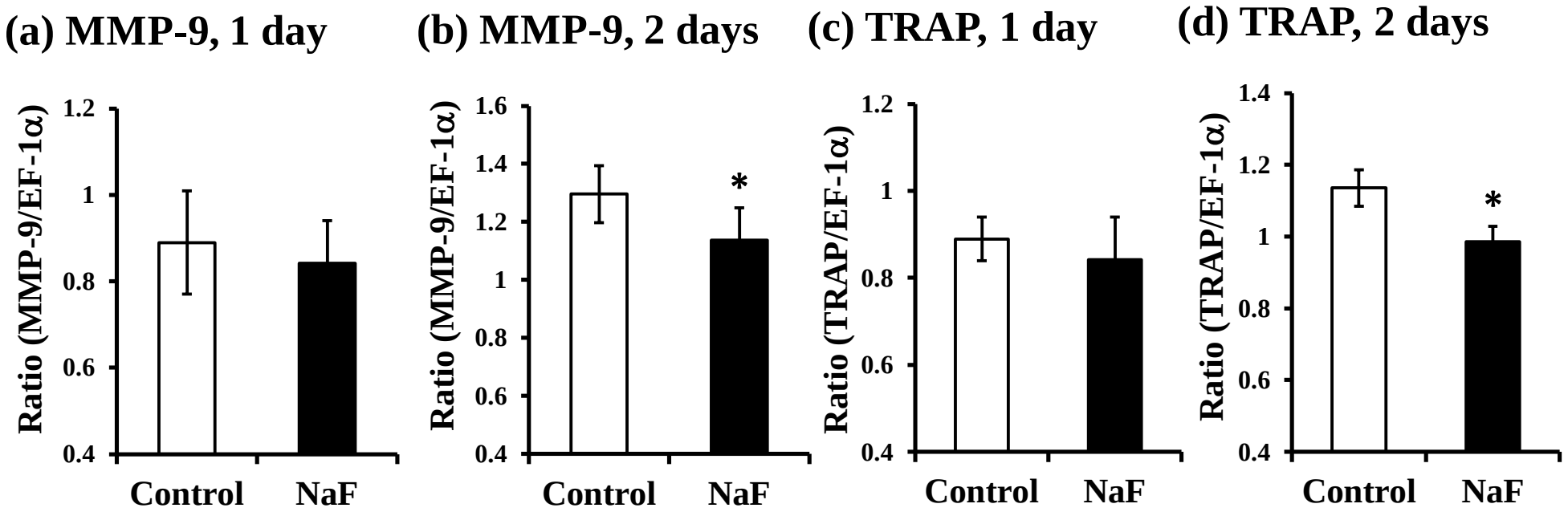


Sato et al., Figure 2



Sato et al., Figure 3

Sato et al., Figure 4



Sato et al., Figure 5

Table 1. Primer sequences for real-time quantitative PCR

Name	Forward primer	Reverse primer	Accession number
MMP-9	TGTGGTGCTCAACCACCTACAACT	ATCCCTGCCTTGAGTGGTGCAT	LC198841
TRAP	TGGATGAGAAGCCCAGAGG	CCGCAGAGGTAAGCAGTGG	AB874604
EF-1α	GTATGGTCGTCACCTTTGCTC	GTGGGTCGTTCTTGCTGTC	AB874605

MMP-9: matrix metalloproteinase-9; TRAP: tartrate-resistant acid phosphatase; EF-1α: Elongation factor-1α