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Melatonin suppresses the plasma calcium level in female goldfish during the late and post-reproductive stages

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In vertebrate females, plasma calcium concentrations decrease after the reproductive period to prevent calcium from leaking from the body. Goldfish scales store calcium internally, which is released into the blood by osteoclast resorption. Previously, we found that melatonin increases calcitonin (CT), an osteoclast inhibitor, through melatonin receptors in goldfish scales, and inhibits osteoclast activity. However, the physiological function of the melatonin-dependent suppression of osteoclast activity is unclear. In this study, we found that plasma calcium levels increased during the reproductive stage in female goldfish and decreased during the post-reproductive stage. Histological and mRNA expression analyses of osteoclast marker molecules revealed that osteoclasts in the scales were inactivated during the late and post-reproductive stages when the expression of CT and its receptor increased in female goldfish scales. Moreover, exogenous melatonin suppressed plasma calcium levels in female goldfish during their late reproductive stage in cases of increased melatonin receptor expression. Taken together, these results suggest that melatonin inactivates scale osteoclast resorption by increasing CT levels in the scales and prevents calcium leakage from the body during the late and post-reproductive stages of female goldfish.

Keywords Calcium, Female reproduction, Goldfish, Melatonin, Osteoclast

Seasonal changes have serious consequences for an organism's survival and species conservation^{1–3}. Therefore, organisms maintain their species by limiting their reproductive stages to certain seasons and by adapting to environmental changes⁴. For example, horses have a gestational period of approximately 1 year, whereas hamsters have a gestation period of a few weeks, and birds experience a short breeding period during spring^{5,6}. They all give birth during spring and summer, and their pups are programmed to develop when provided plenty of food.

Melatonin is a small, amphiphilic, indoleamine molecule that exhibits a variety of biological activities^{7,8}. It was originally considered an endocrine hormone that is primarily synthesized in the pineal gland and released into the blood to interact with melatonin receptors throughout the body^{9,10}. However, melatonin is also produced in small amounts in other tissues and organs, including the brain, retina, intestine, ovary, skin, crystalline lens, and bone^{11,12}. Furthermore, it regulates diverse physiological functions, such as the sleep–wake cycle^{10,13}, circadian clocks^{14,15}, glucose metabolism^{16,17}, immune system activity¹⁸, and bone metabolism^{19,20}. In particular, evidence suggests that melatonin acts as a reproduction regulator^{21–23}.

Previous studies in quails reported that type 2 deiodinase (Dio2), which is involved in the seasonal reproduction (season-dependent testis development) of males, is induced by the mediobasal hypothalamus of the brain during reproduction²⁴. It converts the low active form of thyroid hormones in the hypothalamus into an active form [*i.e.*, triiodothyronine (T3)], which then promotes testicular development²⁵. Notably, in hamsters exhibiting seasonal reproduction²⁶, Dio2 expression has been reportedly suppressed by daily injections of

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exogenous melatonin²⁷. The antigenadotrophic effect of melatonin has also been reported in female mammals²¹. For example, daily injection of melatonin into female rats decreases the incidence of estrus and reduces ovary weight²⁸. In humans, melatonin receptors are expressed in various cell types of the female reproductive tract^{29–33}. Furthermore, clinical and *in vitro* studies have provided evidence that melatonin inhibits human ovulation^{34–37}.

Proper control of plasma calcium concentrations is important for numerous physiological functions, such as neurotransmission, muscle contraction, and blood coagulation^{38,39}. In vertebrate females, calcium demands are exacerbated during the reproductive stage. For example, a study using a mouse model with hypocalcemia revealed that calcium deficiency results in a significantly reduced number of oocytes after ovarian stimulation, as well as a reduced ability to mature⁴⁰. This suggests that serum calcium levels play an important role in determining the number and maturation of oocytes in mammals. In nonmammalian vertebrates, including bony fishes, plasma calcium levels increase exactly at the same time as reproductive development in females^{41–44}. Conversely, the calcium supply is terminated immediately after reproduction to prevent it from leaving the body. Whether this occurs and how the plasma calcium concentration decreases from the late reproductive to non-reproductive stages is unclear.

Mammalian bone tissue consists of osteoblasts, osteoclasts, osteocytes, and calcified bone matrices⁴⁵. It stores calcium and is involved in regulating blood calcium levels⁸. Parafollicular cells in the thyroid gland produce calcitonin (CT)⁴⁶, a hormone that binds to the CT receptor on osteoclasts and inhibits their activity to reduce the blood calcium level⁴⁷. In contrast, the parathyroid gland secretes parathyroid hormone (PTH), which promotes osteoclast activity to increase blood calcium levels⁴⁸.

The regulatory mechanisms for internal calcium are similar between bony fishes and mammals^{8,49}. Freshwater bony fishes, such as goldfish and rainbow trout, store internal calcium in their scales⁵⁰, which, like mammalian bone, are comprised of osteoblasts, osteoclasts, osteocytes, and calcified bone matrix and are involved in regulating calcium levels in the blood^{51–53}. Notably, freshwater bony fishes rely on their internal calcium stores in their scales to increase calcium availability during the reproductive period.

In freshwater bony fish scales, PTH activates osteoclasts⁵⁴, whereas CT acts as an inhibitor of osteoclastic activity^{55,56}. Previously, we found that melatonin increases CT levels and suppresses osteoclastic resorption in the goldfish scale^{57,58}. Therefore, we hypothesized that melatonin-induced CT-dependent suppression of osteoclast activity in scales may reduce plasma calcium concentrations following reproduction in female goldfish. This study provides evidence supporting our hypothesis, and we propose that the anti-calcium effect of melatonin is required for the efficient transition from the reproductive to non-reproductive stages in these fish.

Results and discussion

The plasma calcium level increases during the reproductive stage in female goldfish and sharply decreases after the reproductive stage

The reproductive stage of goldfish was reported to be from March to May^{59–61}. It was also reported that the level of the gonadosomatic index (GSI), an indicator of gonadal development, was low in goldfish around August, demonstrating that August is the non-reproductive stage of goldfish^{59–61}. To identify the reproductive stage of female goldfish in our experimental setting and to determine whether blood calcium levels change during the reproductive stage, the GSI and the plasma calcium levels in female goldfish were measured from December to August. Figure 1A displays that the GSI level was highest in March, which was significantly higher than those in December, June, and August, but not when compared with the other months tested. Based on this result, the reproductive stage of female goldfish in our experimental setting was confirmed to occur from January to May. In addition, December was defined as the pre-reproductive stage, and June to August as the post-reproductive stage in our experimental setting.

The plasma calcium level was highest in May and sharply decreased thereafter (Fig. 1B). The plasma calcium level in May was significantly higher than those in December, January, June, and August, but not when compared with the other months tested. These results indicate that plasma calcium levels increase during the reproductive stage in female goldfish and decrease at the post-reproductive stage. Consistent with our results here, previous studies showed that plasma calcium, which is required for the formation of yolk proteins⁶², is elevated in reproductive stage bony fishes^{43,50}. Saltwater fish use calcium transport mechanisms in their gills and gastrointestinal tract to obtain calcium from the environment and regulate plasma calcium levels^{63,64}. Freshwater fish such as goldfish and rainbow trout rely on internal calcium stores in their scales as well as environmental sources to increase calcium availability during the reproductive period^{65,66}. There is also evidence that osteoclasts detected in bony fish scales regulate plasma calcium levels^{49,67,68}. To determine whether scales are involved in elevated plasma calcium during the reproductive stage of female goldfish, we evaluated osteoclast activity in female goldfish scales during and after the reproductive stage, as described below.

Osteoclasts in the scales of the female goldfish are activated during the reproductive stage

Tartrate-resistant acid phosphatase (TRAP) is the characteristic marker of osteoclast activity⁶⁹. We first examined whether the osteoclastic activities in the female goldfish scales are higher in the reproductive stage (January to April) than in the post-reproductive stage (June) by TRAP staining assay. Consistent with a previous study⁵⁴, TRAP was detected in the scales of female goldfish (Fig. 2A). Ratios of scales expressing TRAP in female goldfish were determined from January to August (Fig. 2B). The ratio at the late reproductive stage (April) was significantly higher than that at the early reproductive stage (January). Then, the ratios of TRAP-negative scales in the female goldfish were determined. The ratio at the post-reproductive stage (June) was significantly higher than those at the reproductive stage (February and April).

Next, the gene expression levels involved in the osteoclast activation, such as cathepsin K (*cathK*)⁷⁰, Vacuolar-ATPase subunit d2 (*V-ATPase*)⁷¹, and receptor activator of NF- κ B ligand (*rankl*)⁷², were examined in the scales of female goldfish before (December), during (from January to May), and after (June and/or August) the

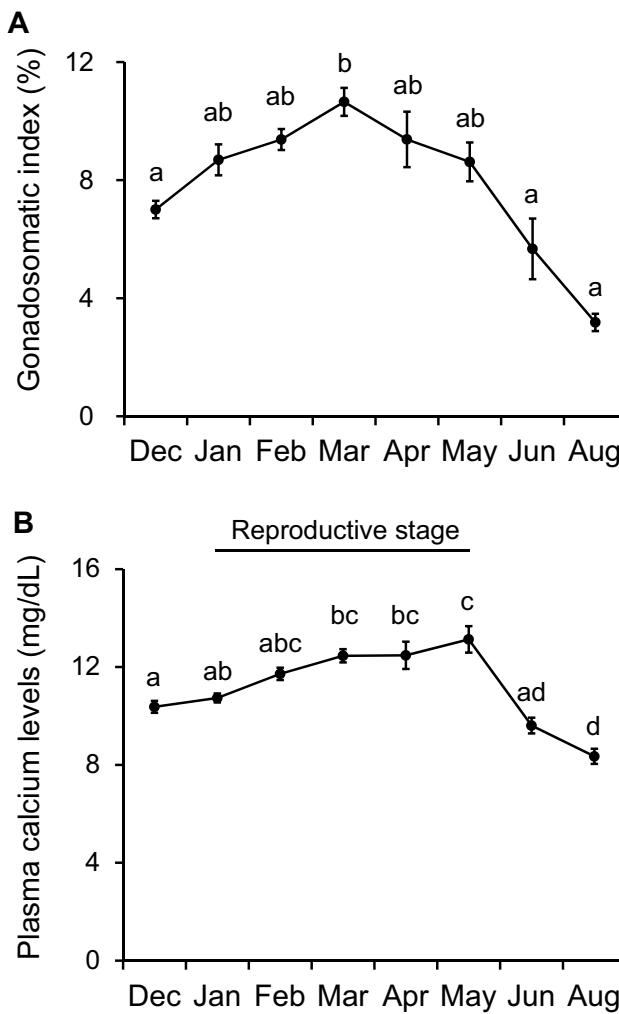


Fig. 1. Seasonal changes in gonadosomatic index (GSI) (A) and plasma calcium levels (B) of female goldfish were measured from December 2008 to August 2009 (each month $n = 10-23$). Different letters indicate significant differences ($P < 0.05$). Abbreviations: Dec, December; Jan, January; Feb, February; Mar, March; Apr, April; Jun, June; Aug, August.

reproductive stage (Fig. 2C). The *cathK* expression during the late reproductive stage (April) was significantly higher than that in other months tested. Additionally, *cathK* expression during the reproductive stage (January to April) was significantly higher than during the pre-reproductive stage (December). *V-ATPase* expression during the mid-reproductive stage (March) was significantly higher than during the pre- and post-reproductive stages. Furthermore, *V-ATPase* expression during the early reproductive stage (January to March) was significantly higher than during the post-reproductive stage. *Rankl* expression during the reproductive stage (January to April) was significantly higher than that of the pre-reproductive stage. Furthermore, the *rankl* expression at the part of the reproductive stage (January and April) was significantly higher than that of the post-reproductive stage (June). Overall, our results reveal that osteoclasts in female goldfish scales are activated during the reproductive stage, and further, this activation is suppressed during the final part of the stage. The activated osteoclasts in the scales are speculated to contribute to increased plasma calcium levels^{49,60}, which is required for the formation of the egg yolk protein in female individuals during the reproduction stage^{73,74}.

17β -estradiol (E_2) is a female-dominant sex hormone that plays a critical role in reproduction and sexual differentiation in vertebrates^{75,76}. Studies have shown that E_2 receptor is expressed in scale osteoclasts of sea bream (*Sparus auratus*) and Mozambique tilapia (*Oreochromis mossambicus*)⁷⁷, and that E_2 increases the osteoclast activity in rainbow trout scales⁶⁸. E_2 receptor has also been shown to be expressed in goldfish scales⁷⁸. Additionally, plasma E_2 concentrations have been shown to increase during the reproductive stage in female goldfish⁵⁹. Based on these findings, we tested whether E_2 activates osteoclasts in female goldfish scales, as described below.

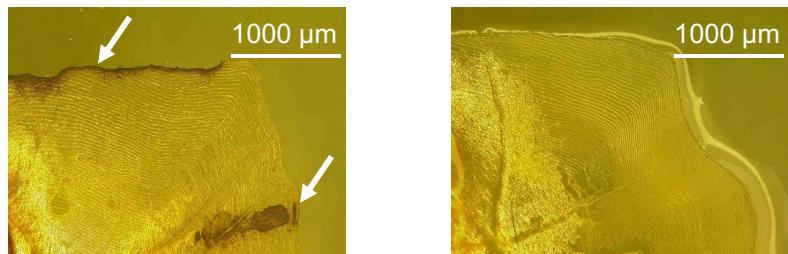
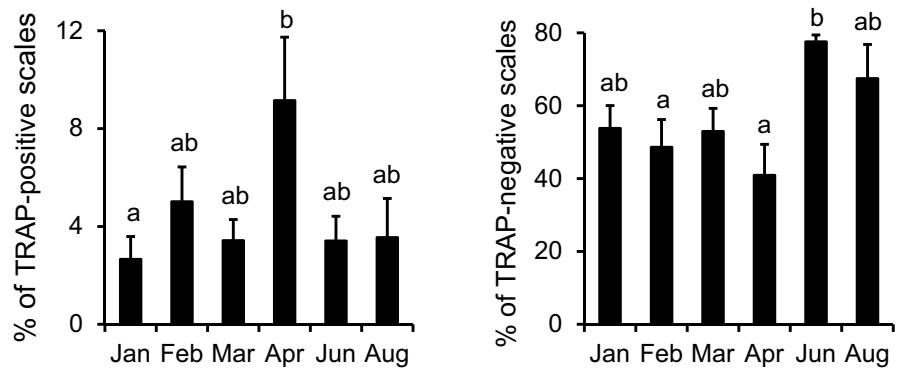
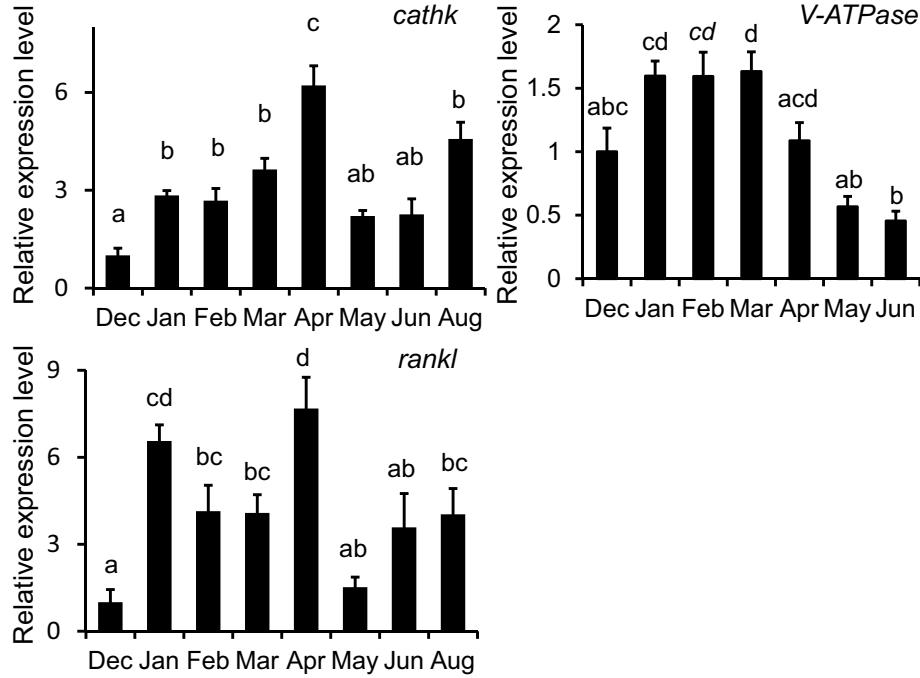
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Fig. 2. Seasonal changes in osteoclast activity in female goldfish scales. (A) Representative samples of goldfish scale expressing tartrate-resistant acid phosphatase (TRAP) (left panel) and the TRAP negative scale (right panel); white arrows indicate TRAP-stain-positive area. (B) Ratios of TRAP-positive scales in female goldfish (left graph) and the ratios of TRAP-negative scales in female goldfish (right panel) from January 2009 to August 2009 (each month $n = 4-6$; 200 scales were analyzed for each individual). Different letters indicate significant differences ($P < 0.05$). (C) Expression levels of genes involved in osteoclast activation were examined in female goldfish scales from December 2008 to August 2009 [for cathepsin K (*cathK*) and receptor activator of nuclear factor κB ligand (*rankl*)] or June 2009 [for vacuolar-ATPase subunit d2 (*V-ATPase*)] (each month $n = 8$ for each gene examined). Different letters indicate significant differences ($P < 0.05$). Average expression levels in December (Dec) were set to 1. Abbreviations: Dec, December; Jan, January; Feb, February; Mar, March; Apr, April; Jun, June; Aug, August.

Plasma E₂ level in female goldfish increases during the reproductive stage, and exogenous E₂ promotes expression of gene involved in osteoclast activation

We compared plasma E₂ levels during and after the reproductive period. As shown in Fig. 3A, the E₂ level in late March (March 24th) was significantly higher than that in early March (March 8th). It was also significantly higher than those of late and post-reproduction stages. On the other hand, the E₂ level in late March (March 24th) was not significantly different from those of April. Although the number of goldfish used in this measurement was small (n=3-5 at each point), these results are consistent with a previous study, which showed elevated plasma E₂ levels during the reproductive period in female goldfish⁵⁹.

In October, during the non-reproductive stage, exogenous E₂ (4 μ g/g of body weight) was injected intraperitoneally into female goldfish. Expression of the gene involved in osteoclast activation, *cathK*⁷⁰, was determined in the scales. As shown in Fig. 3B, exogenous E₂ significantly increased the expression of *cathK* in the scales, evidence that E₂ increases osteoclast activity in goldfish scales. It is important to note that the number of goldfish used in this measurement was small (4 in the control group and 3 in the E2-administered group). Our results are consistent with those of previous studies where E₂ treatment increased scale osteoclastic activity and decreased scale calcium content, indicating an E₂-mediated increase of scale resorption in rainbow trout^{66,68}. In freshwater bony fishes, including goldfish⁶⁵, killifish⁶⁵, and rainbow trout⁶⁶⁻⁶⁸, the increased demand for calcium during the reproductive stage is met through mobilization of internal calcium stores, predominantly from the scales, so bone is initially preserved. E₂ treatment has been shown to decrease pharyngeal bone

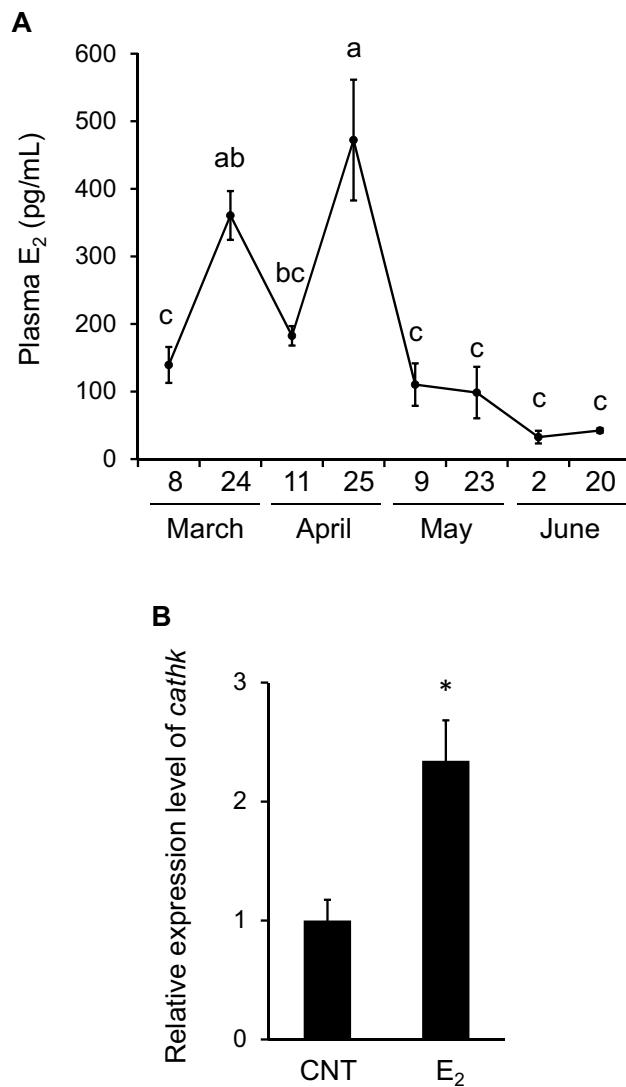


Fig. 3. Changes in plasma 17 β -estradiol (E₂) levels in female goldfish and effects of exogenous E₂ on expression of osteoclastic gene in the scales of female goldfish. (A) Plasma E₂ levels of female goldfish were measured from March 2016 to June 2016 (each month, n = 3-6). Different letters indicate significant differences ($P < 0.05$). (B) mRNA expression of *cathepsin K* (*cathK*) in the scales of goldfish without (CNT) and with injection of E₂ (E₂). The female goldfish were injected intraperitoneally with 4 μ g/g body weight of exogenous E₂ in October 2016. * $P < 0.05$ indicates a significant difference vs. control.

resorption in rainbow trout⁶⁶, consistent with E₂'s physiological role in mammals and birds, where E₂ decreases bone resorption^{79,80}. It appears that in freshwater teleosts, E₂ promotes scale resorption and supplies calcium to protect the skeleton during the reproductive stage.

mRNA expression of calcitonin (CT) and its receptor (CTR) in the scales of female goldfish increases during the late and post-reproductive stages

CT binds to CTR on osteoclasts and inhibits their activity, reducing the amount of calcium that enters the bloodstream⁸. In addition, osteoblast lineage cells produce CT, which inhibits the activity of osteoclasts in scales of bony fish, including goldfish^{57,58}. To examine whether CT is involved in the decreased osteoclast activity in the scales of female goldfish during the late and post-reproductive stages, the mRNA expression of CT and CTR was determined from female goldfish scales during the reproductive (March to May) and post-reproductive (June) stages. As shown in Fig. 4, the mRNA expression of CT was highest at late April (April 25th), and this was significantly higher than that at late March (March 24th) and early April (April 11th). In addition, the mRNA expression of CTR at late April (April 25th) and early May (May 9th) was significantly higher than that in March (March 8th and 24th) and early April (April 11th). These mRNA expression profiles of CT and CTR support the

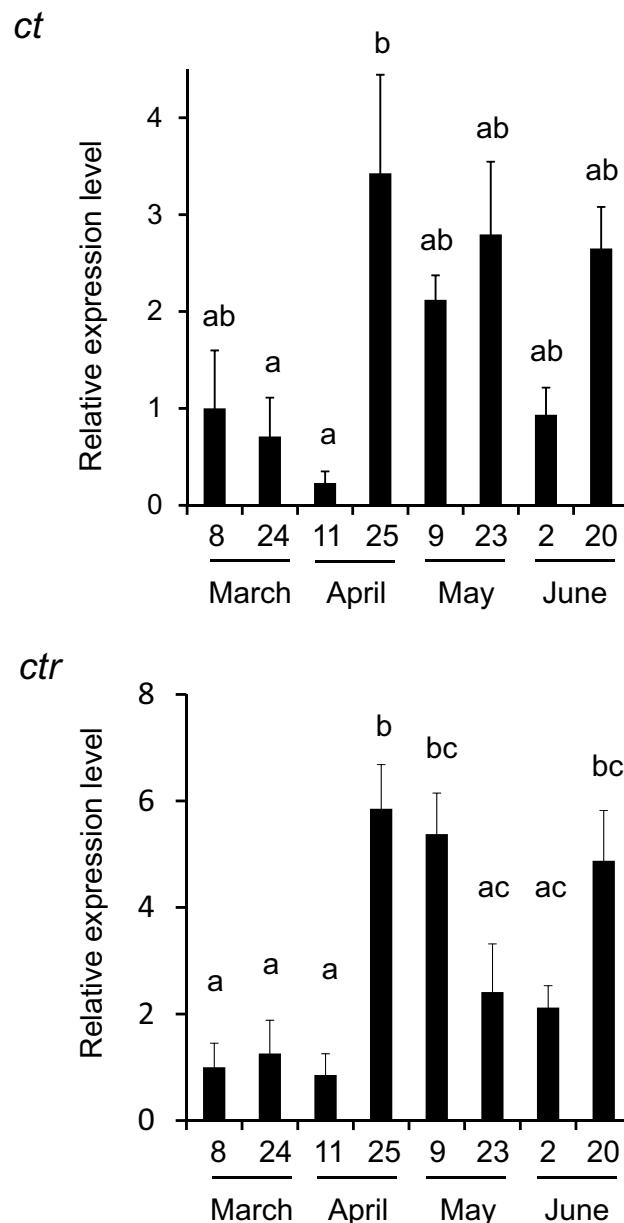


Fig. 4. Changes in mRNA expression of calcitonin (*ct*) and its receptor (*ctr*) in female goldfish scales from March 2016 to June 2016 (each month, $n = 4$). Different letters indicate significant differences ($P < 0.05$). The average expression on March 8 was set to 1.

idea that CT function in goldfish scales increases during the late and post-reproductive stages, which could decrease goldfish scale osteoclast activity and plasma calcium levels in female goldfish.

mRNA expression of the melatonin receptor *Mel1a1.4* in the scales of female goldfish increases during the late reproductive and post-reproductive stages

In a previous study, we found that melatonin treatment increases CT expression and suppresses osteoclast activity in cultured goldfish scales⁵⁸, suggesting that melatonin may play a role in reducing the scales' osteoclast activity and the plasma calcium level in female goldfish during the late reproductive stage. To test this idea, melatonin levels were measured during (March to May) and after (June) the reproduction stage. As melatonin levels have been known to change during day and night^{9,81}, they were separately measured at different times of the day and night. As shown in Fig. 5A, melatonin levels were higher at night than during the day. Furthermore, melatonin levels both at daytime and nighttime were unchanged during and after the reproduction stage. Accordingly, in the current study, plasma melatonin levels did not correlate with reproductive activity in female goldfish. It is important to note that the number of goldfish used in this measurement was small (n=4 at each point).

Melatonin shows its effects by binding to melatonin receptors in the plasma membrane to activate the intracellular signaling pathways^{82,83}. In addition, our previous study reported that melatonin acts on osteoblast lineage cells by binding to melatonin receptors, which promotes CT production in goldfish scales⁵⁸. Accordingly, the melatonin receptor *mel1a1.4* expression levels in the scales of female goldfish were determined during and after the reproduction stage. As shown in Fig. 5B, nighttime *mel1a1.4* expression levels reached their peak during the late reproductive stage [from late April (April 25th) to early May (May 9th)]. These levels were significantly higher than during the middle reproduction stage [from early March (March 8th) to early April (April 11th)]. In addition, daytime *mel1a1.4* expression level was highest at late April (April 25th). The *mel1a1.4* expression level at late April (April 25th) was significantly higher than that in early March (March 8th), but not when compared with the other time points tested. In particular, the nighttime *mel1a1.4* expression level supports the idea that melatonin suppresses osteoclast activity in the scales to reduce plasma calcium levels in female goldfish during the late part of the reproductive stage. The expression of melatonin receptors during reproduction has been enhanced in organisms other than goldfish. For example, the mRNA levels of the melatonin receptors MT1 and MT2 in the developing ovarian follicles of mares were higher during the reproductive than during the non-reproductive periods⁸⁴. The mRNA levels of two of the three melatonin receptors in turbots, a flatfish species, were also elevated in the female gonads during the late reproductive stage⁸⁵. A similar seasonal regulation of melatonin receptor expression was also observed in the testes of birds⁸⁶. These findings suggest that the seasonal regulation of physiological functions' response to melatonin is driven by reproduction-related changes in melatonin receptor expression.

Exogenous melatonin suppresses plasma calcium levels in female goldfish during the late reproductive stage

To gather further evidence of the role of melatonin in the regulation of plasma calcium levels in female goldfish during the reproductive stage, the effects of intraperitoneally administered exogenous melatonin on plasma calcium levels in female goldfish were measured (Fig. 6A). Various melatonin doses were intraperitoneally injected into female goldfish during the late reproductive stage. Plasma calcium levels were then measured in melatonin-treated goldfish. High-dose melatonin (10 µg/g of body weight) suppressed plasma calcium levels, whereas low-dose melatonin (0.1 µg and 1 µg/g of body weight) did not. Previous studies have used high exogenous melatonin concentrations to demonstrate its remarkable effects on physiological processes, including bone metabolism in rats⁸⁷, hypnotic effects on humans^{88,89}, and human fertilization^{90,91}. Next, the effects of intraperitoneally administered exogenous melatonin on plasma calcium levels in female goldfish were measured at various time points during the reproduction stage (Fig. 6B). Exogenous melatonin significantly suppressed plasma calcium levels when administered during the late reproductive stage (April). Conversely, exogenous melatonin did not affect plasma calcium levels when administered at the early reproductive stage (February). Previously, we found that melatonin increases CT levels, an inhibitor of osteoclastic resorption^{53,56}, in goldfish scales^{57,58}. Thus, the suppression of plasma calcium concentration by exogenous melatonin may be mediated by the CT-dependent suppression of osteoclast activity in scales. In addition, *mel1a1.4* expression at night in female goldfish scales was higher during the late reproductive stage (April) compared with the mid-reproductive stage (March) (Fig. 5B). The upregulation of *mel1a1.4* expression was induced during the late reproductive stage, which suggests a mechanism for the period-dependent suppressive effect of melatonin on plasma calcium levels in female goldfish (Fig. 6B).

In freshwater bony fish, such as goldfish and rainbow trout, E₂ increased osteoclastic activity in the scales and released calcium stored in the scales into the blood to increase its availability during the reproductive period^{49,67,68}. The plasma E₂ levels were enhanced during reproduction in female goldfish (Fig. 3A). In addition, exogenous E₂ injection increased the expression of *cathK*, which is involved in osteoclast activation in female goldfish scales⁷⁰ (Fig. 3B). These results suggest that scales contribute to the plasma calcium supply during reproduction in female goldfish. We also found that plasma calcium levels decreased during the post-reproductive stage. Previously, we reported that melatonin acts on osteoblast lineage cells by binding to melatonin receptors and promoting CT production, an inhibitor of osteoclast resorption^{55,56}, in goldfish scales^{57,58} (Fig. 7). We propose that the melatonin-induced CT-dependent suppression of osteoclast activity in scales contributes to a decrease in plasma calcium levels after reproduction in female goldfish, based on the following findings (Fig. 7). First, osteoclast marker expression in the scales decreased during the late and post-reproductive stages in female goldfish (Fig. 2). Second, the expression of CT and CTR in female goldfish scales increased during the late and post-reproductive stages (Fig. 4). Third, the expression of the melatonin receptor *mel1a1.4* in female goldfish scales increased during the late reproductive and post-reproductive stages (Fig. 5B). Finally, exogenous

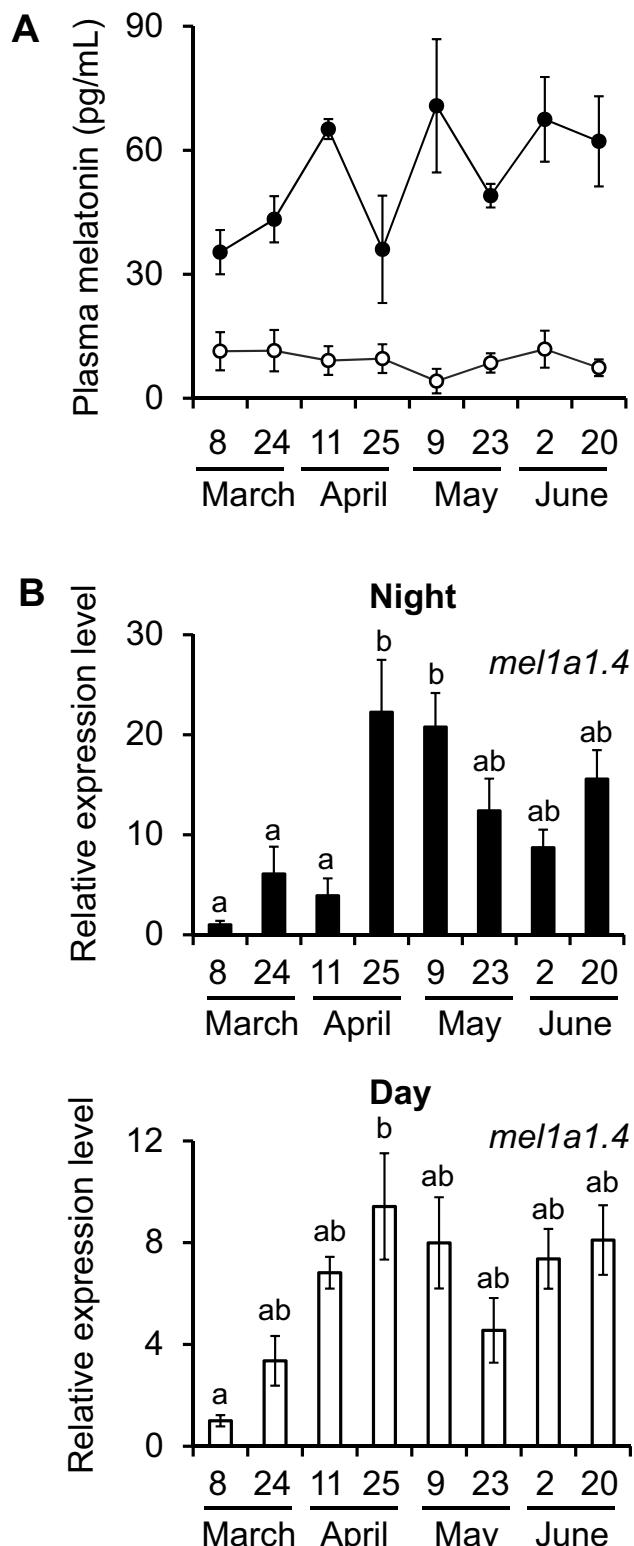


Fig. 5. Seasonal changes in plasma melatonin levels and melatonin receptor *Mel1a* 1.4 (*mel1a* 1.4) expression levels of female goldfish scales. (A) Plasma melatonin levels in goldfish at midnight (closed circle, each time point $n = 4$) and at midday (open circle, each time point $n = 4$) from March 2016 to June 2016. (B) mRNA expression levels of *mel1a* 1.4 of goldfish scales at midnight (upper graph; each time point $n = 6$) and at midday (lower graph; each time point $n = 6$) from March 2016 to June 2016. Different letters indicate significant differences ($P < 0.05$). The average expression on March 8 was set to 1.

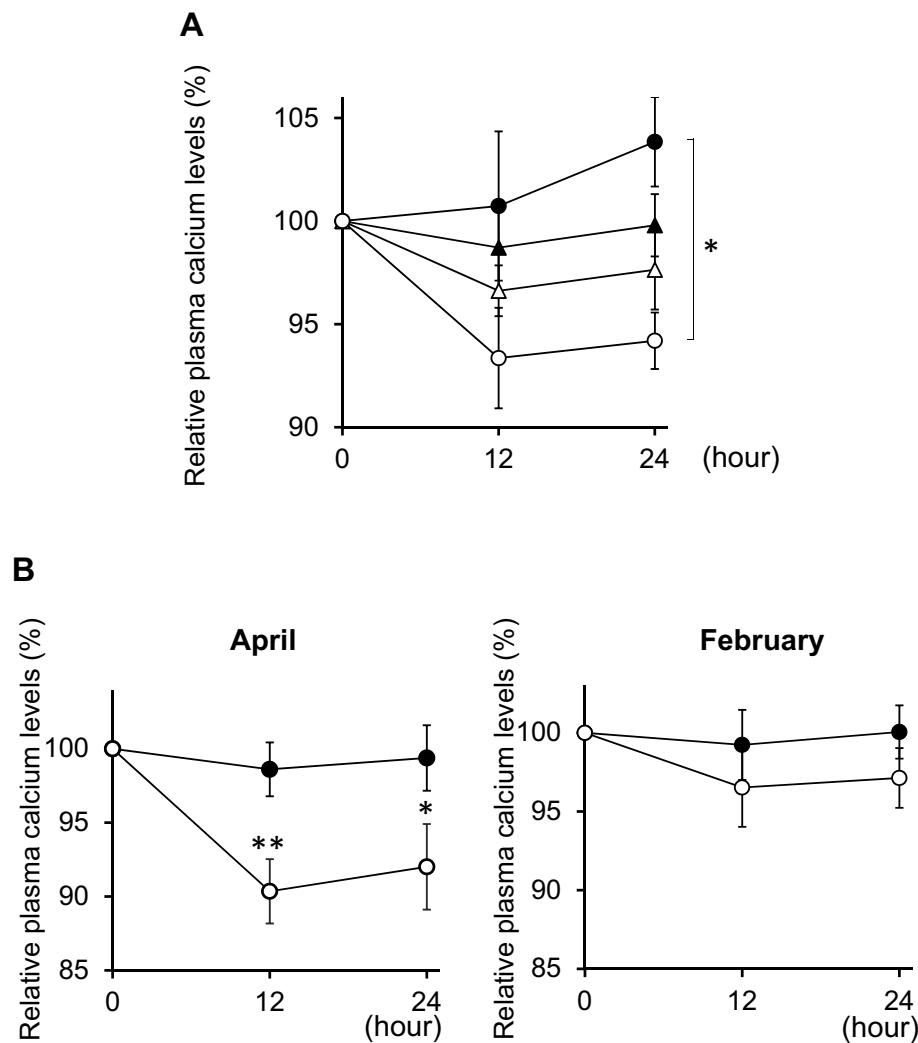


Fig. 6. Effects of melatonin on plasma calcium levels in female goldfish. **(A)** Effects of different doses (0.1 µg, 1 µg, and 10 µg/g body weight) of exogenous melatonin on plasma calcium levels in female goldfish were evaluated at midnight ($n = 9$ or 10) in April 2010. The horizontal axis in the graph depicts the time (hours) after exogenous melatonin administration. Value at time point 0 was set as 100% for each result of the melatonin doses. $*P < 0.05$ indicates significant difference vs. control at 24 h after melatonin (10 µg/g body weight) injection. Close circle, control group; close triangle, 0.1 µg/g body weight melatonin-administered group; open triangle, 1 µg/g body weight melatonin-administered group; and open circle, 10 µg/g body weight melatonin-administered group. **(B)** Effects of exogenous melatonin (10 µg/g body weight) on plasma calcium levels in female goldfish were evaluated (each time point $n = 8$ –12) in April 2009 (left graph) and February 2009 (right graph). The horizontal axis in each graph shows the time (hours) after exogenous melatonin administration. The value at time point 0 was set as 100% for each graph. $*P < 0.05$ and $**P < 0.01$ indicate significant differences vs. control at each time point. Close circle: control groups, open circle: melatonin-administered groups.

melatonin suppressed plasma calcium levels in female goldfish during the late reproductive stage in cases of increased melatonin receptor expression (Fig. 6). Because calcium is needed for eggshell protein formation in fish^{73,74}, the melatonin-mediated decrease in plasma calcium levels could halt the reproduction stage, potentially preventing calcium excretion from the body.

Methods

Ethical treatment of animals

This study was conducted in accordance with the recommendations in the ethical guidelines of Tokyo Medical and Dental University (TMDU). All experimental protocols were approved by the Animal Welfare Committee of TMDU (permit number: 0170339 A). Additionally, the protocols were in accordance with the ARRIVE guidelines 2.0⁹². All experiments were performed in a manner that minimized pain and discomfort.

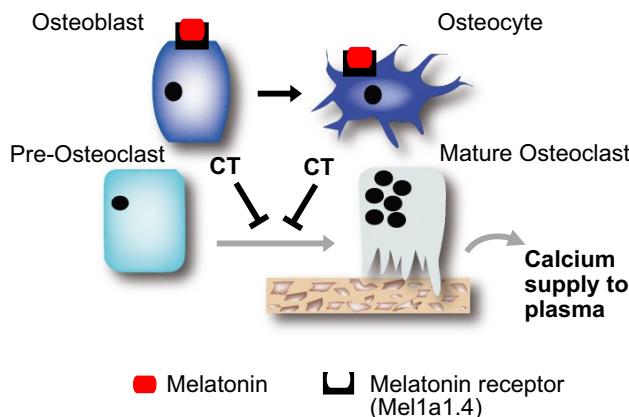


Fig. 7. Proposed model of the mechanism underlying the suppressive effect of melatonin on plasma calcium level. T lines denote inhibitory effects on target pathways. Suppressed pathways are indicated in gray. CT: calcitonin.

Goldfish

Female goldfish (*Carassius auratus*) were purchased from Higashikawa Fish Farm (Yamatokoriyama, Japan). The origin of the female goldfish used in the current study was of the Wakin variety (Japanese goldfish). All of the fish were bred for generations in the Higashikawa Fish Farm and had typical Wakin characteristics, which are the closest to the original goldfish species, with a carp-like tail, carp-like shape, and red body color. The mean and standard error for the weight of the goldfish used in this study were 41.85 ± 1.24 g.

Goldfish were maintained under outdoor conditions during experimental periods (described in each figure) at an open site on the rooftop of the third building of College of Liberal Arts and Sciences of the TMDU, Ichikawa. Here, seasonal changes in the gonadosomatic index (GSI), plasma calcium concentrations, and scale osteoclast activity were examined.

Determination of the GSI

Goldfish were weighted and sacrificed with benzocaine (Sigma-Aldrich, St Louis, MO, USA). Ovaries were collected and weighed. Based on these measurements, the GSI, *i.e.*, the ovarian (gonad)-to-body weight ratio (gonad weight/body weight $\times 100$) was calculated. The number of goldfish used in this analysis was 20 in December, 16 in January, February, March, and April, 23 in May, 14 in June, and 10 in August.

Measurement of plasma calcium, E_2 , and melatonin concentrations

To collect plasma, goldfish were first anesthetized with benzocaine, and whole blood was collected from the caudal vein using a 1-mL heparinized syringe with a 21-G needle (Terumo Corporation, Tokyo, Japan). Plasma samples were obtained after blood centrifugation at $1000 \times g$ for 5 min at 4°C and then stored at -80°C to measure the plasma calcium or melatonin concentration.

Plasma calcium concentrations were measured using the Calcium E Test Wako (FUJIFILM Wako Pure Chemical Industries, Osaka, Japan) following the manufacturer's instructions. The number of goldfish used in this measurement was 20 in December, 16 in January, February, March, and April, 23 in May, 14 in June, and 10 in August.

Plasma E_2 concentrations were measured using the estradiol enzyme-linked immunosorbent (ELISA) assay kit (Cayman Chemical, Ann Arbor, MI, USA) following the manufacturer's instructions. The number of goldfish used in this measurement was 4 on March 8th, 6 on March 24th, April 11th, April 25th, May 9th and June 2nd, 5 on May 23rd, and 3 on June 20th.

For the measurement of plasma melatonin concentration, four times the volume of acetone was added to the goldfish plasma and stirred 10 min using a vortex mixer, followed by centrifugation at $20,800 \times g$ for 10 min. Supernatants were then transferred into clean test tubes and dried at 65°C under a stream of nitrogen gas. After dissolving residues into 100- μL of Milli-Q water, mixtures were filtered through 0.22 μm -pore filters (Centrifugal Filter Units Ultrafree-MC-GV 0.22 μm , Merck Millipore, Darmstadt, Germany) and stored at -80°C until liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis. The number of goldfish used in this measurement was 4 for each time point.

For the LC-MS/MS analysis of melatonin, 10 μL samples were injected into an HPLC system (AC30AD, Shimadzu Corporation, Kyoto, Japan) equipped with a C18 2.0 \times 150-mm, 3 μm Kinetex column (Tosoh, Tokyo, Japan). The mobile phase comprised 10 μM ammonium acetate in 0.05% (v/v) acetic acid with varying MeOH concentrations. The linear gradient was run for over 20 min from 5% to 50% and then maintained at 100% MeOH for 10 min. The flow rate was 0.3 mL/min, and the auto sampler and column oven were maintained at 4°C and 25°C , respectively. Melatonin was detected using a triple quadrupole mass spectrometer (LC-MS-8050, Shimadzu) and quantified using the multiple reaction monitoring method with transitions of parent ions to product ions. The transition for melatonin was m/z 233.0–130.0. The limit of sensitivity for melatonin was 11.1 fg for a 2:1 signal-to-noise ratio. Intra- and inter-assay variation coefficients were 3.94% and 4.60%, respectively.

Evaluation of the effect of exogenous melatonin on plasma calcium levels in female goldfish

Sexually mature female goldfish were anesthetized with benzocaine and intraperitoneally injected with melatonin (FUJIFILM Wako Pure Chemical Industries, Ltd.) in February or April. Melatonin was dissolved in dimethyl sulfoxide (FUJIFILM Wako Pure Chemical Industries). Goldfish were kept continuously under outdoor conditions. Their plasma calcium levels were determined at 12 and 24 h after the injection of melatonin, as described in the previous section. The number of goldfish used in the evaluation in Fig. 6A was 9 for the control group and 0.1 µg/g body weight melatonin-administered group, 10 for 1 µg/g body weight melatonin-administered group and 10 µg/g body weight melatonin-administered group. The number of goldfish used in the February 2009 evaluation in Fig. 6B was 12 in the control group and 11 in the melatonin-administered group, respectively. The number of goldfish used in the April 2009 evaluation in Fig. 6B was 8 for each of the control and melatonin-administered groups.

Evaluation of the effect of exogenous E₂ on osteoclastic gene expression in female goldfish scales

Sexually mature goldfish were maintained under outdoor conditions prior to the experiment. They were then acclimated for 1 week under indoor conditions with a 12L:12D photoperiod and a controlled temperature of 26 °C ± 1°C. The female goldfish were anesthetized with benzocaine and injected intraperitoneally with 4 µg/g body weight of E₂ (Sigma-Aldrich). Osteoclastic gene expression in the scales was analyzed with RT-PCR at 3 days after injection of E₂. The number of goldfish used in the evaluation was 4 in the control group and 3 in the E₂-administered group.

Histological observations of tartrate-resistant acid phosphatase (TRAP) activity in goldfish scales

Two hundred scales on the right side of the body were collected from each fish and then fixed in 10% formaldehyde in a 0.05 M cacodylate buffer (pH 7.4). Simultaneously, eight scales on the left side of the body of each fish were collected in ISOGEN (Nippon Gene, Tokyo, Japan) for quantitative real-time polymerase chain reaction (RT-PCR) analysis, as described in the next section.

The scales were stained to check for the presence of TRAP using a commercial acid phosphatase leukocyte kit (Sigma-Aldrich). The scales were washed using distilled water and incubated with TRAP-staining solution for 1 h at 37 °C under dark conditions. The assay components comprised naphthol AS-BI phosphate solution, diazotized fast garnet GBC base solution, sodium nitrite solution, acetate solution (pH 5.2), and 10 mM of L(+)-tartrate solution.

The scales were examined under two different conditions based on TRAP staining and scale erosion level: TRAP-stained negative and TRAP-stained positive scales. The former group was characterized by the total absence of TRAP staining points inside the scale, whereas the latter group was stained inside the scale and absorbed over five ridges (incremental line of scale) by the osteoclasts. The numbers of TRAP-stained negative and TRAP-stained positive scales were counted, and the proportion of each scale was determined. The number of goldfish used in the histological observations was 6 in January, February, March, April, and June, and 4 in August.

RT-PCR

Total RNA was extracted from goldfish scales using ISOGEN (Nippon Gene) following the manufacturer's instructions. Then, the total RNA was reverse-transcribed into cDNA using PrimeScript RT-PCR kit (Takara, Shiga, Japan). Each quantitative RT-PCR was performed using the MX3000 P ° QPCR System (Agilent, Tokyo, Japan). For a 10 µL PCR reaction, 1-µL containing the cDNA template was combined with 9 µL of the SYBR® Premix Ex Taq™ (Takara) and mixed with appropriate primers to a final concentration of 200 nM. The reaction mixture was incubated at 90 °C for 10 s, followed by 40 cycles at 95 °C for 30 s and at 60 °C for 40 s. β-actin was used for normalization. PCR primer sequences used in the current study are listed in Supplementary Table S1. The number of goldfish used in the RT-PCR at each time point was 8 for *cathk*, *V-ATPase*, and *rankl* and 6 for *ct*, *ctr*, and *mella1.4*.

Statistical analysis

The data are presented as means ± standard errors. Means of more than two groups were compared using analysis of variance. Multiple comparisons were evaluated with Tukey's honestly significant difference tests. Differences between the two groups were assessed with a Student's t-test. *P*-values <0.05 were considered statistically significant.

Data availability

Data that support the study findings are available from the corresponding authors upon reasonable request.

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Author contributions

Y.M., N.S. and A.H. contributed to the study conception. Y.M., K.W., R.M., and K.K. performed experiments. Y.M., K.W., R.M., K.K., H.M., N.S., J.H., and A.H. analyzed data. The first draft of the manuscript was written by J.H., K.W., Y.M., and A.H. All authors commented on it. All authors read and approved the final manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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