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(Citation)

Ecotoxicology, 30(6):1138-1149

(Issue Date)

2021-08

(Resource Type)

journal article

(Version)

Accepted Manuscript

(Rights)

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(URL)

<https://hdl.handle.net/20.500.14094/90008464>



Influence of salinity on physiological development and zinc toxicity in the marine medaka *Oryzias melastigma*

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Abstract

To determine whether the marine medaka *Oryzias melastigma* is a suitable model organism for evaluating the effects of environmental chemicals on marine teleosts, we examined the effect of salinity on physiological development and zinc toxicity. Growth as measured by total body length was significantly lower in fresh water compared to brackish water. Reproductive success was also significantly reduced in fresh water, although we observed cells in the pituitary producing gonadotropins such as Gpa (common glycoprotein hormone α), Fshb (follicle stimulating hormone β), and Lhb (luteinizing hormone β) at all salinities. These results indicate that *O. melastigma* is adaptable to various salinities from fresh to seawater, and brackish water is best for physiological processes including growth performance and reproduction. When zinc was dissolved in saltwater, a white precipitate formed immediately, and the dissolved concentration decreased in the supernatant and increased at precipitate. We performed zinc toxicity tests on early life stage and adult stage in fresh water, brackish water, and seawater. Among adults, the lowest observed effect concentration for mortality in freshwater (15.3 mg/L) was lower than in brackish water (> 48 mg/L) or seawater (> 48 mg/L). Similarly, among embryos and larvae, the lowest observed effect concentration for mortality in freshwater (4.8 mg/L) was lower than in brackish water (48 mg/L) or seawater (48 mg/L). These results highlight the importance of using marine organisms to evaluate the ecological effects of marine pollutants.

Key words: ecotoxicity, embryo development, reproduction, marine fish, physiology, salinity

Declarations

Data Availability

The authors confirm that all data underlying the findings are fully available without restriction.

Animal Research (Ethics)

The fish which was used in the present study were handled according to guidelines of Akita Prefectural University.

Consent to Participate (Ethics)

This research did not involve human subjects, so clinical trial registration is not applicable.

Consent to Publish (Ethics)

The authors certify that this manuscript is our original unpublished work, has not been published elsewhere, and is not under consideration by another journal. All authors have approved the manuscript and agree with its submission.

Plant Reproducibility

The authors confirmed reproducibility.

Author Contribution

All authors listed on the current study contributed to the experimental design or data analysis. (Yoshifumi Horie; All experiment except chemical analysis: Chiho Takahashi; chemical analysis).

Conflict of Interest

The authors declare that they have no conflict of interest.

Funding

This study was supported partly by the Salt Science Research Foundation, Japan (grant no. 1915), the Kurita Water and Environment Foundation, Japan (grant no. 18K007), and a grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan (Grant-in-Aid for Scientific Research [B] grant no. 19H04294) to Y.H..



1. Introduction

The potential adverse effects in aquatic ecosystems caused by environmental chemical substances are a serious international problem. Because environmental chemicals discharged from land will eventually flow into the sea via rivers or groundwater (Li and Barry, 1999; Martin and Whitfield, 1983; Saniewska et al., 2014), it is necessary to clarify their impacts not only on freshwater organisms but also on marine organisms. At the Organization of Economic Cooperation and Development (OECD), various test methods (OECD TGs) have been established for clarifying the influence of environmental chemicals on living aquatic organism. OECD TGs have been established using the Japanese medaka (*Oryzias latipes*), the zebrafish (*Danio rerio*), and the fathead minnow (*Pimephales promelas*) as model freshwater fish species and are frequently used in ecological risk assessment for environmental chemicals (Ankley et al., 2001; Mihaich et al., 2015; Horie et al., 2017). In contrast, convenient model marine fish for ecological risk assessment in seawater are still virtually unavailable in OECD TG, although acute toxicity using marine fish including topsmelt (*Atherinops affinis*), mummichog (*Fundulus heteroclitus*), Pinfish (*Lagodon rhomboides*), inland silversides (*Menidia beryllina*) and Sheepshead Minnow (*Cyprinodon variegatus*) were established in US-EPA (EPA-821-R-02-012).

Oryzias melastigma lives in brackish waters and estuaries, and it is generally found in shallow lagoons and swamps among roots and mangrove trees along coastal margins (Menon, 1999). The species is highly tolerant of changes in salinity and readily acclimates to freshwater and seawater environments (Inoue and Takei, 2002). Recent studies have posited that the species may be a useful seawater model for assessing the toxicity of environmental chemicals (Cong et al., 2017; Hong et al., 2015; Lee et al., 2014; Li et al., 2014; Yan et al., 2017; Yi and Leung, 2017) thanks to its short generation time, small size, ease of breeding in the laboratory, and sexual dimorphism. In addition, breeding conditions for *O. melastigma* are similar to those of its congener *Oryzias latipes* (Takehana et al., 2005), which is a freshwater model for the assessment of chemical impacts in tests established by the OECD. Although there has been a recent increase in efforts to evaluate the effects of chemicals on *O. melastigma*, salinities used in these studies have varied broadly from 15 to 35 (Chen et al., 2016; Dong et al., 2017, 2018; Lee et al., 2018; Yi and Leung, 2017). In OECD TGs, test conditions such as test temperature, photoperiod and stocking density are set because these conditions affect embryo development, growth, or reproduction in fish and also affect chemical toxicities (Crisp, 1993; Newman, 1993; Imsland et al., 2001; Davis et al., 2002; Sibly and Hone, 2002; Li et al., 2014, 2015; Kim et al., 2017). It is still unclear whether salinity

conditions adversely affect aspects of the *O. melastigma* life cycle such as embryo development, growth, or reproduction, although the species is capable of surviving in freshwater and seawater (Inoue and Takei, 2002).

To date, there have been numerous studies on the relationship between salinity and chemical toxicity (Grosell et al., 2007). For example, Li et al (2008) have reported that the 96-h LC₅₀ of boron is lower at 3‰ salinity than at 20‰ in *Litopenaeus vannamei*. Martins et al. (2011) have reported that acute copper toxicity is higher at 2‰ salinity than at 30‰ in *Callinectes sapidus*. Kwok and Leung (2005) have reported that copper and tributyltin toxicity declines with increasing salinity in *Tigriopus japonicus*. Finally, Bielmyer et al (2012) and Yung et al (2017) reported that Zn toxicity in *Fundulus heteroclitus*, *Kryptolebias marmoratus*, and *Thalassiosira pseudonana* decreased (LC₅₀ or IC₅₀ values increased) as salinity increased. These results indicate that salinity is an important environmental factor affecting chemical toxicity. However, the specific effect of salinity on toxicity of environmental chemicals in *O. melastigma* remains unclear.

The present study, therefore, revealed any influence of salinity on the life cycle in *O. melastigma*, especially on embryo development, growth, reproduction, and gonadal development. Next, we examined whether various salinities influenced acute zinc toxicity in adults, or influenced the effect of zinc on embryo development and hatching. The findings of this study will be useful for further ecotoxicological test assays established with marine fish.

2. Materials and Methods

2.1. Test chemicals and chemical analysis

Zinc chloride (CAS No. 7646-85-7, purity 98.0%) was obtained from the FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). The exposure concentration of zinc was set to one of six concentrations: control, 0.48, 1.53, 4.8, 15.3, and 48 mg/L. In the present study, we used dechlorinated tap water. The water quality of each salinity was as follows; Freshwater [0 PSU]; calcium ion concentration was 7.2 mg/L, magnesium ion concentration was 2.3 mg/L, water hardness was 27 mg/L. Brackish water [17 PSU]; calcium ion concentration was 270 mg/L, magnesium ion concentration was 630 mg/L, water hardness was 3300 mg/L. Seawater [34 PSU]; calcium ion concentration was 450 mg/L, magnesium ion concentration was 1200 mg/L, water hardness was 6200 mg/L.

Total zinc concentrations were detected by using an inductively coupled plasma-mass spectrometer (ICP-MS; Agilent 7700x, Agilent Technologies Japan, Ltd, Tokyo, Japan). Each water sample (25 mL) was placed in a fluororesin beaker, to which

68 % nitric acid (0.25 mL) was added. The beaker was heated at 150 °C on a hot plate. After leaving it to cool to room temperature, it was transferred to a test tube, and used for analysis. Analytical conditions were as follow; Sampling depth was 10 mm; Plasma gas flowrate was 15 L/min; Carrier gas flowrate was 0.35 L/min; HMI gas flowrate was 0.60 L/min; Collision cell gas (He) flowrate was 10 mL/min; Nebulizer Pump rotation speed was 0.1 rps; temperature of the spray chamber was 2°C. Internal standard method (internal standard element was Co) was used in ICP-MS analysis. Blank value was 0.000249 mg/L. Zinc and Cobalt standard solution were obtained from the FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

2.2. Study animals

Oryzias melastigma purchased from a local pet shop and confirmed by using 12S and 16S ribosomal RNA genes as reported before (Takehana et al., 2005). Fish were maintained in our laboratory at Akita Prefectural University from 2017. Artificial seawater was made based on Marine ART Hi (Osaka Yakken Co. Ltd, Osaka, Japan). The fish which was used in the present study were handled according to guidelines of Akita Prefectural University.

2.3. Influence of salinity on embryonic development

The influence of salinity (freshwater [0 PSU], brackish water [4, 8, and 17 PSU], and seawater [34 PSU]) on the embryonic stage of *O. melastigma* was conducted by using OECD TG 212 (OECD, 1998), with minor modifications. To assess the effects of salinity on embryonic development in *O. melastigma*, we used four replicate 100-mL glass vessels for each of five salinities [34, 17, 8, 4, and 0 PSU] at 25 ± 2 °C, for a total of 20 vessels. Fertilized eggs were transferred to each salinity within 2 hours post fertilization (10 fertilized eggs per vessel), and cultured until hatching (water exchange rate, once per 2 days). Embryos were observed at least once daily under a stereomicroscope. We used the following endpoints for observing the influence of salinity: mortality, hatching rate (% of fertilized eggs), and time to hatching. Dead embryos can decompose rapidly, so they were removed soon.

2.4. Influence of salinity on growth and gonadal sex differentiation

The effects of salinity (fresh water [0 PSU], brackish water [17 PSU], and seawater [34 PSU]) on growth and gonadal sex differentiation was conducted by using OECD TG 234 (OECD, 2011), with minor modifications. We used three salinities - fresh water [salinity = 0 PSU], brackish water [17 PSU], and seawater [34 PSU] - 25 ± 2 °C and an artificial

photoperiod of 14 h light:10 h dark. Fertilized eggs were transferred to each salinity within 2 h post fertilization and cultured until hatching in 100-mL glass vessels (water exchanged every day). After hatching, hatched larvae were transferred to 5-L cubic glass tanks at a density of 25 fishes per tank and cultured using a recirculating system. At 42 days post hatching (dph), fish were transferred to new 5-L cubic glass tanks at a density of 15 fishes per tank and cultured using recirculated water. After absorption of the yolk sac, the larvae were fed live, freshly-hatched brine-shrimp nauplii at approximately 10% of the fish body weight per day, divided into two feedings. Culture of embryos and post-hatch fish continued at the same salinities.

At 0, 30, 60, 90, and 120 dph, fish were anesthetized by using MS-222 (ethyl 3-aminobenzoate methanesulfonate; Sigma-Aldrich, St. Louis, MO, USA). Secondary sexual phenotype was determined on the basis of the shapes of the anal fin. Total body length and wet body weight were measured by using digital calipers (CD-S10C; Mitsutoyo, Kanagawa, Japan) and an electronic balance (ATX224; Shimadzu, Kyoto, Japan), respectively.

2.5. Influence of salinity on reproductive success

The effect of salinity (fresh water [0 PSU], brackish water [17 PSU], and seawater [34 PSU]) on reproductive success was conducted by using OECD TG 229 (OECD, 2009), with minor modifications. *O. melastigma* at 14-15 weeks post-fertilization were transferred to 5-L cubic glass tanks at a density of nine fishes (three females and six males) per tank. To assess the effects of salinity on reproductive success, we used four replicate 5-L cubic glass tanks for each of three salinities [fresh water, salinity = 0 PSU; brackish water, 17 PSU; seawater, 34 PSU] at 25 ± 2 °C with recirculating seawater and an artificial photoperiod of 14 h light:10 h dark, for a total of 12 tanks. Fish were cultured at each salinity for 21 d. Spawned eggs from the tanks with six males and three females were collected every morning, and the total number of eggs, number of fertilized eggs, and number of unfertilized eggs were counted under a stereomicroscope.

2.6. Immunohistochemistry

The antisera of common glycoprotein hormone α (Gpa) (Ikeuchi et al., 1999), follicle stimulating hormone β (Fshb) (Shimizu et al., 2012), and luteinizing hormone β (Lhb) (Shimizu and Yamashita, 2002) were used in this study. The specificities of these antisera and Immunohistochemistry (IHC) method have been described previously (Horie et al., 2014). Heads were fixed in Bouin's fixative solution for 15 h, respectively. After fixation, the heads were dehydrated using an ethanol series and embedded in Paraplast Plus

(McCormick Scientific, St. Louis, MO, USA). Five-micrometer-thick serial sagittal sections were obtained. For IHC, sections were rinsed in phosphate-buffered saline (PBS) and incubated with the primary antibodies anti-Gpa (1:1000), anti-Fshb (1:1000), or anti-Lhb (1:1000) overnight at 4 °C. After three washes with PBS, sections were incubated for 30 min at room temperature (RT) in biotinylated goat anti-rabbit IgG solution (Nichirei Biosciences, Tokyo, Japan). After three washes in PBS, peroxidase activity was visualized using 0.05% 3,30-diaminobenzidine (DAB) in Tris–HCl buffer (pH 7.6) and 0.01% H₂O₂. Sections were lightly counterstained with Carazzi's hematoxylin.

2.7. Adult medaka acute toxicity test

For these tests, we examined adult *O. melastigma* (i.e., 5–6 months post-hatching). Acute toxicity tests were conducted using three replicate 5-L glass tanks. Four *O. melastigma* were placed in each tank (i.e., a total of 12 fishes per treatment concentration) and exposed to the test solution for 96 h. Fish were not fed during the test period, and the test solution was exchanged every 48 h. We used mortality endpoint to determine the toxicological effect. Dead fish were removed as soon as they were observed because they can decompose rapidly. Tests were conducted three salinities [fresh water, salinity = 0 PSU; brackish water, 17 PSU; seawater, 34 PSU] at a temperature of 25 ± 2 °C and under a light:dark cycle of 16 h light, 8 h dark.

2.8. Embryo and sac fry short-term toxicity test

Short-term toxicity tests were conducted in accordance with methods described in previous studies (Horie et al., 2017). The following experiment was conducted to evaluate the effect of salinity on zinc toxicity. For each treatment concentration, we used four replicate 100-ml glass vessels (i.e., a total of 24 vessels per treatment). Eggs were selected under a microscope <2-h post-fertilization and exposed to each treatment concentration. After exposure, 10 fertilized eggs were distributed to each 100-ml glass vessel (i.e., a total of 40 fertilized eggs per treatment concentration) and cultured until 5 dph. Embryos and larvae were observed under a microscope every 24 h, and the test solution was exchanged every 48 h. We used mortality and hatching rate endpoints to determine the toxicological effects. Dead embryos and larvae were removed as soon as they were observed because they can decompose rapidly. Tests were conducted three salinities [fresh water, salinity = 0 PSU; brackish water, 17 PSU; seawater, 34 PSU] at a temperature of 25 ± 2 °C and under a light:dark cycle of 16 h light, 8 h dark.

2.9. Statistical analysis

Statistical analyses were conducted as reported previously (Horie et al., 2017; Kobayashi et al., 2000). All data were analyzed using Excel software (Microsoft, Redmond, WA, USA), R software ver 3.5.1, and the R package " Rcmdr " (Fox and Bouchet-Valat, 2018). Statistical analyses were conducted as follow; (1) Bartlett's test is used as a test for the equality of k variances (significance level, 5%); (2) Then, if the k sampled populations have equal variances assumed (i.e., the data are homoscedastic) ($p > 0.05$ by the Bartlett's test), Dunnett's multiple comparison test is performed; (3) If the k sampled populations have equal variances not assumed (i.e., the data are heteroscedastic) ($p < 0.05$ by the Bartlett's test), Steel's test is used. The lowest-observed-effect concentration (LOEC) is the lowest test concentration at which the substance is observed to have a statistically significant effect.

3. Results

3.1 Effects of salinity on embryonic development

The average times to hatching of the groups exposed to salinities of 4 and 8 PSU were significantly shorter than those in the group at 34 PSU ($p < 0.05$) (Fig. 1a). However, hatching rate between groups was not significantly affected by the salinities tested (Fig. 1b). During the embryonic development stage, mortality was negligible (<3%) at all salinities tested (data not shown). Furthermore, there were no abnormal embryos (e.g., small eyes or body malformations) observed at any of the salinities tested (data not shown).

3.2 Effects of salinity on growth

The wet body weight at 0 dph could not be determined with our electronic balance because the weight of fish was below the minimum measurable weight of 10 mg. The growth rates in terms of total body length in the freshwater exposure groups were significantly lower than that of brackish water groups (Fig. 2a). In terms of wet body weight, the growth rates of the freshwater exposure groups were also significantly lower than that of brackish water (Fig. 2b), but those in the seawater exposure groups were significantly higher.

3.3 Effects of salinity on reproduction

The fecundity and fertility in freshwater exposure groups were significantly lower than those in the brackish water groups (fecundity, $p < 0.05$; fertility, $p = 0.0205$) (Fig. 3a, b). The proportion of spawning females tended to be lower in both seawater and freshwater compared to brackish water, but none of these differences was significant

(Fig. 3c). In the seawater and freshwater groups, the number of fertilized eggs was well below that of the brackish water groups throughout the test period (Fig. 3d); in the seawater groups, the number of fertilized eggs increased following day 11 (Fig. 3d). Beginning at 30 dph, Gpa-, Fshb-, and Lhb-immunoreactive cells were observed in the pituitary of all medakas tested, both male and female (Fig. 4). At 60 dph, number of Fshb-immunoreactive cells in the freshwater group tended to be fewer than in the brackish water and seawater groups (Fig. 4k-2, l-2).

3.4 Effects of salinity on gonadal sex differentiation

In females at 30 dph, oocytes were observed at each stage of meiotic division, and in ovarian cavities in fish from all salinities (Fig. 5a, e, i). In males at 30 dph, initiation of spermatogenesis was seen in all salinity treatments (Fig. 5b, f, j). Interestingly, one of ten males in the freshwater group had testis-ova (Fig. 5m). At 60 dph, the ovaries in the brackish water and seawater groups contained many large, growing oocytes at the diplotene stage; in the freshwater group, the ovaries had only a few growing oocytes at the diplotene stage (Fig. 5c, g, k). There were no apparent differences between the testis at any of the salinities tested (Fig. 5d, h, l).

3.5. Water analysis

Table 1 shows zinc concentrations measured in the present study. In the brackish water and seawater groups (except in the controls), zinc concentrations were measured in the supernatant or precipitate due to the presence of white zinc precipitates in the 15.3 and 48 mg/L concentration groups. White precipitates were not observed in the freshwater exposure group and in the controls; therefore, zinc concentrations for these groups were measured in the supernatant. Zinc concentrations of 0.011 mg/L were measured in the freshwater and brackish water controls, and the concentration in the seawater control groups was 0.013 mg/L. In the brackish water and seawater groups where we observed the presence of white precipitates (zinc concentrations of 15.3 and 48 mg/L), zinc concentrations in precipitate exceeded those of the supernatant. In the lower zinc concentration groups (0.48, 1.53, and 4.8 mg/L), however, zinc concentrations did not differ between the supernatant and precipitate (Table 1).

3.6. Zinc acute toxicity test

Figure 6 shows the results of zinc acute toxicity testing on adult fish. In freshwater, all fish died within 24 h in the 48 mg/L treatment group, and they died within 48 h in the 15.3 mg/L treatment group. In the 4.8 and 0.48 mg/L treatment groups, 2 of 12 and 1 of

12 fish died, respectively. No dead fish were observed in the control or 1.53 mg/L treatment groups (Fig. 6a). In brackish water, 2 of 12 fishes died in the 48 mg/L treatment group, and no dead fish were observed in the control, 0.48, 1.53, 4.8, and 15.3 mg/L treatment groups (Fig. 6b). In seawater, 1 of 12 fishes died in the 48 mg/L treatment group, but no dead fish were observed in the control, 0.48, 1.53, 4.8, and 15.3 mg/L treatment groups (Fig. 6c).

3.7. Zinc short-term toxicity test

Figure 7 shows the results of zinc short-term toxicity testing on embryos and larvae. In freshwater, all fish died without hatching in the 48 mg/L treatment group (Fig 7a, b), and all fish died in the 15.3 mg/L concentration group (Fig 7b). In the 4.8 mg/L concentration group, hatching and survival rates were significantly reduced compared to controls (hatching rates, $p < 0.001$; survival rates, $p < 0.001$) (Fig 7a, b). In brackish water and seawater, hatching and survival rates in the 48 mg/L treatment group were significantly reduced compared to controls (hatching rates in brackish water, $p = 0.0122$; survival rates in brackish water, $p < 0.001$; hatching and survival rates in sea water, $p < 0.05$) (Fig 7c-f).

3.8. Lowest observed effect concentrations (LOECs) for each endpoint at each salinity treatment

LOECs for each endpoint at each salinity treatment are shown in Table 2. For all endpoints, the LOEC of zinc exposure was lower in freshwater than in brackish water or seawater. The LOEC for lethality was lower for embryos and larvae than for adults in every salinity group.

4. Discussion

Oryzias melastigma inhabits the coastal water and freshwater in Pakistan, India, Myanmar, and Thailand (Dong et al., 2014). Inoue and Takei (2002) have reported that *O. melastigma* adults are capable of surviving in waters with varying salinity values ranging from freshwater to seawater, although increases in salinity delayed the time to hatching and reduced the hatching rate. The present study showed that hatching rate in *O. melastigma* was not affected by salinity, although the time to hatching at salinities of 4, 8, and 17 (brackish water) tended to be faster than at 0 (freshwater) or 34 (seawater). We found that salinity did not affect embryonic development or hatching rate in *O. javanicus* (Horie et al., 2019), this species of *Oryzias* is highly adaptable to salinity ranges from freshwater to seawater, species of the genus *Oryzias* are highly adaptable to salinity

ranges from freshwater to seawater.

Although embryonic development is unaffected by salinity, the present study showed that salinity affects growth performance in *O. melastigma* as fish mature. Salinity condition on growth and development is closely related in fish. For example, Kim et al. (2017) have reported in sablefish *Anoplopoma fimbria* that at low salt concentration, the daily growth rate was significantly decreased. Brown and Hartwick (1988) have also reported, using Pacific oyster, *Crassostrea gigas*, that a significant reduction in the growth in low salinity. In contrast, Imsland et al. (2001) have reported that growth performance in turbot, *Scophthalmus macimus*, significantly increased at lower salinity. These reports indicated that the best salinity for growth performance varies by species. In the present study, growth rate, measured by total body length, tended to be greater in the brackish water groups than that in the seawater and freshwater groups. This suggested that brackish water results in the best growth performance in *O. melastigma*.

In teleosts, the regulation of growth, osmoregulation, metabolism, reproduction, development, and feeding behavior are controlled by the growth hormone (GH)/insulin-like growth factor-I (Igfl) axis (Madsen and Bern, 1992; McLean et al., 1993; Duan, 1997; Shepherd et al., 1997; Kawauchi and Sower, 2006). In the present study, we found that growth performance in freshwater was lower than in brackish water or seawater. Moorman et al. (2016), studying Mozambique tilapia (*O. mossambicus*), have reported that pituitary GH mRNA expression changed with salinity and that there was a strong positive correlation between pituitary GH mRNA expression and growth. On the other hand, Mohammed-Geba et al. (2016), using gilthead sea bream (*Sparus aurata* L.), reported that Igfl expression correlated negatively with increasing environmental salinity and positively with growth at isosmotic salinity, whereas the relationships with GH were less clearly defined. These results suggest that GH/Igfl axis expression is also involved in regulating differences in growth performance with salinity in *O. melastigma*. However, this relationship is still unclear and requires future study.

In the present study, salinity affected reproduction in *O. melastigma*, with fish in brackish water showing greater reproductive success than those in seawater or freshwater. In general, Fsh and Lh are involved in reproduction in vertebrates; Fsh is a trigger for vitellogenic hormone production, and Lh is related to final gonadal maturation and ovulation (Pierce and Parsons, 1981; Suzuki et al., 1988; Horie et al., 2014). In this study with IHC method, we described the ontogenic expression profiles of Gpa, Fshb, and Lhb in the pituitary of *O. melastigma*, and Gpa-, Fshb-, and Lhb-producing cells were

observed in both males and females from 30 dph in all salinities tested. In freshwater, however, oocyte development tended to be delayed and Fshb-immunoreactive cells also tended to be fewer compared with brackish water or seawater. In their review, Yaron et al. (2003) note that Fsh determines the number of oocytes at each developmental stage. These observations suggest that the delayed oocyte development was induced by fewer Fsh-producing cells in the pituitary. However, quantitative of gonadotropin expression is still unclear from present study, future studies should, therefore, study the relationship between salinity and gonadotropin expression in the pituitary using quantitative real-time PCR method.

The gonadal sex differentiation observed in *O. melastigma* in this study is similar to that in Japanese medaka (*O. latipes*) (Sato and Egami, 1972), although the initiation of spermatogenesis was not seen until after 70 dph in male Japanese medaka (Horie et al., 2014). In the present study, one male *O. melastigma* in the freshwater group had testis-ova, although salinity did not affect gonadal sex differentiation.

In the present study, white zinc precipitates were observed at high treatment concentrations (15.3 and 48 mg/L) in brackish water and seawater. Park et al. (2014) have also reported that dissolved Zn saturation concentrations decrease as salinity increases. This result indicates that metal saturation concentrations differ between seawater and freshwater. Therefore, it is necessary to use marine organisms to study the ecological effects of metals in the ocean.

Interestingly, the LOEC for mortality was lower in freshwater than in brackish water or seawater in both the 96-h acute toxicity test and the short-term toxicity test. Moreover, the LOEC for mortality in brackish water and seawater exceeded the LOEC for mortality in freshwater. Previous studies have examined the relationship between chemical toxicity and water salinity. For example, Park et al. (2014) have revealed that elevated salinity decreases the dissolved concentration of Zn ions, thereby reducing acute toxicity in *Tigriopus japonicus*. Ardiansyah et al. (2012) have reported 96-h zinc LC₅₀s for *Litopenaeus vannamei* Boone of 3780 µg/L at 5‰ salinity, 5520 µg/L at 15‰, and 8910 µg/L at 27‰. The implication is that the LC₅₀ increases with elevated salinity. Similarly, Bielmyer et al (2012) also reported that acute Zn toxicity in both *Fundulus heteroclitus* and *Kryptolebias marmoratus* generally decreased (LC₅₀ values increased) as salinity increased. Taken together with our study results, these findings indicate that metal toxicities change with salinity difference. However, the relationship between toxicity and salinity difference is unclear. Therefore, further research is needed in the near future.

Although recent studies have increasingly used *O. melastigma* as a model organism to examine the effects of chemicals on aquatic organisms, those studies were

carried out at various salinities ranging from 15 to 35 (Chen et al., 2016; Dong et al., 2017, 2018; Lee et al., 2018; Yi and Leung, 2017). However, our results show that brackish water is the best for growth performance and reproduction, and also the LOEC of lethality for zinc differs among salinities. Therefore, it is necessary to establish appropriate test salinities for *O. melastigma*, in part to help understand how salinity affects the toxicity of various substances including metals, pesticides, pharmaceuticals, and endocrine disruptors, etc.

5. Conclusions

To conduct ecological risk assessment of a chemical for marine organisms, it is essential to first know whether salinity affects the physiological processes and ecotoxicology of that chemical. This is the first study to clarify the effects of salinity on physiological processes such as embryo development, growth, reproduction, and gonadal development in a marine medaka (*O. melastigma*) using tests based on OECD methods. *Oryzias melastigma* is highly adaptable to various salinities from freshwater to seawater. However, our results show that brackish water is the best for growth performance and reproduction, although salinity did not affect gonadal sex differentiation. These findings suggest that brackish water provides the best salinity conditions for physiological processes. Ours is the first study to clarify the influence of salinity on zinc toxicity in *O. melastigma*. Zinc was observed to form a white precipitate upon dissolving in saltwater, and zinc toxicity was lower in brackish water and seawater than in freshwater. These findings underline the importance of using marine organisms to evaluate the ecological effects of chemicals in the ocean.

Acknowledgments

This study was supported partly by the Salt Science Research Foundation, Japan (grant no. 1915), the Kurita Water and Environment Foundation, Japan (grant no. 18K007), and a grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan (Grant-in-Aid for Scientific Research [B] grant no. 19H04294) to Y.H..

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Figures

Figure 1. Effect of salinity on embryonic development in *Oryzias melastigma* as assessed by (a) average time to hatching and (b) hatching rate. Columns represent mean values and error bars represent SD ($n = 4$). Asterisks indicate statistically-significant differences compared to the respective values at salinity 34 (Steel's test; $*P < 0.05$).

Figure 2. Effect of salinity on growth in *Oryzias melastigma*. (a, b) Growth measured by total body length and body wet-weight, respectively, during ontogeny. Could not be calculated because the wet body weight at 0 dph was below the minimum measurable weight for our balance of 10 mg. Columns and symbols represent mean values and error bars represent SD ($n = 4$). BW, brackish water; SW, seawater; FW, fresh water.

Figure 3. Effects of salinity on reproduction in *Oryzias melastigma*. (a) Fecundity, (b) fertility, (c) proportion of spawning females the during 21-d assay, and (d) the cumulative number of fertilized eggs per female. Columns and symbols represent mean values and error bars represent SD ($n = 4$). Asterisks indicate statistically-significant differences compared to the respective values in brackish water (BW) ((a) fecundity and (c) proportion of spawning females were by Steel's test; (b) fertility was by Dunnett's test, $*P < 0.05$). SW, seawater; FW, fresh water.

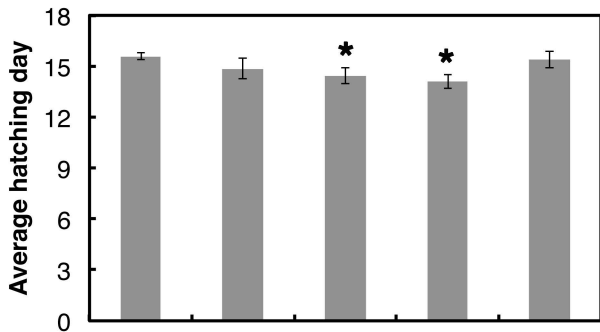
Figure 4. Gpa-, Fshb-, and Lhb-immunoreactive cells in the pituitary of individual male and female *Oryzias melastigma* during ontogeny. Cells were observed by 3,30-diaminobenzidine (DAB) staining (brown color). a–f, 30 days post hatching (dph); g–l, 60 dph. a, c, e, g, i, k, Males; b, d, f, h, j, l, females. a, b, g, h, brackish water (BW); c, d, i, j, seawater (SW); e, f, k, l, fresh water (FW). 1, Gpa; 2, Fshb; 3, Lhb. Arrowheads, immunoreactive cells. Scale bar, 50 μm .

Figure 5. Gonadal sex differentiation at each test salinity in *Oryzias melastigma*. Gonads were cross-sectioned serially at 5- μm thickness. a–d, brackish water (BW); e–h, seawater (SW); i–m, fresh water (FW). a, c, e, g, i, k, m, ovary; b, d, f, h, j, l, testis; m, testis-ova. Scale bar, 100 μm .

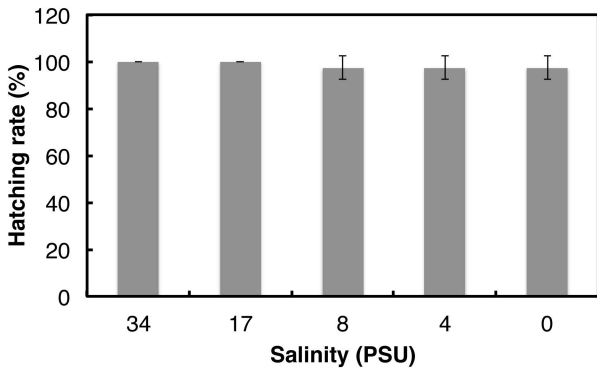
Figure 6. Results of 96-h zinc acute toxicity tests on adult fish at three salinity conditions: (a) freshwater, (b) brackish water, and (c) seawater. Columns and error bars represent means $\pm$ SEM ($n = 3$).

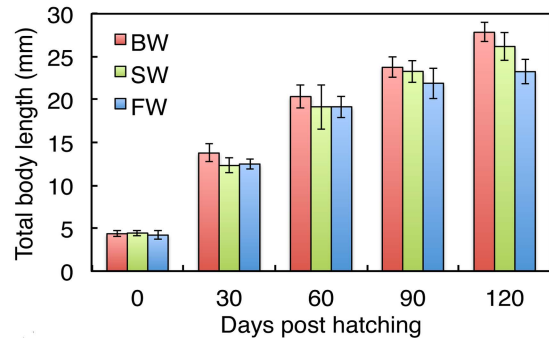
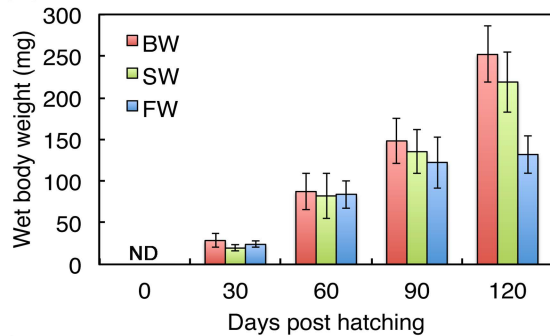
Figure 7. Results of short-term zinc toxicity tests on embryos and larvae at three salinity conditions: (a, b) freshwater, (c, d) brackish water, and (e, f) seawater. (a, c, e) Hatching percentage. (b, d, f) Survival rate at the end of the test assay. Columns and error bars represent means $\pm$ SEM ($n = 4$). Asterisks indicate significant differences compared with controls ((e, f) seawater was by Steel's test; (a, b) freshwater and (c, d) brackish water were by Dunnett's test, $*P < 0.05$).

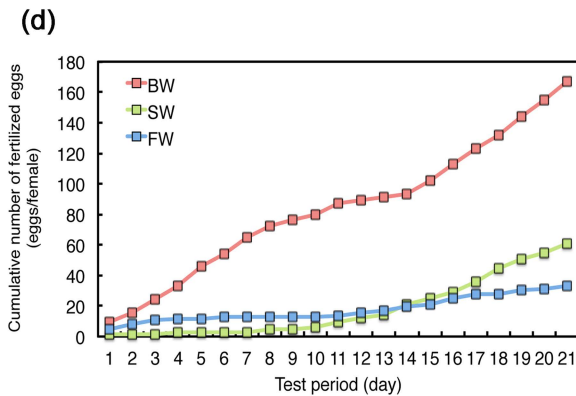
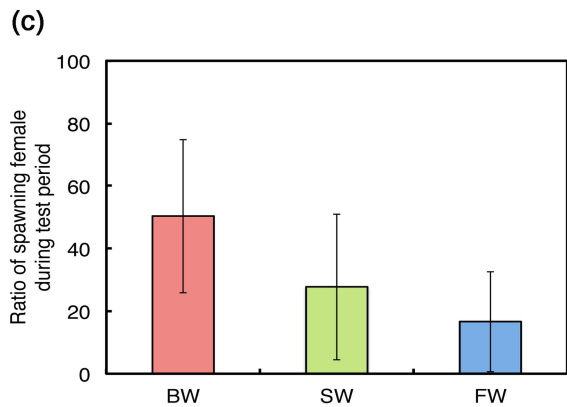
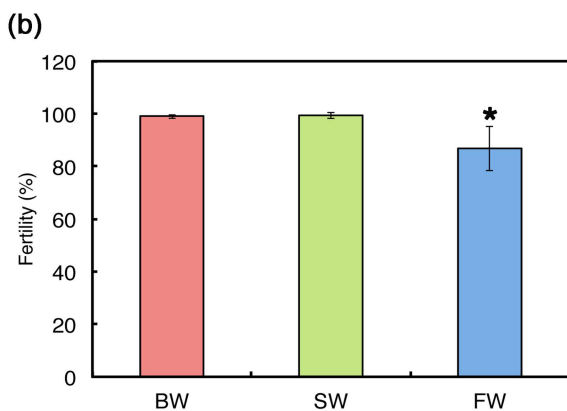
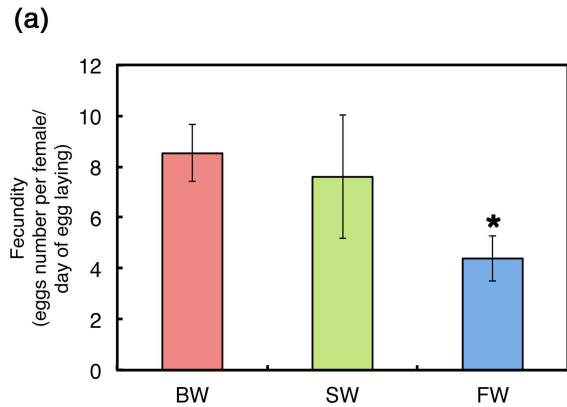
(a)

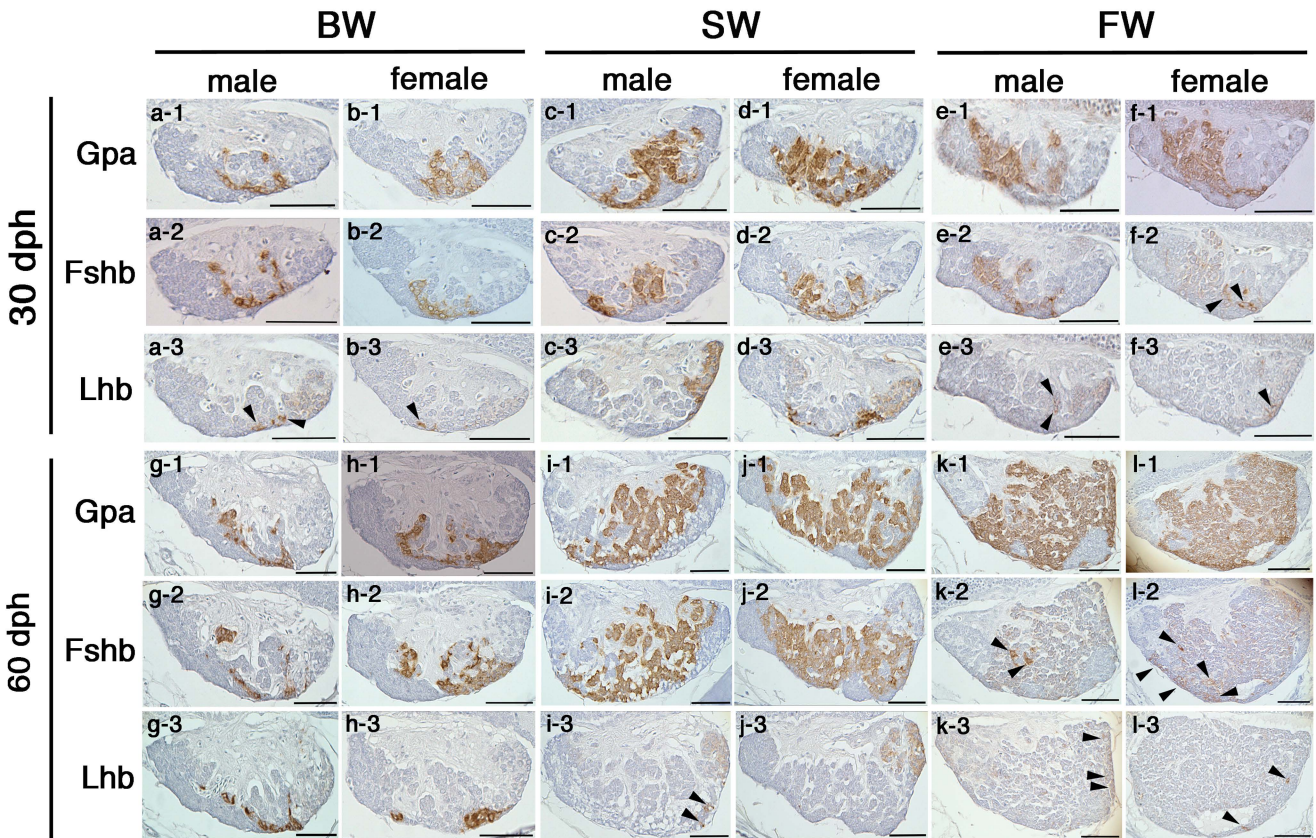


(b)



(a)**(b)**

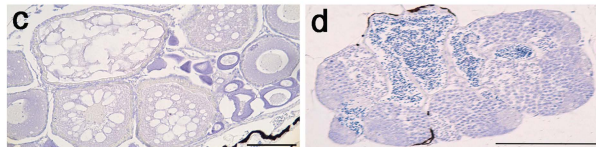
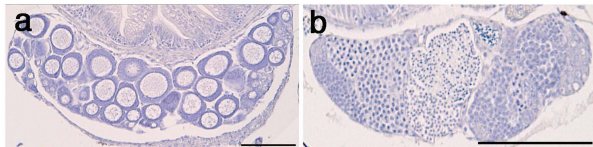




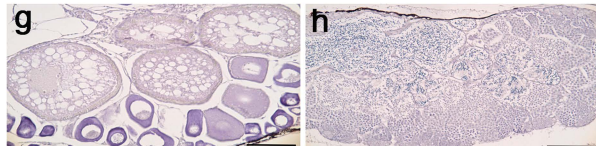
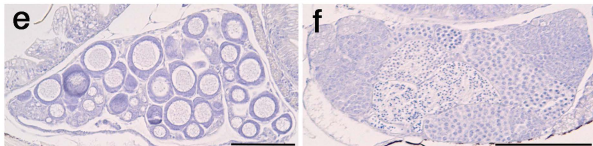
30 dph

60 dph

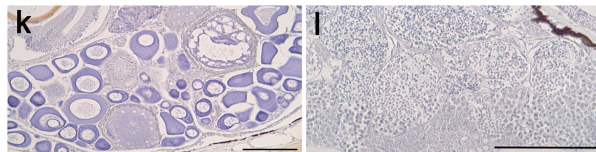
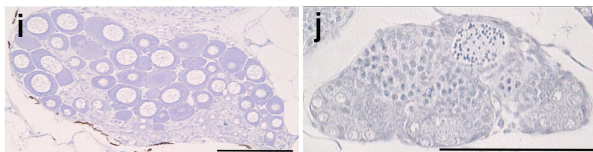
BW



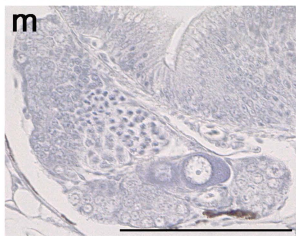
SW



FW

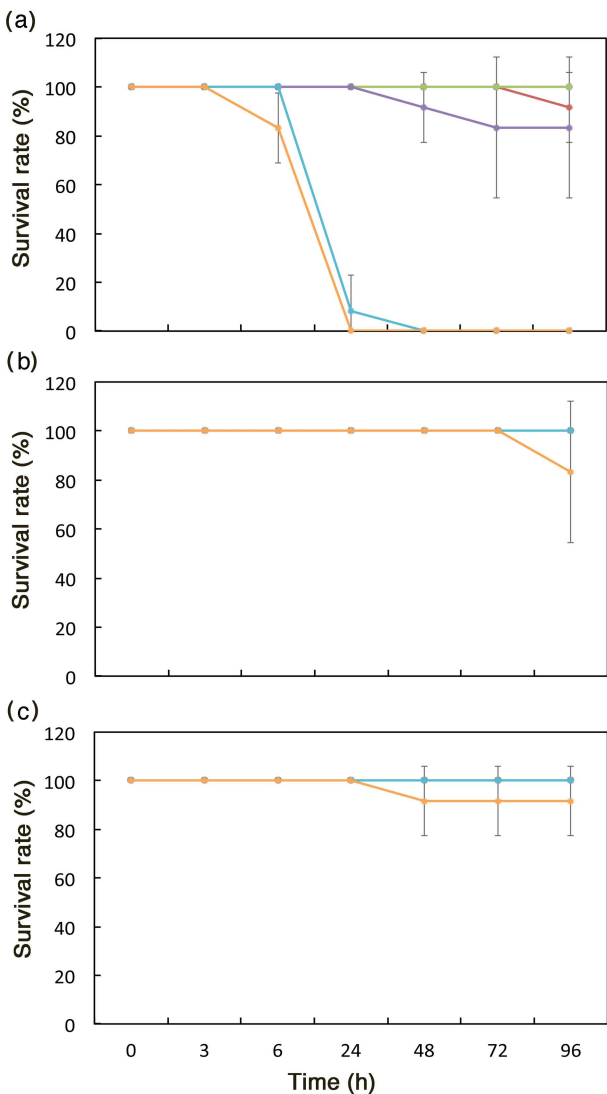


FW



Nominal Zn concentration (mg/L)

Control 0.48 1.53 4.8 15.3 48



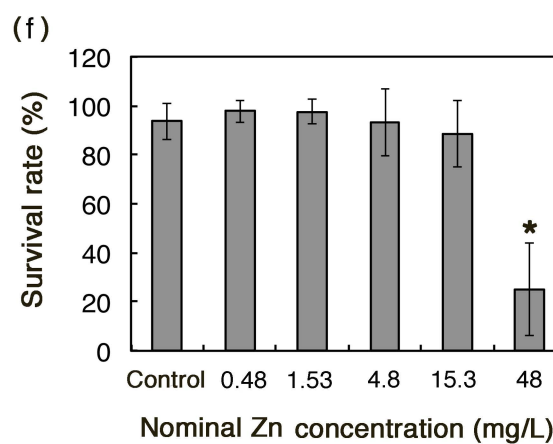
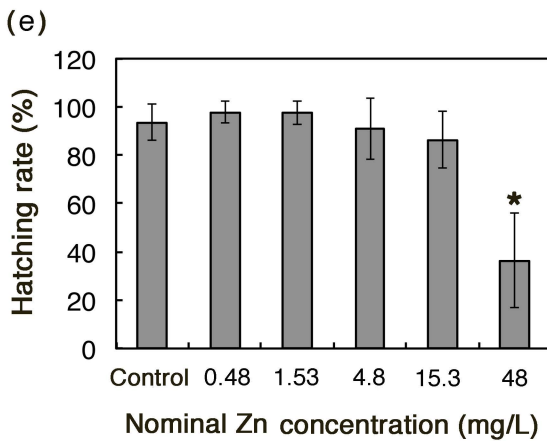
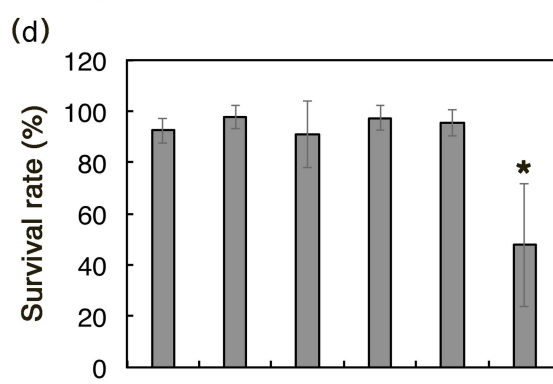
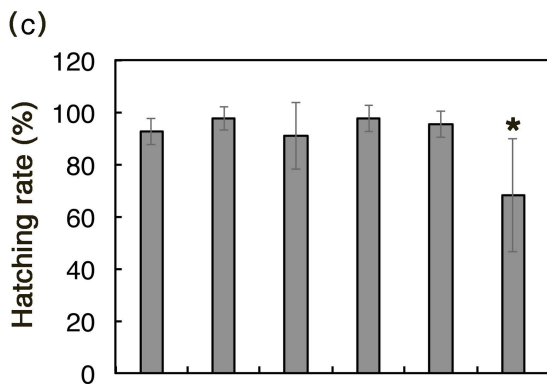
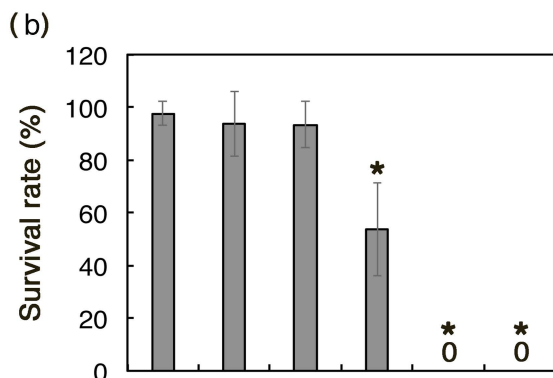
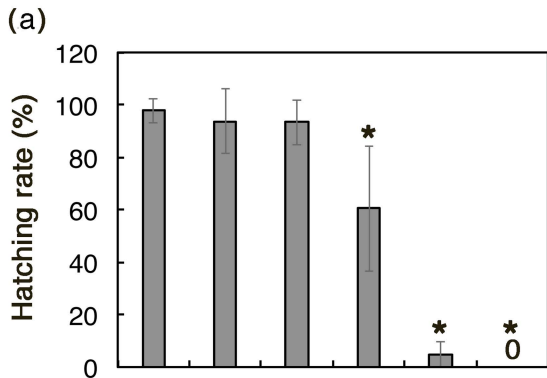


Table 1. Nominal and measured Zn concentrations for test assays

Salinity treatment	Nominal ZnCl ₂ concentration (mg/L)	Measured Zn concentration (mg/L)	
		Supernatant	Precipitate
FW	Control	0.011	—
	0.48	0.39	—
	1.53	1.4	—
	4.8	5.5	—
	15.3	15	—
	48	42	—
BW	Control	0.011	—
	0.48	0.51	0.51
	1.53	1.6	1.6
	4.8	4.6	4.7
	15.3	10	34
	48	31	99
SW	Control	0.013	—
	0.48	0.53	0.52
	1.53	1.7	1.7
	4.8	4.7	4.7
	15.3	10	34
	48	19	130

—: No data.

FW: freshwater; BW: brackish water; SW: seawater.

Table 2. Lowest observed effect concentrations (LOECs) for each endpoint at each salinity treatment

Endpoint	Salinity	LOEC (mg/L)
Mortality (adult)	FW	15.3
	BW	>48
	SW	>48
Mortality (embryo and larvae)	FW	1.53
	BW	48
	SW	48
Hatching	FW	4.8
	BW	48
	SW	48

FW: freshwater; BW: brackish water; SW: seawater.