<Aquat. Toxicol.>

Iron reproductive toxicity of marine rotifer sibling species: Adaptation to temperate and tropical habitats

Chengyan Han[a], Hee-Jin Kim[a,*], Jae-Seong Lee[b], Yoshitaka Sakakura[a,c], Atsushi Hagiwara[a,c]

[a] *Graduate School of Fisheries and Environmental Sciences, Nagasaki University, Bunkyo 1-14, Nagasaki 852-8521, Japan*

[b] *Department of Biological Sciences, College of Science, Sungkyunkwan University, Suwon 16419, South Korea*

[c] *Organization for Marine Science and Technology, Nagasaki University, Bunkyo 1-14, Nagasaki 852-8521, Japan*

*Corresponding author. E-mail heejin@nagasaki-u.ac.jp (H.-J. Kim)

Abstract

Iron (Fe), a trace metal in coastal waters has increased significantly due to anthropogenic activities, however, few studies have examined its toxicity to marine organism reproduction and associated mechanisms. We employed two marine rotifers, the temperate *Brachionus plicatilis,* and tropical *B. rotundiformis* to investigate the toxicity of iron ($FeSO_4 \cdot 7H_2O$) and its deleterious effects on reproductive features in females (sexual fecundity, abnormal resting eggs, and swimming speed) and males (lifespan, swimming speed, and spermatozoa quality) under lethal and sub-lethal exposure. The 24-h median lethal concentration (LC_{50}) of iron was determined as 0.9 and 1.7 μg/mL per ng of dry weight for *B. plicatilis* and *B. rotundiformis*, respectively. During sub-lethal iron (20–75 μg/mL) exposure, higher iron (≥ 20 μg/mL for *B. plicatilis* and ≥ 45 μg/mL for *B. rotundiformis*) induced rotifer sexual toxicity especially in normal resting egg development and production. These were supported by the data of male shorter lifespan, poor sperm vitality, and rotifer behavioral changes as the iron concentration increased. Iron effects on swimming behavior, slower males and faster females, should reduce male/female encounter rates associated with inactive fertilized egg (resting egg) production. Two rotifer species exhibited different iron-response patterns in genetic and enzymatic activities including iron homeostasis-maintaining related *Fe-S protein*, and oxidative/antioxidant related lipid peroxidation product (MDA), *superoxidase dismutase*/SOD, *catalase*/CAT, and *cytochrome P450* under acute iron exposure. Antioxidant activities were vulnerable in *B. plicatilis* but kept activities in *B. rotundiformis*, which may attribute to their temperate and tropical habitat adaptations.

Keywords: Rotifera, Iron toxicity, Reproductivity, Sperm, Climatic habitat

1. Introduction

Iron (Fe) as a trace essential nutrient is involved in the metabolism of multiple functional proteins: (1) hemoglobin for oxygen transport, (2) transferrin and ferritin for immune regulation and spermatogenesis, (3) cytochromes, superoxide dismutase (SOD), and catalases (CAT) for cellular respiration, electron transfer, and reactive oxygen species (ROS) metabolism (Brock and Mulero, 2000; Pantopoulos et al., 2012). However, iron has negative effects on the growth and reproduction of aquatic animals when it exceeds the threshold level (Cadmus et al., 2018). Iron exists in two forms in biological systems: redox-active ferrous iron Fe (II) and oxidized ferric iron Fe (III). A high dosage of labile Fe (II) is more toxic, as it can directly react with hydrogen peroxide (H_2O_2) via Fenton reaction, inducing the generation of hydroxyl free radicals (Winterbourn, 1995). To date, iron is not considered a priority pollutant due to low concentrations in water systems. However, the increased anthropogenic iron application such as industries (mining and ore processing, canning) (Ito and Shi, 2016), agriculture (Pahlavan-Rad and Pessarakli, 2009), and aquaculture such as 1.3–10 μg/mL of iron is added to microalgae culture as a supplement, which is used in the cultivation of rotifers and fish larvae (Brown, 2002) is raising iron levels in aquatic environments. Therefore, higher levels (> 13.0 μg/mL) of iron have been detected in coastal waters near the beach (Jonathan et al., 2011), which could place aquatic animals at the risk of overloading since species have different tolerance of iron exposure. For example, 4.1 μg/mL of iron causes abnormal morphological development in the marine abalone *Haliotis rubra* larvae (Gorski and Nugegoda, 2006), while 0.14–0.33 μg/mL of iron induces mortality in minute marine rotifer *Proales similis* (Rebolledo et al., 2021). Furthermore, increased iron can be bio-accumulated in marine organisms through respiration, absorption, and ingestion, and may pose a health risk to consumers via the food web (Ahmed et al., 2019).

The monogonont rotifer *Brachionus plicatilis* species complex is widely used as live food for rearing fish larvae in aquaculture as well as a cost-effective marine toxicity assessment model species, because of the features of easy to culture, growing rapidly, and sensitive to numerous contaminants (Dahms et al., 2011; Hagiwara et al., 1998). We previously described iron ($FeSO_4 \cdot 7H_2O$) species-specific effects on reproduction of the temperate *B. plicatilis* and tropical *B. rotundiformis* in the *B. plicatilis* species complex and determined the underlying

mechanisms via ROS pathway and lipid accumulation (Han et al., 2021). In this study, we hypothesized that iron-induced rotifer sexual reproductive sensitivity might be attributed to the vulnerability of male functionality including swimming behavior and sperm vitality to iron exposure. Spermatogenesis has been demonstrated to be an iron-dependent process. However, mature spermatids and spermatozoa are susceptible to iron imbalance-induced oxidative stress, as the sperm cell membranes contain lots of polyunsaturated fatty acids, which are easily attacked by hydroxyl free radicals (Aitken et al., 1993). The toxicological and nutritional iron importance in regulating sperm activity has been documented. For instance, the beneficial concentrations of iron in human semen are 9.0–30.0 mmol/L, while iron-overload (median value 37.0 mmol/L) triggers oxidative stress, causing sperm DNA damage and the deterioration of sperm motility (Perera et al., 2002). In the marine mytilid *Brachydontes variaibilis*, 10.4 μg/mL of iron exposure induced sperm malformations including pathological alteration in spermatogonia, spermatocytes, and spermatozoa (Desouky, 2009).

Based on these considerations, the following three experiments were conducted under lethal and sub-lethal iron exposure to investigate the sexual reproductive sensitivity especially in male functionality and its underlying mechanisms in *B. plicatilis* and *B. rotundiformis*: (1) 24 h LC_{50} of iron to the two *Brachionus* rotifer species; the intracellular ROS levels, lipid peroxidation (malondialdehyde [MDA]), acetylcholinesterase (AChE), and antioxidant (SOD and CAT) enzymatic activities, and the mRNA expression of associated biomarkers respond to iron acute exposure; (2) the patterns of lifespan and fecundity of two types of sexual females (unfertilized and fertilized mictic females) and the lifespan of males under different iron concentrations in an individual culture; and (3) reproductive parameters, swimming speed of female and male rotifers, and the quality of male spermatozoa in response to seven days of chronic iron exposure. In summary, we show iron toxicity on male and female reproductive ability, as well as interspecific endurance adaptations in terms of genetic and enzymatic activities between temperate *B. plicatilis* and tropical *B. rotundiformis* rotifers.

2. Materials and methods

2.1. Rotifers stock culture

The two rotifer species employed were temperate *B. plicatilis* (NH17L strain, mean lorica size 275 μm) and tropical rotifer *B. rotundiformis* (Kochi strain, lorica size 179 μm) that have been maintained at the Aquaculture Biology Laboratory, Nagasaki University, Japan, for over two decades. Rotifers were cultured in filter/autoclaved artificial seawater (ASW) (Marine Art Hi, Tomita Pharmaceutical, Tokushima, Japan) at 17 parts per thousand (ppt) at 25 °C and fed on the microalga *Nannochloropsis oculata* at 7.0×10^6 cells/mL in darkness.

2.2. Chemical preparations

Ferrous sulfate ($FeSO_4 \cdot 7H_2O$, purity > 99%; Wako, Osaka, Japan) was used in this study. The iron stock solution was prepared with distilled water at 10 mg/mL and then diluted with ASW to reach the target concentrations. The iron concentrations in the test solution (Suppl. Table 1) were measured using Palintest Iron MR based on standard laboratory instructions (Photometer 8000, Palintest Ltd, Gateshead, England). To minimize hydrolytic precipitation and adsorption, samples were maintained with high-purity nitric acid at pH < 2 before testing.

2.3. Short-term observation

The acute toxicity of iron was evaluated by the 24 h LC_{50} of neonates (< 2 h) from amictic eggs. The neonates were prepared by applying approximately 10,000 stock female rotifers carrying amictic eggs into a screw-capped vial containing 10 mL ASW and then agitated to shake off the eggs. The separated eggs were collected and incubated in a well of a 6-well microplate containing 5 mL ASW at 25 °C, and the hatchlings were observed every 30 min. Based on the preliminary tests, the following concentrations of $FeSO_4 \cdot 7H_2O$ were set: 0, 300, 400, 450, 500, 550 and 600 μg/mL for *B. plicatilis*, and 0, 150, 250, 300, 350, 400, 500 and 600 μg/mL for *B. rotundiformis*, respectively. Eight rotifers were inoculated into a well of a 6-well microplate with 5 mL of working solution. The culture conditions were the same as those used for the stock culture, but no food was supplied during observation. The living and dead rotifers were counted after 24 h of exposure using a stereomicroscope at 60× magnification (SZ-STS, Olympus, Tokyo, Japan). Mortality was calculated as the number of dead individuals with no movement of cilia and mastax for over 30 s. The data of 24 h LC_{50} and 95% confidence intervals

were obtained by Probit analysis, by fitting a log-dose response curve to a line regression, and then estimating the models by least squares or maximum likelihood (Sakuma, 1998). All experiments were performed in triplicate.

2.4. Reactive oxygen species levels and biomarkers/enzymatic activities

The effects of short-term iron exposure were compared between two rotifer species, with the intracellular ROS level after 12 h of iron exposure at the concentration of 24 h LC_{50}. The rotifers were subjected to the following three treatments: 0 (control), Fe, and Fe + N-acetylcysteine (NAC). The ROS inhibitor NAC (purity > 99%, Sigma-Aldrich, St. Louis, MO, USA) was added to check whether the iron-induced oxidative stress could be scavenged. Rotifers in the Fe + NAC group were treated with NAC (0.5 mM) for 6 h before co-incubation with iron solution, based on the preliminary test. A cell-permeable fluorogenic probe 2',7'-dichlorofluorescein diacetate (H_2DCFDA, Sigma-Aldrich), which can be oxidized to produce fluorescent 2',7'-dichlorofluorescein (DCF) by ROS, was used to estimate intracellular ROS levels. The detection methods are described in detail in the Supplementary Information.

Oxidative/antioxidant mechanisms were determined in response to short-term iron exposure. Enzymatic (MDA, AChE, SOD, and CAT) activities and transcript abundance of *NADPH oxidase 5* (*Nox* 5), *heat shock protein 70* (*Hsc70 like 1*), *copper/zinc sod* (*CuZnSOD*), *cat*, *cytochrome P450* (*CYP*), and three iron-metabolism-related genes (*iron oxidase*, *Fe-S protein,* and *Fe-S cluster*) were measured after 12 h of iron exposure.

Rotifers (approximately 8,000 individuals) were used for RNA extraction. Total RNA was extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Subsequently, genomic DNA was removed using TURBO DNA-free™ (Ambion®, Carlsbad, CA, USA), and cDNA was synthesized using the PrimeScript™ II 1st strand cDNA Synthesis Kit (Takara Bio, Kusatsu, Shiga, Japan). The cDNA was preserved at −20 °C until use. The quality and quantity of RNA and cDNA were assessed by measuring the absorbance at 230, 260, and 280 nm, and the ratios of A260/280 and A230/260 using a spectrophotometer (NanoDrop™ 2000, Thermo Scientific™). For real-time quantitative polymerase chain reaction (PCR), 1 μL cDNA template, 0.5 μL forward and reverse primers (10 μM)

and 10 μL TB Green Premix Ex Taq (2×) (Takara Bio) were combined to a total volume of 20 μL. The analyzed genes are listed with primer sequences in Suppl. Table 2. Primer sequences used in this study were designed using Primer Premier 6.0 (PREMIER Biosoft, San Francisco, CA, USA). Thermal cycling was carried out at 94 °C for 4 min, followed by 39 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. The assay was conducted using a LightCycler® 96 real-time PCR system (Roche Life Science, Basel, Switzerland). The melting curve cycles were run under the following conditions: 95 °C for 10 s, 55 °C for 1 min and 80 cycles at 55 °C/10 s, with an increase of 0.5 °C per cycle. We tested two housekeeping genes, elongation factor 1-α (*EF1-α*) and *18S* rRNA in a pilot analysis, and the *EF 1-α* was chosen as a reference gene to normalize the transcript levels, as it showed the least variation among and within the experimental groups. The $2^{-\Delta\Delta CT}$ method was used to calculate the transcriptional levels. All treatments were performed in triplicate.

MDA activity was measured via the reaction of MDA with thiobarbituric acid to form a colorimetric product, which can be detected at the absorbance of optical density (OD) at 532 nm. AChE assay uses 5,5-dithiobis (2-nitrobenzoic acid) to quantify the amount of thiocholine produced by AChE during the hydrolysis of acetylthiocholine. The signal was proportional to the AChE activity, which can be detected by measuring the absorbance at 410 nm. SOD activity was estimated by the reduction of formazan dye produced by the reaction of superoxide anions and WST-1 (2-[4-Iodophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium, monosodium salt). SOD catalyzes the dismutation of the superoxide anion into hydrogen peroxide (H_2O_2) and O_2; the greater the SOD activity, the lesser formazan is formed, which is detected at an absorbance of 440 nm. The CAT present in the samples reacted with H_2O_2 to produce H_2O and O_2, and unconverted H_2O_2 reacted with the probe to produce a product, which could be measured colorimetrically at an absorbance of 570 nm. The Supplementary Information includes a full description of the measurements.

2.5. Patterns of lifespan and fecundity of sexual females and males

Based on the observed rotifer reproduction responses to different concentrations of iron in our previous study (Han et al., 2021), the following six concentrations of $FeSO_4 \cdot 7H_2O$ (0, 20,

30, 45, 60, and 75 μg/mL) were used in the current study for investigation. The following parameters were measured to assess iron effects on rotifer sexual reproduction: the fecundity and lifespan of unfertilized and fertilized females, and the alteration of male lifespan under each iron concentration. Thirty female neonates (< 2 h) hatched from amictic eggs and twenty newborn males (< 2 h) hatched from mictic eggs, were cultured together in a well of a 6-well microplate containing 5 mL food suspension and iron. These batches were incubated at 25 °C in total darkness and checked every 12 h until the mictic egg-bearing and the resting egg-bearing females appeared. Twelve individuals of each mictic female type were individually transferred into a well of a 24-well microplate, and the rotifers were checked every 12 h to record the number of male and resting eggs produced. The maternal females were transferred daily to new well-containing food and iron solutions. This procedure was continued until all the maternal rotifers died. In addition, the effects of iron concentration on male lifespan were investigated using the same method.

2.6. Long-term observation

Rotifer neonates (< 2 h) were inoculated into 100 mL screw-capped bottles containing 80 mL of iron solution at the initial density of 1 ind./mL. They were daily fed on *N. oculata* (7.0×10^6 cells/mL) and incubated in complete darkness at 25 °C for 7 days. The applied $FeSO_4 \cdot 7H_2O$ concentrations were the same as those used in the above individual reproduction investigations. To minimize the precipitation of iron and phytoplankton cells, horizontal shaking was carried out at 85 ± 1 rpm using a shaker (Triple Shaker NR-80, TAITEC, Saitama, Japan). During the culture period, four types of females: non-ovigerous females, amictic females carrying large female eggs (FF), unfertilized mictic females carrying small and numerous male eggs (MF), and resting egg females carrying yellowish fertilized eggs (RF) were daily counted. These numbers were used to calculate the population growth rate (r), mix induction (%), and fertilization (%). Half of the culture medium was renewed daily to maintain the iron concentration. On the final day of cultivation, rotifer feeding was stopped, and a three-day starvation period was performed, after which the resting eggs (RE) were harvested and counted. All treatments were performed in triplicate and the mean of each number was used to calculate the following reproductive parameters:

Population growth rate (r): ln (Nt/N_0)/t (where t represents the culture day, Nt is the number of female rotifers on day t, and N_0 is the number of female rotifers on day 0).

Mixis (%): [(MF + RF)/(FF + MF + RF)] × 100

Fertilization (%): [RF/(MF + RF)] × 100

Ratio (%) of abnormal resting eggs: [(abnormal RE)/(normal RE + abnormal RE)] × 100

2.7. Swimming activity analysis

Twenty female rotifers bearing one amictic egg and twenty males (< 2 h) were sampled from each treatment on the last day of culture (day 7). The selected individuals were placed in a glass-bottom dish (Matsunami, Osaka, Japan) with a thickness of 1.5 mm and a diameter of 14 mm containing 200 μL of culture solution, and the swimming behavior was recorded for 20 s under a stereomicroscope (HAS-UZ, Olympus, Tokyo, Japan) equipped with a digital camera HAS-X Viewer Ver. 12.1201; DITECT, Tokyo, Japan). The swimming speed (mm/s) was measured using the tracking software Dipp Motion Pro Ver. 1.1.31 (DITECT, Tokyo, Japan). To obtain males, unfertilized mictic females from each treatment were released into a well of a 6-well microplate containing 5 mL iron solution and incubated at 25 °C. The rotifers were checked every 30 min, and the newborn males were collected for analysis.

2.8. Viability assessment of male sperm cells

The effects of iron on the viability of male spermatozoa were investigated using a LIVE/DEAD® Sperm Viability Kit (Molecular Probes, Thermo Fisher Scientific, Waltham, MA, USA) with dual DNA staining with SYBR-14 and propidium iodide (PI), which were associated with staining for cell membrane permeability (Garner and Johnson, 1995). For each treatment, five newborn males (< 30 min) were prepared using the same methods as described in the previous section. In detail, a male individual in 10 μL diluted (40×) HEPES buffer (pH 7.4) containing 10 mM HEPES, 150 mM NaCl, and 10% bovine serum albumin was placed onto a glass slide, and 5 μL of diluted SYBR-14 (50×) was added and co-incubated at 25 °C for 15 min. And then the sample was slightly compressed by a coverslip to expel sperm after the addition of 2.5 μL PI followed by incubation at 25 °C for another 15 min. The stained samples

were observed under a fluorescence microscope (Ts2-FL, Nikon, Tokyo, Japan) at an excitation/emission wavelength of 488/516 nm for SYBR-14 and 535/617 nm for PI. Fluorescence intensity was quantified using ImageJ 1.53e software (National Institutes of Health, USA).

2.9. Statistics

The Bartlett test of variance homogeneity was used to test the normality of the data. Significant differences were analyzed using a one-way analysis of variance followed by Tukey's honest significant difference (HSD) test. The nonparametric Kruskal-Wallis test followed by the Wilcoxon rank-sum test (Bonferroni adjustment) was used to analyze the data that showed non-normal distribution. Iron effects on the transcript levels of biomarkers and enzymatic activities were analyzed by Student's t-test. The results are presented as mean ± standard error. All statistical analyses were conducted using R version 3.6.3 (https://cran.r-project.org/bin/windows/base/old/3.6.3/).

The sigmoid dose-response regression-based median effective concentrations (EC_{50}) (Beasley et al., 2015) for reproductive parameters, fecundity, lifespan, and swimming speed were applied to summarize iron chronic effects.

3. Results

3.1. 24 h LC_{50} concentrations

The 24 h iron LC_{50} concentration to temperate *B. plicatilis* and tropical *B. rotundiformis* was 469.6 and 363.6 μg/mL, respectively (Table 2).

3.2. Reactive oxygen species levels and enzymes activities

Acute exposure of iron to two rotifer species significantly increased intracellular ROS levels ($p < 0.05$) (Fig. 1A). The ROS levels showed a 1.3 folds increase in both *B. plicatilis* and *B. rotundiformis*, compared to the control. Co-incubation with the ROS scavenger NAC decreased the iron-induced ROS levels and that of the Fe + NAC group in both rotifer species, with no

300 significant difference from the control.

301 Different response patterns in the oxidative/antioxidant enzymatic activities were observed
302 in the two rotifer species in response to acute iron exposure. The relative MDA levels in the
303 iron-exposed group were significantly suppressed (a 1.3 folds reduction, $p < 0.05$) in *B. plicat-*
304 *ilis*, while a 1.3 folds increase was observed ($p < 0.05$) in *B. rotundiformis* compared with the
305 control. The AChE enzymatic activity was significantly inhibited by 1.5 and 1.3 folds ($p < 0.05$)
306 by iron in *B. plicatilis* and *B. rotundiformis*, respectively (Fig. 1B). Meanwhile, the antioxidant
307 SOD enzymatic activity was inhibited 1.0 folds in *B. plicatilis*, but it was stimulated 1.1 folds
308 in *B. rotundiformis*. CAT activity showed no variation in *B. plicatilis*, but was significantly
309 upregulated (a 1.4 folds increase, $p < 0.05$) in *B. rotundiformis* in response to iron exposure
310 (Fig. 1C).

311

312 ***3.3. Molecular biomarkers analysis***

313

314 The mRNA expression of iron metabolism-related genes, *iron oxidase,* and *Fe-S protein*
315 were significantly downregulated by 1.8 and 1.9 folds ($p < 0.05$) in *B. plicatilis* in response to
316 iron, respectively. For *B. rotundiformis*, the *Fe-S protein* and *Fe-S cluster* were significantly
317 upregulated 1.4 and 7.1 folds, respectively ($p < 0.05$) (Fig. 2A). The oxidative stress biomarker
318 *Hsc70 like 1* was significantly upregulated 1.7 and 1.3 folds ($p < 0.05$) in *B. plicatilis* and *B.*
319 *rotundiformis*, respectively. The *NADPH oxidase 5* was significantly upregulated in both two
320 species, 1.7 folds in *B. plicatilis* and 14.5 folds in *B. rotundiformis* ($p < 0.05$). The expression
321 of *AChE* was significantly downregulated 1.4 and 1.8 folds ($p < 0.05$) in *B. plicatilis* and *B.*
322 *rotundiformis*, respectively (Fig. 2B). The transcript levels of antioxidant responses related *to*
323 *CuZnSOD* and *CYP* were downregulated 1.34 and 2.15 folds ($p < 0.05$) in *B. plicatilis*, while
324 they were significantly upregulated 1.73 and 3.56 folds in *B. rotundiformis*, respectively, after
325 iron incubation. There was no difference in the transcript level of *CAT* after iron exposure in *B.*
326 *plicatilis*, compared to the control, while it was significantly upregulated 2.57 folds ($p < 0.05$)
327 in *B. rotundiformis* (Fig. 2C).

328

329 ***3.4. Lifespan and fecundity of sexual female and male***

330

The responses of lifespan and fecundity in two types of sexual females (unfertilized and fertilized mictic females) to iron were similar in both two species. An insignificant decrease in the lifespan and fecundity of unfertilized mictic females was observed as iron concentration increased, the lifespan and fecundity of the control *B. plicatilis* were 8.16 ± 0.63 d and 15.33 ± 0.99 males/female, respectively, and decreased to 6.29 ± 0.46 d and 10.67 ± 1.38 males/female at 45 μg/mL of iron (Fig. 3A). The fecundity of unfertilized mictic females decreased from 12.50 ± 1.48 (control) to 8.92 ± 1.26 males/female (30 μg/mL iron) in *B. rotundiformis* (Fig. 3B). The parameters of fertilized mictic females showed a similar decreasing pattern in both species: the lifespan and fecundity decreased from 8.82 ± 0.10 to 7.32 ± 0.71 d, 3.88 ± 0.61 to 1.64 ± 0.24 resting eggs/female in *B. plicatilis* (Fig. 3C) and from 5.24 ± 0.83 to 3.55 ± 0.51 d, 4.50 ± 0.52 to 2.58 ± 0.40 resting eggs/female in *B. rotundiformis* (Fig. 3D).

The male lifespan of both species was substantially shortened by iron treatment: reduced from 2.56 ± 0.32 to 1.28 ± 0.06 d in *B. plicatilis* (Fig. 3E), and from 2.19 ± 0.18 to 1.02 ± 0.06 d in *B. rotundiformis* (Fig. 3F).

3.5. Reproductive parameters

There was no significant difference in the asexual population growth rate of temperate *B. plicatilis* rotifer at 0–45 μg/mL iron concentration, while the number of sexual resting eggs was significantly inhibited ($p < 0.05$) when the iron concentration was higher than 20 μg/mL. Higher levels (60–75 μg/mL) of iron showed negative effects on both the population growth rate and resting egg production. Tropical *B. rotundiformis* rotifer showed a significant reduction in both sexual and asexual reproduction at ≥ 45 μg/mL iron concentration, while iron concentrations within 0–30 μg/mL did not induce negative effects on the population growth and resting egg production (Table 1).

3.6. Swimming speed analysis

Iron induced different swimming responses in male and female rotifers (Fig. 4-1). The swimming speed of male rotifers showed a decreasing pattern at the tested iron concentrations. The pattern was similar in both species: speed decreased from 1.07 to 0.76 mm/s in *B. plicatilis*,

and from 0.78 to 0.36 mm/s in *B. rotundiformis*. For female rotifers, the swimming speed was stimulated by iron: a significant increase ($p < 0.05$) from 0.65 (control) to 0.80 mm/s (20 μg/mL of iron) in *B. plicatilis*, and an insignificant increase from 0.67 (control) to 0.81 mm/s (30 μg/mL of iron) in *B. rotundiformis*.

The computed EC_{50} values for reproductive parameters, fecundity, lifespan, and swimming speed were shown in Table 2.

3.7. Viability of male spermatozoa

Spermatozoa with high viability showed comparatively strong green fluorescence, while those with low vitality showed weak signals (Fig. 4-2). High iron affected the vitality of spermatozoa in both rotifer species, as demonstrated by a decrease of SYBR-14 fluorescence intensity as the iron concentration increased, compared to the control. No dead PI-stained spermatozoa were observed in the tested sperm specimens.

The normal and abnormal resting eggs produced by the two species are shown in Suppl. Figs. S1A and B. The ratio of abnormal resting eggs in *B. plicatilis* was significantly increased ($p < 0.05$) at all iron concentrations tested. *B. rotundiformis* showed a substantial (4.1 and 8.0 folds) increase ($p < 0.05$) in the abnormal resting egg rate at 60 and 75 μg/mL of iron (Suppl. Fig. S1C).

4. Discussion

The current study determined iron reproductive toxicity of two marine rotifers, the temperate *B. plicatilis* and tropical *B. rotundiformis*, with the following parameters which may evolve as habitat adaptations: the sensitivity of male performances i.e., sperm vitality, and rotifer swimming behaviour to iron chronic (sub-lethal) exposure, as well as genetic and enzymatic activity associated with acute (lethal) exposure. The tested 24 h LC_{50} value of $FeSO_4 \cdot 7H_2O$ to *B. plicatilis* and *B. rotundiformis* was 469.6 and 363.6 μg/mL, respectively. Because there was a correlation between animal size and toxicants tolerance, the larger the species, the higher toxicity tolerance, and vice versa (Sarma et al., 2007), thus based on the different dry weight between the two species, *B. plicatilis* (495 ng/ind), and *B. rotundiformis* (220 ng/ind) (Yúfera

et al., 1997), we transformed the 24-h LC_{50} data to μg/mL per ng with a divide by rotifer dry weight, which showed 0.9 for *B. plicatilis* and 1.7 for *B. rotundiformis*, respectively. Furthermore, during chronic exposure, *B. rotundiformis* exhibited greater iron tolerance in sexual reproductivity than *B. plicatilis*, with higher EC_{50} values in mixis, fertilization, sexual female fecundity, and lifespan (Table 2). These obtained data, on the other hand, were higher than iron to other freshwater zooplanktons, such as 0.1–1.3 μg/mL (24 h LC_{50}) to the rotifers *B. calyciflorus*, *Lecane inermis*, *E. dilatata* (Couillard and Pinel-Alloul, 1989; Santos-Medrano and Rico-Martínez, 2013), and 0.3 μg/mL to the cladoceran *Daphnia magna* (Santos-Medrano and Rico-Martínez, 2015). Meanwhile, iron chronic tolerance in the population growth rate (*r*) of the two marine rotifers tested was 24 folds higher than the freshwater rotifer *E. dilatate* (EC_{50} 2.49 μg/mL) (Hernández-Flores et al., 2020). Freshwater rotifer species were proved to be more sensitive to pollutants than marine species, especially metals. For instance, cadmium (Cd) toxicity was reduced 87.4 folds in the marine rotifer *B. plicatilis* compared to the freshwater rotifer *B. calyciflorus* (Snell et al., 1991). The factors contributing to the difference in metal sensitivity between freshwater and marine species include salinity, metal complexation, and the variable alkalinity and pH in the water ecosystems. Salinity has a substantial impact on metal toxicity in aquatic invertebrates, with metal toxicity increasing as salinity decreases due to the ample supply of free metal ions and the reduced formation of chloro-complexes at lower salinities (Hall and Anderson, 1995). A recent study found that lower salinities increased Fe, Zn, Cd, and Cu toxicity in the rotifer *P. similis*, not only for single metals but also co-effects of mixed metals (synergistic effects) (Rebolledo et al., 2021). In contrast, metal inorganic (Cl^-, CO_3^{2-} and PO_4^{3-}), organic, proteins and humic acid complexations reduced free available metals, resulting in metal bioavailability and toxicity inhibition (Neubecker and Allen, 1983). Iron in the ocean is mainly complexed with organic matter produced by microorganisms such as bacteria and phytoplankton, inducing low dissolved iron levels and reduced iron availability (Van den Berg, 1995). While in freshwater ecosystems, ferrous iron is usually found as a dissolved ion in anoxic waters and the redox reaction of ferric iron can occur even under oxygenated conditions (Emmenegger et al., 2001), potentially increasing the dissolved ferrous iron concentrations and enhancing iron availability for freshwater organisms. In addition, pH-dependent Fe (II) oxidation is also important for the regulation of iron bioavailability. Fe (II) oxidation was faster in high pH conditions, the half time of Fe (II) oxidation was reduced one order of magnitude from

37.1 to 3.3 min when the pH increased from 7.2 to 8.2 under the same temperature (González et al., 2010). The pH of most natural freshwater ranges from 6 to 8.5, and fluctuates significantly over daily and seasonal timeframes (Tucker and D'Abramo, 2008), while for marine waters it remains stable at around 8.2. Thus, lower salt ions, poor organic complexation capacity, and variable pH in freshwater ecosystems may contribute to the vulnerability of metal toxicity including iron to freshwater species.

The temperature variation affects iron speciation (Breed et al., 1999) and the metabolic rate of rotifers (Li et al., 2014). Increased temperature reduces oxygen concentration in aquatic waters and regulates Fe (II)/Fe (III) ratio, which goes from 10^{-10} (in oxygenated seawater) to 10^{-7} (in mildly anoxic seawater) (Shaked, 2008). This has potential influences on iron bioavailability and toxicity. The employed two rotifer species *B. plicatilis* and *B. rotundiformis* are originally collected from temperate and tropical regions, respectively, and have different temperature tolerances for growth. While there is water temperature overlap (20–30 °C) for both species due to the diurnal/seasonal temperature variations (Gómez et al., 1997; Walczyńska and Serra, 2014). For example, *B. plicatilis* has stronger low-temperature tolerance than *B. rotundiformis*, they can reproduce well at 15–25 °C with an increased population growth rate (*r*) from 0.43 to 0.74. *B. rotundiformis* exhibits active growth at 20–35 °C with an increased *r* from 0.2 to 1.8, however it cannot reproduce at 15 °C (Hagiwara et al., 1995b; Jung et al., 1997). Both species can coexist and maintain active reproduction at 25 °C, with an *r* value of 0.74 and 1.20 to *B. plicatilis* and *B. rotundiformis*, respectively (Hagiwara et al., 1995a, b; Ito et al., 1981), and thus our laboratory keeps this temperature for rotifer stocks. Considering the aspects of both rotifer metabolism and iron ion state, we subjected the two rotifer species to the same temperature of 25 °C and compared the iron response patterns. Nevertheless, the habitat-originated adaptations could affect species toxicant responses. As previously reported, the tropical *B. rotundiformis*, which may experience more ROS caused by active metabolic activities under high temperature evolved a greater anti-oxidative strategy than the temperate *B. plicatilis* in response to stressful conditions (Han et al., 2021; Tanaka et al., 2009). The same pattern was observed in this study after iron 24 h acute exposure. Regarding the oxidative and antioxidant responses following acute iron exposure, the increased intracellular ROS concentrations, stimulated stress biomarkers of *NADPH oxidase 5* (Lambeth, 2004) and *Hsc70 like 1*

(Hartl, 1996), modified MDA activity, as well as inhibited AChE activity, implying that oxidative stress is triggered by acute iron exposure. Whereas, the two rotifers exhibited different antioxidant activities. Increased SOD and CAT enzymatic activity, as well as their encoding genes, indicating the effectively worked oxidative defences in the tropical *B. rotundformis*. By contrast, the temperate *B. plicatilis* showed substantial downregulation of *CuZnSOD* and *CYP*, and inhibited SOD enzymatic activity. Furthermore, downregulation of iron homeostasis-regulating genes, *iron oxidase*, and *Fe-S protein* in *B. plicatilis* should represent iron stresses. As a product of lipid peroxidation (MDA) was significantly increased in *B. rotundiformis*, but decreased in *B. plicatilis* after iron exposure. This phenomenon might be induced by the interaction of lipid and oxidative responses (Chen et al., 2017), and thus it is expected that iron might catalysis lipid depletion in *B. plicatilis*, reducing MDA enzyme levels (Han et al., 2021).

The variance of rotifer swimming speed under sub-lethal iron exposure were detected with an increasing trend in females but decreasing in males. Meanwhile, males exhibited shorter lifespans when the iron concentration increased. The swimming activity of female and male rotifers is important for mating initiation, which is a starter of the following mating process: the male circling attempt, copulation, and insemination. The ratio of copulation/mating attempts is only 2–5%, and only 10% of sperm numbers can be transferred in each insemination (Snell and Childress, 1987). Thus, males with longer lifespans and faster swimming speeds can search for females many times and choose appropriate individuals for insemination. Under the situation described as slower males and faster females in swimming speed, the mate encounter rate is likely decreased, which could suppress rotifer sexual reproductivity. Moreover, the inhibited *AChE* gene and associated AChE enzymatic activity after iron exposure might be served as evidence of iron regulate-effects on the locomotion of rotifers. AChE is proved to be involved in behavioral regulation (i.e., muscle movement and contraction) of aquatic species (Jin et al., 2019). The inhibition of AChE has a detrimental effect on the central nervous system of species, resulting in behavior disorders such as hyperactivity, paralysis, and loss of coordination (Roast et al., 2001). The increased female rotifer swimming speed under iron exposure may be due to hyperactivity. In this study, the swimming speed of control male rotifers in large type *B. plicatilis* was higher than the super small type *B. rotundiformis*, which is contrary to the common knowledge that rotifer species with small body sizes swim faster than large ones. This may be attributable to the incubation temperature (25 °C) used, which was higher than the

485 inhabit condition of 20 °C for *B. plicatilis*. Higher temperatures may improve their metabolic
486 rates, making them more active (Li et al., 2014).

487 Furthermore, as we hypothesized that higher iron exposure affected the quality of male sper-
488 matozoa. SYBR-14 and PI are two fluorescent nucleic stains that are used to evaluate the vi-
489 tality of sperm cells by staining the nuclei of living and dead sperm, respectively (Garner and
490 Johnson, 1995). The SYBR fluorescent signals gradually decreased in *B. plicatilis* under both
491 iron treatments, and in *B. rotundiformis* after iron concentrations exceeding 30 μg/mL, indicat-
492 ing deteriorated vitality or quantity of sperm cells. No PI-fluorescent signal was detected in
493 either treatment, which may be due to the use of youngest (< 30 min) males, as sperm cell
494 mortality and low quality increased with age (Snell and Hoff, 1987). Moreover, as the iron
495 concentration increased, the number of abnormal resting eggs increased. The poor sperm qual-
496 ity and a higher frequency of abnormal resting eggs, contribute to the deleterious effects of iron
497 on the sexual reproductivity of rotifers.

498

499 **5. Conclusions**

500 As summarised in Fig. 5, the present study defined the variation in iron ($FeSO_4 \cdot 7H_2O$) oxi-
501 dative stress triggered oxidative/antioxidant enzymatic responses, such as MDA, AChE, SOD,
502 and CAT between the two marine *Brachionus* sibling rotifers in different climatic regions.
503 These findings could help to better understand rotifer reproductive adaptations under stressful
504 conditions: vulnerability of male performances such as shortened lifespan, reduced sperm via-
505 bility, and swimming speed.

506

507 **Acknowledgments**

508

509 This research was partly supported by JSPS KAKENHI Grant numbers JP20H03063 and
510 JP20K21340 to AH, and 19K15897 to HK, the China Scholarship Council (CSC), and Naga-
511 saki university special research fellowship to CH. We would like to express our gratitude to
512 Professor Shigenobu Takeda and Yoshiko Kondo for providing us with knowledge on iron spe-
513 ciation.

514

515 **References**

Ahmed, A.S.S., Rahman, M., Sultana, S., Babu, S.M.O.F., Sarker, M.S.I., 2019. Bioaccumulation and heavy metal concentration in tissues of some commercial fishes from the Meghna River Estuary in Bangladesh and human health implications. Mar. Pollut. Bull. 145, 436–447. https://doi.org/10.1016/j.marpolbul.2019.06.035.

Aitken, R.J., Harkiss, D., Buckingham, D.W., 1993. Analysis of lipid peroxidation mechanisms in human spermatozoa. Mol. Reprod. Dev. 35, 302–315. https://doi.org/10.1002/mrd.1080350313.

Beasley, A., Belanger, S.E., Brill, J.L., Otter, R.R., 2015. Evaluation and comparison of the relationship between NOEC and EC10 or EC20 values in chronic Daphnia toxicity testing. Environ. Toxicol. Chem. 34, 2378–2384. https://doi.org/10.1002/etc.3086.

Breed, A.W., Dempers, C.J.N., Searby, G.E., Gardner, M.N., Rawlings, D.E., Hansford, G.S., 1999. The effect of temperature on the continuous ferrous-iron oxidation kinetics of a predominantly *Leptospirillum ferrooxidans* culture. Biotechnol. Bioeng. 65, 44–53. https://doi.org/10.1002/(SICI)1097-0290(19991005)65:1<44::AID-BIT6>3.0.CO;2-V.

Brock, J.H., Mulero, V., 2000. Cellular and molecular aspects of iron and immune function. Proc. Nutr. Soc. 59, 537–540. https://doi.org/10.1017/S002966510000077X.

Brown, M.R., 2002. Nutritional value and use of microalgae in aquaculture. Avance en Nutrición acuícola VI. Mem. del VI Symposium Internacional de Nutrición Acuícola 3, pp. 281–292.

Cadmus, P., Brinkman, S.F., May, M.K., 2018. Chronic toxicity of ferric iron for North American aquatic organisms: derivation of a chronic water quality criterion using single species and mesocosm data. Arch. Environ. Contam. Toxicol. 74, 605e615. https://doi.org/10.1007/s00244-018-0505-2.

Chen, X., Wang, X., Gu, X., Jiang, Y., Ji, R., 2017. Oxidative stress responses and insights into the sensitivity of the earthworms *Metaphire guillelmi* and *Eisenia fetida* to soil cadmium. Sci. Total Environ. 574, 300–306. https://doi.org/10.1016/j.scitotenv.2016.09.059.

Couillard, Y., Ross, P., Pinel-Alloul, B., 1989. Acute toxicity of six metals to the rotifer *Brachionus calyciflorus*, with comparisons to other freshwater organisms. Environ. Toxicol. Water Qual. 4, 451–462. https://doi.org/10.1002/tox.2540040405.

546 Dahms, H.U., Hagiwara, A., Lee, J.S., 2011. Ecotoxicology, ecophysiology, and mechanistic
547 studies with rotifers. Aquat. Toxicol. 101, 1–12. https://doi.org/10.1016/j.aqua-
548 tox.2010.09.006.
549 Desouky, M.M., 2009. Pathological effects of environmental pollution on the ultrastructure of
550 spermatogenic stages and the mature sperm of *Brachydontes variabilis* (Krauss, 1848) (Bi-
551 valvia-Mytilidae). Aust. J. Basic Appl. Sci. 3, 2627–2636.
552 Emmenegger, L., Schönenberger, R., Sigg, L., Sulzberger, B., 2001. Light-induced redox cy-
553 cling of iron in circumneutral lakes. Limnol. Oceanogr. 46, 49–61.
554 https://doi.org/10.4319/lo.2001.46.1.0049.
555 Garner, D.L., Johnson, L.A., 1995. Viability assessment of mammalian sperm using SYBR-14
556 and propidium iodide. Biol. Reprod. 53, 276–284. https://doi.org/10.1095/biolre-
557 prod53.2.276.
558 Gómez, A., Carmona, M.J., Serra, M., 1997. Ecological factors affecting gene flow in the *Bra-*
559 *chionus plicatilis* complex (Rotifera). Oecologia 111, 350–356.
560 González, A.G., Santana-Casiano, J.M., Pérez, N., González-Dávila, M., 2010. Oxidation of
561 Fe(II) in natural waters at high nutrient concentrations. Environ. Sci. Technol. 44, 8095–
562 8101. https://doi.org/10.1021/es1009218.
563 Gorski, J., Nugegoda, D., 2006. Sublethal toxicity of trace metals to larvae of the blacklip
564 abalone, *Haliotis rubra*. Environ. Toxicol. Chem. 25, 1360–1367.
565 https://doi.org/10.1897/05-060R.1.
566 Hagiwara, A., Jung, M.M., Sato, T., Hirayama, K., 1995a. Interspecific relations between ma-
567 rine rotifer *Brachionus rotundiformis* and zooplankton species contaminating in the rotifer
568 mass culture tank. Fish Sci 61, 623–627. https://doi.org/10.2331/fishsci.61.623.
569 Hagiwara, A., Kotani, T., Snell, T.W., Assava-Aree, M., Hirayama, K., 1995b. Morphology,
570 reproduction, genetics, and mating behavior of small, tropical marine *Brachionus* strains
571 (Rotifera). J. Exp. Mar. Biol. Ecol. 194, 25–37. https://doi.org/10.1016/0022-
572 0981(95)00081-X.
573 Hagiwara, A., Yamamiya, N., De Araujo, A.B., 1998. Effect of water viscosity on the popula-
574 tion growth of the rotifer *Brachionus plicatilis* Müller. Hydrobiologia 387, 489–494.
575 https://doi.org/10.1023/A:1017008208670.
576 Hall, L.W., Anderson, R.D., 1995. The influence of salinity on the toxicity of various classes

577 of chemicals to aquatic biota. Crit. Rev. Toxicol. 25, 281–346.
578 https://doi.org/10.3109/10408449509021613.
579 Han, C., Kim, H.-J., Lee, J.-S., Sakakura, Y., Hagiwara, A., 2021. Species-specific effects of
580 iron on temperate and tropical marine rotifers in reproduction, lipid and ROS metabolisms.
581 Chemosphere 277, 130317. https://doi.org/10.1016/j.chemosphere.2021.130317.
582 Hartl, F.U., 1996. Molecular chaperones in cellular protein folding. Nature 381, 571–579.
583 https://doi.org/10.1038/381571a0.
584 Hernández-Flores, S., Santos-Medrano, G.E., Rubio-Franchini, I., Rico-Martínez, R., 2020.
585 Evaluation of bioconcentration and toxicity of five metals in the freshwater rotifer *Euchlanis*
586 *dilatata* Ehrenberg, 1832. Environ. Sci. Pollut. Res. Int. 27, 14058–14069.
587 https://doi.org/10.1007/s11356-020-07958-3.
588 Ito, A., Shi, Z., 2016. Delivery of anthropogenic bioavailable iron from mineral dust and com-
589 bustion aerosols to the ocean. Atmos. Chem. Phys. 16, 85–99. https://doi.org/10.5194/acp-
590 16-85-2016.
591 Ito, S., Sakamoto, H., Hori, M., Hirayama, K., 1981. Morphological characteristics and suitable
592 temperature for the growth of several strains of the rotifer, *Brachionus plicatilis*. Bull. Fac.
593 Fish. Nagasaki Univ. 51, 9–16.
594 Jin, S., Shao, L., Song, X., Xiao, J., Ouyang, K., Zhang, K., Yang, J., 2019. Fertilization and
595 male fertility in the rotifer *Brachionus calyciflorus* in the presence of three environmental
596 endocrines. Chemosphere 220, 146–154. https://doi.org/10.1016/j.chemo-
597 sphere.2018.12.097.
598 Jonathan, M.P., Roy, P.D., Thangadurai, N., Srinivasalu, S., Rodríguez-Espinosa, P.F., Sarkar,
599 S.K., Lakshumanan, C., Navarrete-López, M., Muñoz-Sevilla, N.P., 2011. Metal concentra-
600 tions in water and sediments from tourist beaches of Acapulco, Mexico. Mar. Pollut. Bull.
601 62, 845–850. https://doi.org/10.1016/j.marpolbul.2011.02.042.
602 Jung, M.M., Hagiwara, A., Hirayama, K., 1997. Interspecific interactions in the marine rotifer
603 microcosm. Hydrobiologia 358, 121–126. https://doi.org/10.1023/A:1003120120931.
604 Lambeth, J.D., 2004. NOX enzymes and the biology of reactive oxygen. Nat. Rev. Immunol.
605 4, 181–189. https://doi.org/10.1038/nri1312.
606 Li, A.J., Leung, P.T., Bao, V.W., Yi, A.X., Leung, K.M.Y., 2014. Temperature-dependent tox-
607 icities of four common chemical pollutants to the marine medaka fish, copepod and rotifer.

608 Ecotoxicology 23, 1564–1573. https://doi.org/10.1007/s10646-014-1297-4.
609 Neubecker, T.A., Allen, H.E., 1983. The measurement of complexation capacity and condi-
610 tional stability constants for ligands in natural waters. Water Res. 17, 1–14.
611 https://doi.org/10.1016/0043-1354(83)90280-4.
612 Pahlavan-Rad, M.R., Pessarakli, M., 2009. Response of wheat plants to zinc, iron, and manga-
613 nese applications and uptake and concentration of zinc, iron, and manganese in wheat grains.
614 Commun. Soil Sci. Plant Anal. 40, 1322–1332.
615 https://doi.org/10.1080/00103620902761262.
616 Pantopoulos, K., Porwal, S.K., Tartakoff, A., Devireddy, L., 2012. Mechanisms of mammalian
617 iron homeostasis. Biochemistry 51, 5705–5724. https://doi.org/10.1021/bi300752r.
618 Perera, D., Pizzey, A., Campbell, A., Katz, M., Porter, J., Petrou, M., Irvine, D.S., Chatterjee,
619 R., 2002. Sperm DNA damage in potentially fertile homozygous β-thalassaemia patients
620 with iron overload. Hum. Reprod. 17, 1820–1825. https://doi.org/10.1093/hum-
621 rep/17.7.1820.
622 Rebolledo, U.A., Páez-Osuna, F., Fernández, R., 2021. Single and mixture toxicity of As, Cd,
623 Cr, Cu, Fe, Hg, Ni, Pb, and Zn to the rotifer *Proales similis* under different salinities. Environ.
624 Pollut. 271, 116357. https://doi.org/10.1016/j.envpol.2020.116357.
625 Roast, S.D., Widdows, J., Jones, M.B., 2001. Impairment of mysid (*Neomysis integer*) swim-
626 ming ability: an environmentally realistic assessment of the impact of cadmium exposure.
627 Aquat. Toxicol. 52, 217–227. https://doi.org/10.1016/S0166-445X(00)00153-3.
628 Sakuma, M., 1998. Probit analysis of preference data. Appl. Entomol. Zool. 33, 339–347.
629 https://doi.org/10.1303/aez.33.339.
630 Sarma, S.S.S., Peredo-Alvarez, V.M., Nandini, S., 2007. Comparative study of the sensitivities
631 of neonates and adults of selected cladoceran (Cladocera: Crustacea) species to acute tox-
632 icity stress. J. Environ. Sci. Health A 42, 1449–1452.
633 https://doi.org/10.1080/10934520701480839.
634 Santos-Medrano, G.E., Rico-Martínez, R., 2013. Lethal effects of five metals on the freshwater
635 rotifers *Asplanchna brigthwellii* and *Brachionus calyciflorus*. Hidrobiologica 23, 82–86.
636 https://doi.org/10.4161/epi.24362.
637 Santos-Medrano, G.E., Rico-Martínez, R., 2015. Acute and chronic effects of five metals in a
638 battery of freshwater planktonic organisms. Fresenius Environ. Bull. 24, 4658–4666.

639 Shaked, Y., 2008. Iron redox dynamics in the surface waters of the gulf of aqaba, red sea. GCA
640 72, 1540e1554. https://doi.org/10.1016/j.gca.2008.01.005.
641 Snell, T.W., Childress, M., 1987. Aging and loss of fertility in male and female *Brachionus*
642 *plicatilis* (Rotifera). Int. J. Invertebr. Reprod. Dev. 12, 103–110.
643 https://doi.org/10.1080/01688170.1987.10510306.
644 Snell, T.W., Hoff, F.H., 1987. Fertilization and male fertility in the rotifer *Brachionus plicatilis*.
645 Hydrobiologia 147, 329–334. https://doi.org/10.1007/BF00025762.
646 Snell, T.W., Moffat, B.D., Janssen, C., Persoone, G., 1991. Acute toxicity tests using rotifers:
647 IV. Effects of cyst age, temperature, and salinity on the sensitivity of *Brachionus calyciflo-*
648 *rus*. Ecotoxicol. Environ. Saf. 21, 308–317. https://doi.org/10.1016/0147-6513(91)90070-6.
649 Tanaka, C., Hashimoto, Y., Nakao, S., Yoshinaga, T., 2009. Effect of juglone on the survival
650 time of two *Brachionus* species (Rotifera): species-specific tolerance against oxidative
651 stress. Fish Sci 75, 191–194. https://doi.org/10.1007/s12562-008-0009-5.
652 Tucker, C.S., D'Abramo, L.R., 2008. Managing high pH in freshwater ponds. South. Reg. Aq-
653 uac Cen., 1–5.
654 Van den Berg, C.M.G., 1995. Evidence for organic complexation of iron in seawater. Mar.
655 Chem. 50, 139–157. https://doi.org/10.1016/0304-4203(95)00032-M.
656 Walczyńska, A., Serra, M., 2014. Inter-and intraspecific relationships between performance
657 and temperature in a cryptic species complex of the rotifer *Brachionus plicatilis*. Hydrobi-
658 ologia 734, 17–26. https://doi.org/10.1007/s10750-014-1859-8.
659 Winterbourn, C.C., 1995. Toxicity of iron and hydrogen peroxide: the Fenton reaction. Toxicol.
660 Lett. 82–83, 969–974. https://doi.org/10.1016/0378-4274(95)03532-X.
661 Yúfera, M., Parra, G., Pascual, E., 1997. Energy content of rotifers (*Brachionus plicatilis* and
662 *Brachionus rotundiformis*) in relation to temperature. Hydrobiologia 358: 83-87.

663 **Table 1.** Iron ($FeSO_4 \cdot 7H_2O$) chronic effects on the reproductive patterns of temperate *Brachionus plicatilis* and tropical *B. rotundiformis* rotifers.

		Iron (µg/mL)					
		0	20	30	45	60	75
B. plicatilis	Population growth rate (*r*)	0.626 ± 0.009^{A}	0.625 ± 0.014^{A}	0.622 ± 0.006^{A}	0.585 ± 0.010^{AB}	0.527 ± 0.006^{B}	0.403 ± 0.034^{C}
	Mixis (%)	29.3±3.7	28.5±3.0	23.7±2.2	19.4±3.1	17.0±1.8	24.2±3.6
	Fertilization (%)	23.9 ± 4.0^{A}	23.6 ± 7.6^{AB}	19.3 ± 2.7^{AB}	21.6 ± 6.1^{AB}	10.9 ± 6.7^{AB}	3.4 ± 1.7^{B}
	Resting eggs/mL	936.7 ± 111.4^{A}	686.7 ± 55.5^{AB}	550.0 ± 20.8^{BC}	476.7 ± 8.8^{BC}	323.3 ± 26.7^{CD}	96.7 ± 18.6^{D}
B. rotundi-formis	Population growth rate (*r*)	0.729 ± 0.017^{a}	0.724 ± 0.014^{a}	0.716 ± 0.011^{a}	0.620 ± 0.013^{b}	0.608 ± 0.016^{b}	0.428 ± 0.008^{c}
	Mixis (%)	19.3 ± 2.1^{ab}	25.1 ± 0.4^{a}	21.8 ± 1.7^{ab}	20.0 ± 1.2^{ab}	18.7 ± 1.7^{ab}	14.0 ± 2.1^{b}
	Fertilization (%)	29.5 ± 0.7^{a}	28.2 ± 1.2^{ab}	34.5 ± 5.8^{a}	26.1 ± 2.9^{ab}	19.1 ± 5.3^{ab}	8.8 ± 5.9^{b}
	Resting eggs/mL	1196.7 ± 133.1^{ab}	1578.3 ± 111.8^{a}	990.0 ± 36.1^{b}	410.0 ± 23.6^{c}	460.0 ± 170.1^{c}	121.7 ± 40.0^{c}

664 Data are mean ± standard Error. The letters indicate significant differences among treatments (A > B > C > D, a > b > c, Tukey HSD test, $p < 0.05$,
665 n = 3).

Table 2. 24-hour median lethal concentrations (24 h LC_{50}, μg/mL) and median effective concentrations (EC_{50}, μg/mL) for reproductive parameters, fecundity, lifespan, and swimming speed of temperate *Brachionus plicatilis* and tropical *B. rotundiformis* in response to iron ($FeSO_4 \cdot 7H_2O$) acute and chronic exposure.

	B. plicatilis	*B. rotundiformis*
Acute exposure (24 h LC_{50})		
LC_{50} (95% confidence intervals)	469.6 (438.3–501.0)	363.6 (323.4–406.0)
LC_{50} /dry weight (μg/mL per ng)	0.9 (0.8–1.0)	1.7 (1.5–1.8)
Chronic exposure (EC_{50})		
Population growth rate (*r*)	59.62	55.59
Mixis	34.54	45.80
Fertilization	58.52	67.98
Resting egg production	33.29	34.37
Unfertilized mictic female		
Fecundity	39.57	20.37
Lifespan	11.40	ND
Fertilized resting egg female		
Fecundity	37.43	41.59
Lifespan	17.66	50.83
Male		
Lifespan	36.13	43.61
Swimming speed	35.73	ND

ND indicates no data.

Figure Legends

Fig. 1. Effects of iron ($FeSO_4 \cdot 7H_2O$) on (A) intracellular reactive oxygen species (ROS) levels with and without N-acetyl-L-cysteine (NAC) treatment, and the enzyme activities related to (B) oxidative stress and (C) antioxidant metabolisms in the temperate *Brachionus plicatilis* and tropical *B. rotundiformis* after 12 h iron ($FeSO_4 \cdot 7H_2O$) exposure (A > B, a > b, Tukey HSD test, $p < 0.05$; *, ** and *** indicate $p < 0.05$, 0.01 and 0.001 respectively, Student's t-test, n = 3).

Fig. 2. Transcriptional levels of genes involved in (A) iron metabolism, (B) oxidative stress, and (C) anti-oxidant responses in temperate *Brachionus plicatilis* and tropical *B. rotundiformis* after 12 h iron ($FeSO_4 \cdot 7H_2O$) exposure (Student's t-test, *, ** and *** indicate $p < 0.05$, 0.01 and 0.001 respectively, n = 3).

Fig. 3. Patterns of lifespan and fecundity of (A–B) unfertilized and (C–D) fertilized mictic females, and (E–F) male lifespan in temperate *Brachionus plicatilis* and tropical *B. rotundiformis* under the different iron ($FeSO_4 \cdot 7H_2O$) concentrations (A > B, a > b > c, Tukey HSD test, $p < 0.05$, n = 12).

Fig. 4-1. The swimming speed of females and males in the (A) temperate *Brachionus plicatilis* and (B) tropical *B. rotundiformis* under the different iron ($FeSO_4 \cdot 7H_2O$) concentrations (A > B > C, a > b, Tukey HSD test, $p < 0.05$, n = 20).

Fig. 4-2. The male and stained living spermatozoa of rotifer (A) temperate *Brachionus plicatilis* and (B) tropical *B. rotundiformis* under the different iron ($FeSO_4 \cdot 7H_2O$) concentrations. The merged image contains the fluorescence of SYBR-14 (green) and PI (propidium iodide, red) staining. Arrow indicated data are the quantified fluorescence intensity. All scale bars represent 50 μm.

Fig. 5. Summary of iron acute and chronic toxicity responses in the two different climatic rotifer species. Blue and green represent the temperate rotifer *Brachionus plicatilis* and the tropical rotifer *B. rotundiformis*, respectively.

Fig. 1

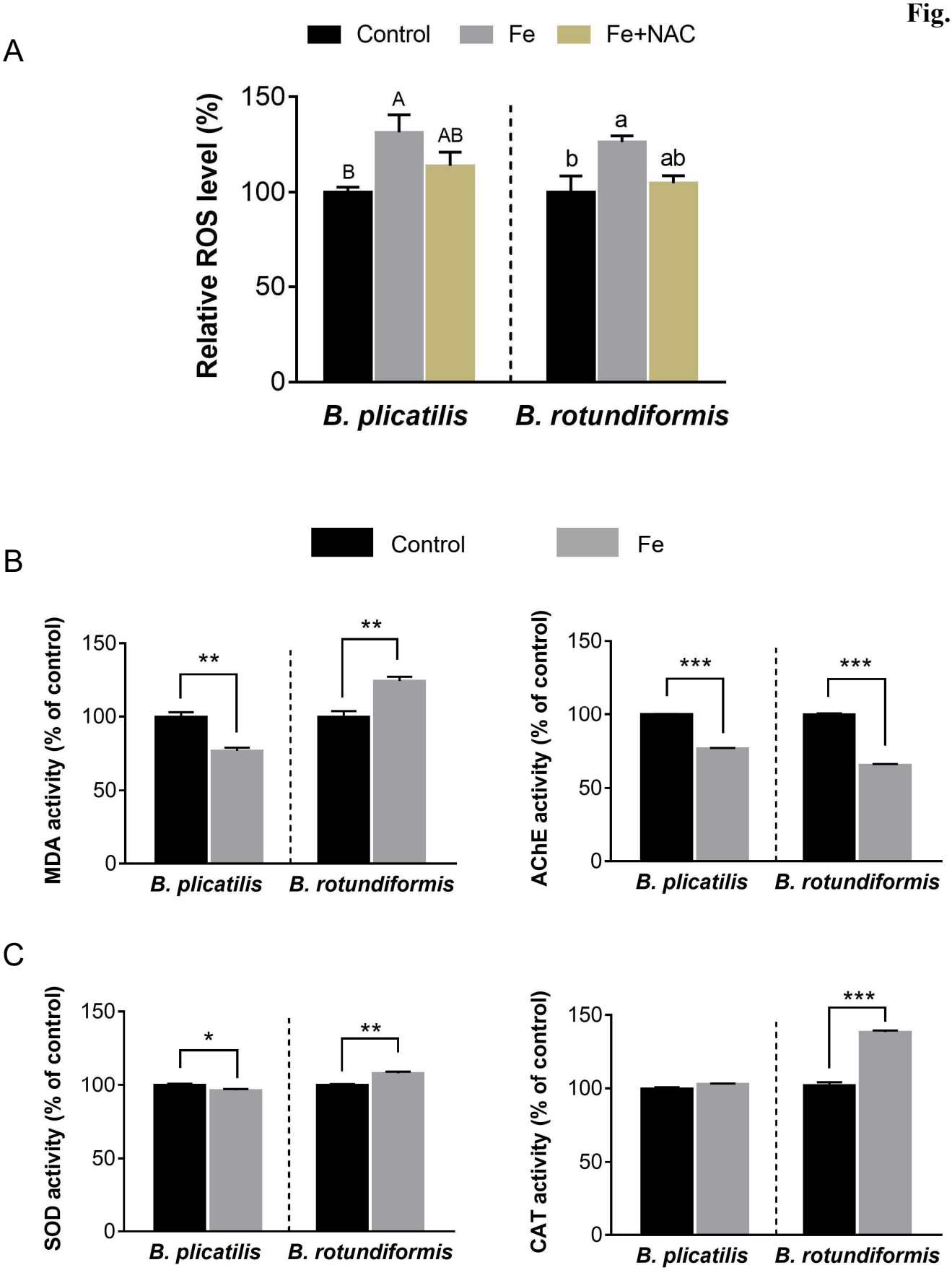

A
Control
Fe
Fe+NAC
Relative ROS level (%)
150
100
50
0
B
A
AB
b
a
ab
B. plicatilis
B. rotundiformis
B
Control
Fe
MDA activity (% of control)
**
**
AChE activity (% of control)
***
***
C
SOD activity (% of control)
*
**
CAT activity (% of control)
***

Fig. 2

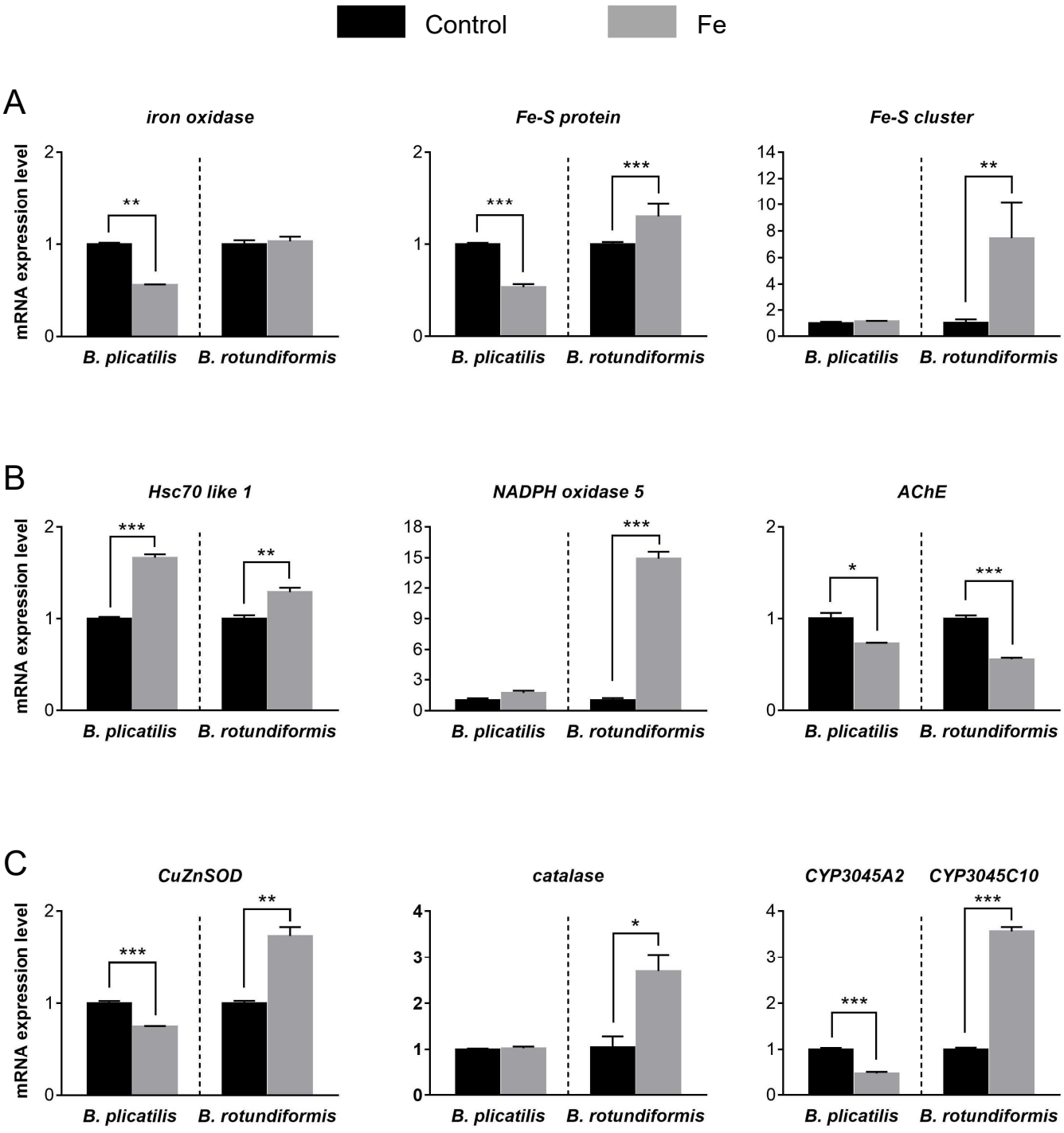

Control
Fe
A
iron oxidase
Fe-S protein
Fe-S cluster
mRNA expression level
B. plicatilis
B. rotundiformis
B
Hsc70 like 1
NADPH oxidase 5
AChE
C
CuZnSOD
catalase
CYP3045A2
CYP3045C10

Fig. 3

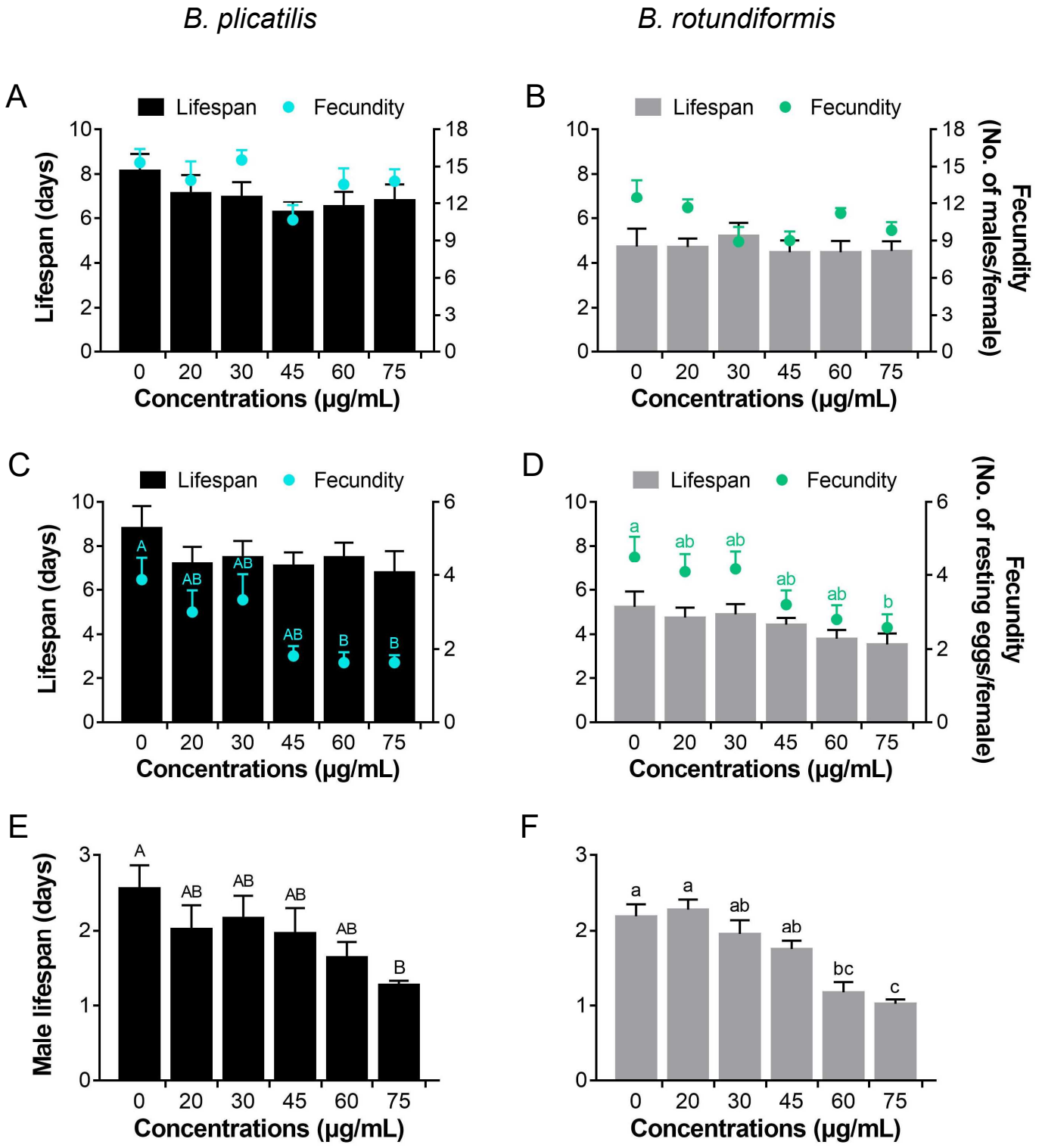

B. plicatilis
B. rotundiformis
A
B
C
D
E
F
Lifespan
Fecundity
Lifespan (days)
Fecundity (No. of males/female)
Fecundity (No. of resting eggs/female)
Male lifespan (days)
Concentrations (μg/mL)
0 20 30 45 60 75
A AB AB AB B B
a ab ab ab ab b
a a ab ab bc c

Fig. 4-1

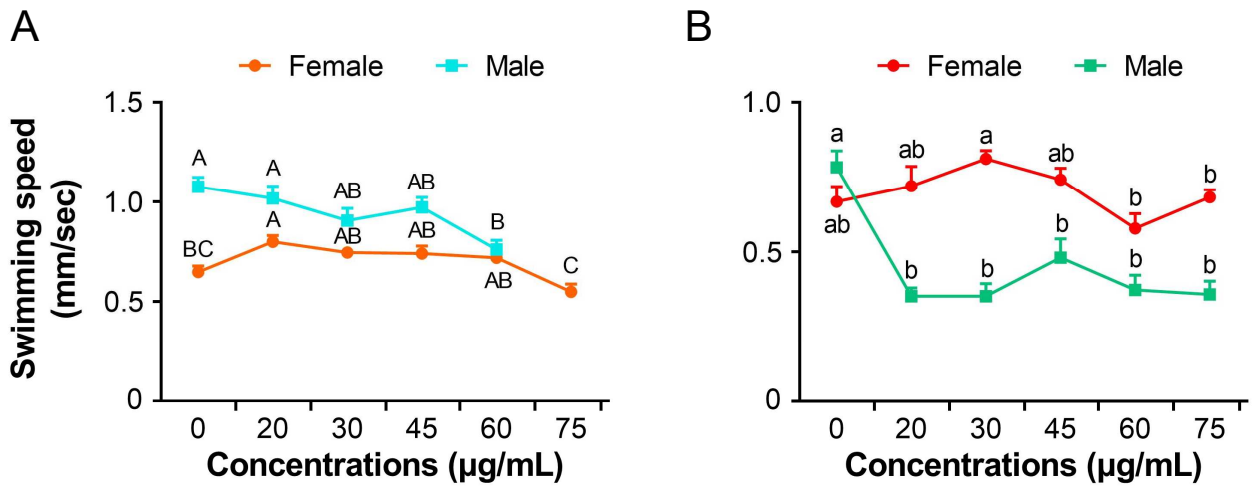

A
Female
Male
Swimming speed (mm/sec)
1.5
1.0
0.5
0
A
A
AB
AB
B
BC
A
AB
AB
AB
C
0
20
30
45
60
75
Concentrations (μg/mL)
B
Female
Male
1.0
0.5
0
a
ab
a
ab
b
b
ab
b
b
b
b
b
0
20
30
45
60
75
Concentrations (μg/mL)

Fig. 4-2

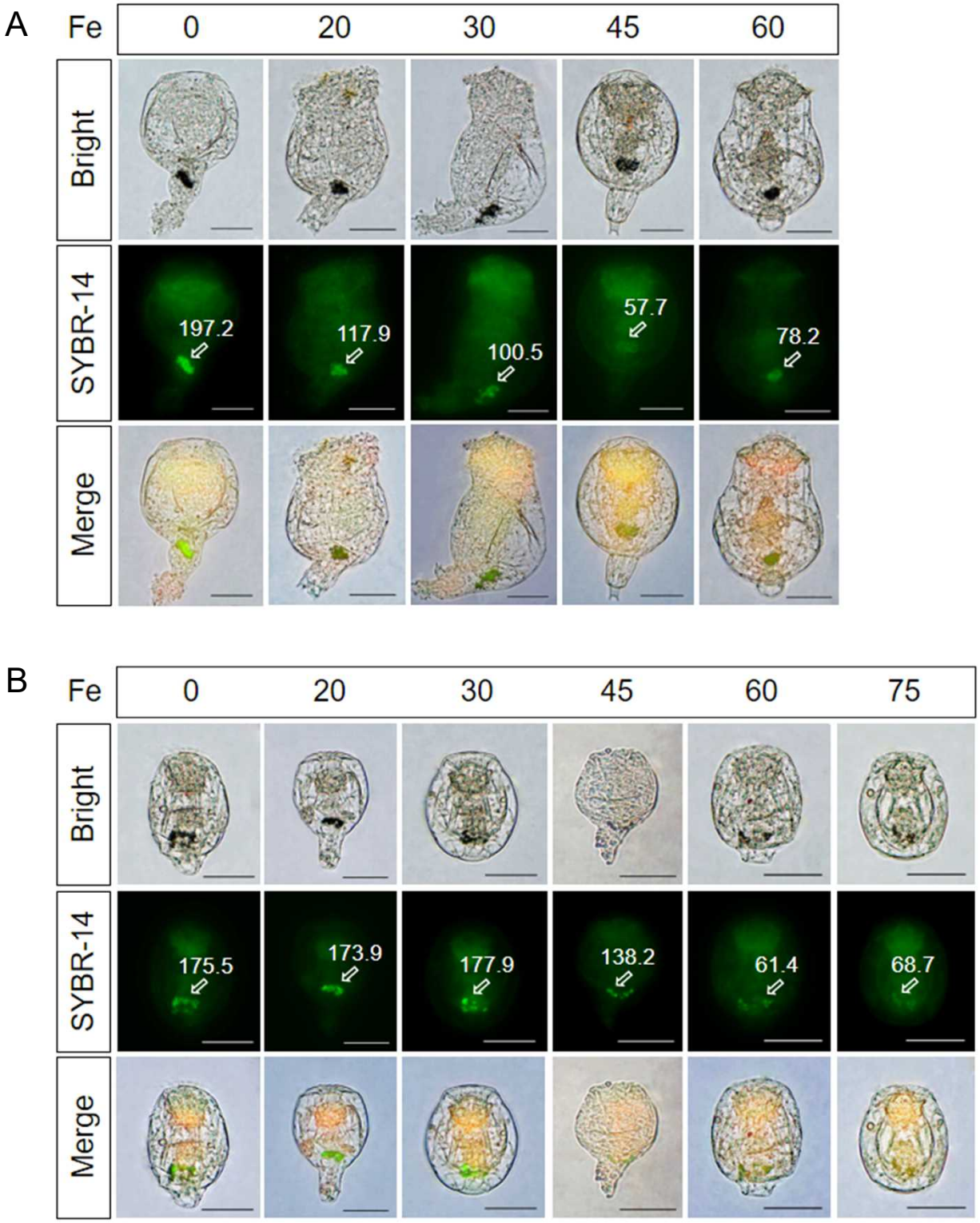
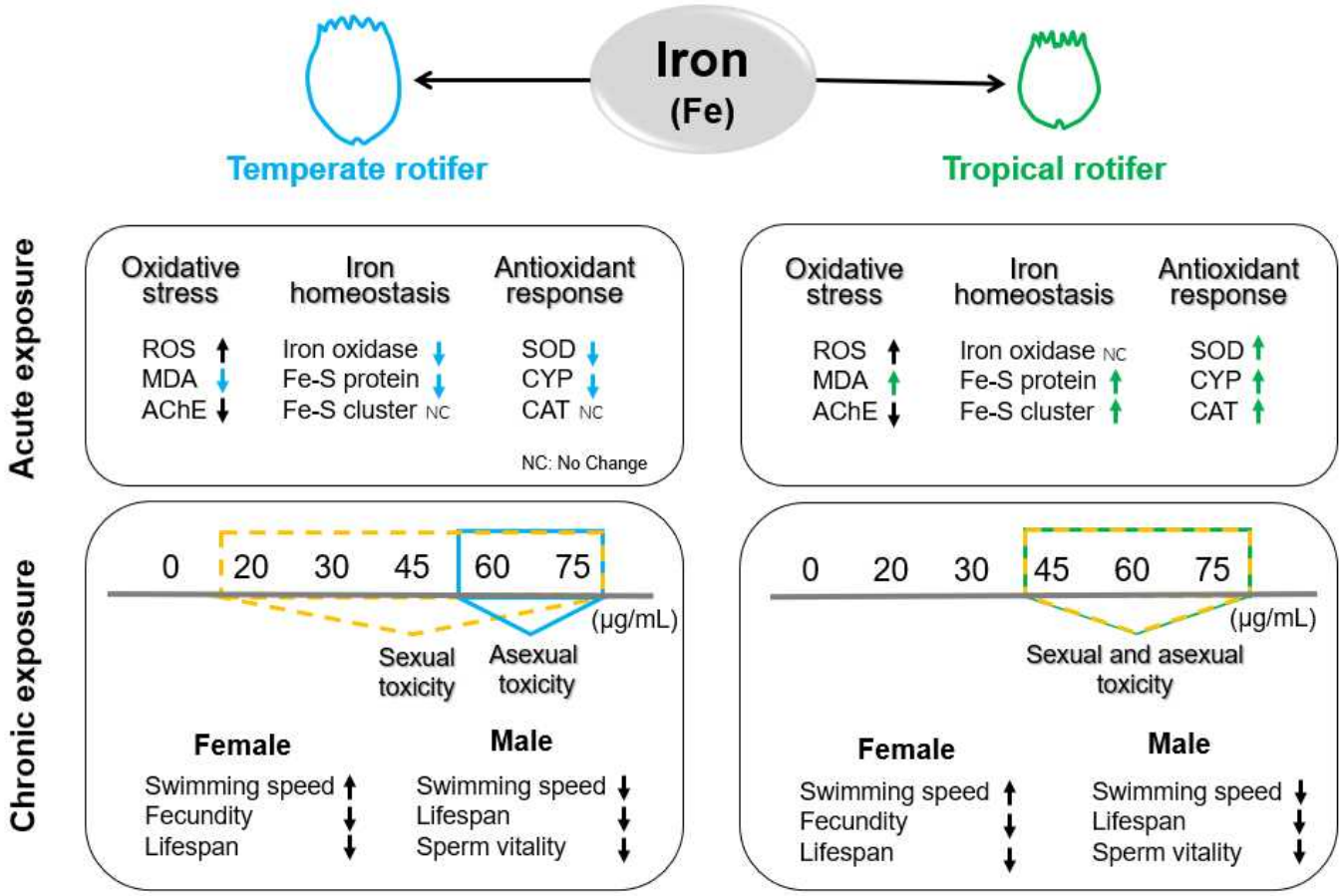

A
Fe
0
20
30
45
60
Bright
SYBR-14
197.2
117.9
100.5
57.7
78.2
Merge
B
Fe
0
20
30
45
60
75
Bright
SYBR-14
175.5
173.9
177.9
138.2
61.4
68.7
Merge

Fig. 5


Iron
(Fe)
Temperate rotifer
Tropical rotifer
Acute exposure
Chronic exposure
Oxidative stress
Iron homeostasis
Antioxidant response
ROS
MDA
AChE
Iron oxidase
Fe-S protein
Fe-S cluster NC
SOD
CYP
CAT NC
NC: No Change
Oxidative stress
Iron homeostasis
Antioxidant response
ROS
MDA
AChE
Iron oxidase NC
Fe-S protein
Fe-S cluster
SOD
CYP
CAT
0 20 30 45 60 75
(μg/mL)
Sexual toxicity
Asexual toxicity
Female
Swimming speed
Fecundity
Lifespan
Male
Swimming speed
Lifespan
Sperm vitality
0 20 30 45 60 75
(μg/mL)
Sexual and asexual toxicity
Female
Swimming speed
Fecundity
Lifespan
Male
Swimming speed
Lifespan
Sperm vitality

Graphical Abstract