Stress responses of reproduction and behavior in the marine rotifer *Brachionus* sibling species: Adaptation to temperate and tropical habitats

海産ワムシ姉妹種の生殖及び行動のストレス応答と温帯・熱帯域への適応

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Contents

Abstract

Rotifers are significant second consumers in hydrosphere food webs, which can transfer both the energy and pollutant to higher trophic levels. Therefore, they have widespread applications in aquaculture and ecotoxicology studies. Rotifer mass production is important for improving aquaculture productivity. However, they may encounter different kinds of stressors during cultivation, such as high levels of unionized ammonia (NH_3-N), increased as the rotifer population expands, and overdosed trace metals. In aquaculture operations, iron (Fe) and zinc (Zn), as essential trace nutrients, are added to the culture medium of marine microalgae. The algae suspension without filtration, known as a "green water" technique, was commonly supplied in the culture tanks of rotifers or fish larvae. Whilst this procedure may put rotifers at a risk of trace metal overexposure or overload, which could induce deleterious effects. Furthermore, some rotifer sibling species from different habitats, such as temperate water species *Brachionus plicatilis* and tropical species *Brachionus rotundiformis*, exhibit distinct tolerance to ecological factors and show different growth characteristics even under similar culture conditions. It is expected that the two species may represent varied reproductive strategies regarding stressful situations because of their habitat-evolutionary experience, nevertheless, the underlying mechanisms have not been uncovered.

Based on these considerations, this study aims to (1) evaluate the stress tolerance and reproductive flexibility of the two rotifer sibling species, *B. plicatilis* and *B. rotundiformis*, and (2) identify the underlying mechanisms that regulate their stress-reproductive adaptations following exposure to different levels of Fe (ferrous sulfate) (Chapter II), Zn (zinc chloride) (Chapter III), and ammonia (ammonium chloride) (Chapter IV), respectively.

First, the median lethal concentrations (LC_{50}) of $FeSO_4 \cdot 7H_2O$, $ZnCl_2$, and NH_4Cl (expressed as NH_3-N form) to the employed two rotifer species were determined after 24 h acute exposure. Because the tolerance of toxicants was significantly affected by the animal size, thus, to compare the toxic tolerance in the two species, I transformed the LC_{50} values by dividing the dry weights (DW) of rotifers. In this transformation, the Fe and NH_3

tolerance in *B. rotundiformis* were greater than in *B. plicatilis*, with 1.9- and 1.5-fold higher LC_{50}/ng DW values, respectively. While in response to Zn exposure, the two rotifers showed similar transformed values.

Second, rotifer reproductive patterns and swimming speed, a sensitive behavior biomarker in rotifer toxicology studies, were investigated following chronic exposure to $FeSO_4 \cdot 7H_2O$ (0–96 mg/L), $ZnCl_2$ (0–2 mg/L), and NH_3-N (0–29.3 mg/L) individually or in batches. The results revealed that the two rotifer species displayed species-specific reproductive sensitivity associated with chemical concentrations, especially in sexual reproduction. The mixis induction and resting egg production in *B. plicatilis* were more susceptible to either the trace elements or NH_3-N exposure. Conversely, *B. rotundiformis* showed higher resilience in response tothe same treatments, such as 6 mg/L of Fe and 1 mg/L of Zn, with enhanced sexual reproductivity. In behavior observations, both species demonstrated increased swimming speed following Zn and NH_3-N exposure. Whereas exposure to Fe affected differently in females and males in both species. Fe concentrations of 20–45 mg/L resulted in a decrease in male swimming speed but an increase in female swimming speed.

Third, to uncover the underlying mechanisms involved in the different reproductive flexibility of the two species, the biological activities that regulate rotifer reproductive fitness were estimated, such as lipid (an energy source) accumulation in female reproductive organs and male sperm viability. Additionally, stress response-associated metabolisms, such as intracellular reactive oxygen species (ROS) production and antioxidant activities were evaluated. Consequently, the two rotifers demonstrated differential modulations in lipid accumulation, sperm viability, and ROS scavenging responses, with overall inhibition in *B. plicatilis* but stimulation in *B. rotundiformis*. The reduced neutral lipid accumulation and downregulated lipid-synthesis-related biomarkers (e.g., *acetyl-CoA carboxylase* and *mitochondrial cytochrome*) were observed in *B. plicatilis*. The suppressed antioxidant activities in *B. plicatilis*, evidenced by inhibited SOD, CAT, and CYP, suggested that stress-induced ROS was not neutralized by its defense systems, thereby negatively affecting rotifer reproduction. Furthermore, Fe and Zn exposure sensitively inhibited the sperm viability of *B. plicatilis*. In contrast, *B. rotundiformis* exhibited active defensive responses, with enhanced antioxidant activity and

lipid synthesis, implying the higher resilience of *B. rotundiformis* in response to stressful situations.

In conclusion, this study determined species-specific stress response patterns in tolerance and reproductive flexibility between the temperate and tropical marine rotifer sibling species. The potential metabolisms involved were established by lipid and ROS scavenging responses. Different sexual reproductive sensitivity associated with inhibited/stimulated lipid metabolic in two rotifers, implied the significance of lipids in regulating rotifer sexual reproduction. Furthermore, stress stimulating effects on rotifer swimming speed suggested an indirect impact of rotifer reproduction via behavior regulations. Additionally, in aquaculture practice, the greater tolerance or resilience of *B. rotundiformis* in response to stress exposure, supported its high-density endurance during mass cultivation. Finally, the different modes of stress response in the two rotifers might be related to the temperate and tropical habitat evolutionary adaptations, which should be investigated further.

Chapter I

General Introduction

Rotifers are widely used live food for rearing fish larvae in aquaculture due to their features of ease of culture, growth rapidly, and exhibit appropriate size distributions for feeding establishment (Lubzens et al., 1989; Hagiwara et al., 1995b, 1998, 2007; Wallace, 2002). Furthermore, rotifers, as significant secondary consumers in hydrosphere food webs, can transfer not only energy but also pollutants, to higher trophic levels, and thus have widespread applications in ecotoxicology research (Snell and Janssen, 1995; Dahms et al., 2011; Snell and Marcial, 2017; Won et al., 2017). The mass production of rotifer promotes aquaculture productivity, however, rotifer propagation is limited by numerous factors, such as food shortage (Yoshimura et al., 1996, 1997), increased unionized ammonia (NH_3-N) and water viscosity (Yu and Hirayama, 1986; Hagiwara et al., 1998), as well as protozoa and bacteria contamination (Yu et al., 1990; Jung et al., 1997; De Araujo et al., 2000, 2001).

The production of rotifers strongly depends on the quality and quantity of their microalgal diet (Lubzens, 1987; Reitan et al., 1997; Lubzens et al., 2001). Trace metals, including iron (Fe) and zinc (Zn), are essential micronutrients for the growth of algae (Sunda, 1989). However, the natural concentrations of dissolved Fe and Zn, especially in seawater, are very low, with Fe concentrations ranging from 0.09 to 3.05 nM (Johnson et al., 1997; Schallenberg et al., 2016) and Zn contents ranging from 0.3 to 2 nM (Ellwood and Van den Berg, 2000), potentially limiting ocean productivity. In the field studies, dissolved Fe supplementation in high-nitrate and low-chlorophyll ocean waters promote phytoplankton biomass (Martin and Fitzwater, 1988; Martin et al., 1990; Tsuda et al., 2003). This kind of investigation has been conducted for several decades (Martin et al., 1989; Sunda and Huntsman, 1995; Trick et al., 2010). In aquaculture operations, certain amounts of Fe (1.3–10 mg/L) and Zn (0.023–2.2 mg/L) are commonly added to the culture medium of microalgae (Guillard, 1975). The algae suspension without filtration is supplied in the cultural tanks of zooplankton (e.g., rotifer) and fish larvae, which is termed as “green water” technique (Naas and Harboe, 1992; Neori, 2011). Nevertheless, Wang et al. (2019)

observed that the application of "green water" enhances rotifer gut algae retention and increases Zn, Fe, and Cu concentrations in rotifers. The algae (*Nannochloropsis* spp.) diet for rotifer has 2-fold higher Fe (298 mg/kg) and Zn (103 mg/kg) concentrations than rotifers (Hamre et al., 2008; Wang and Wang, 2018; Wang et al., 2019). However, it is also suggested that the negative effects of enriched microelements in rotifers should be considered during the practical application of "green water" technique (Wang et al., 2019).

Microelements have deleterious effects on the growth and reproduction of aquatic organisms when exceeding a threshold. The inhibitory effects of Fe and Zn on the reproduction of aquatic vertebrates (Baker et al., 1997; Slaninova et al., 2014; Mohanty and Samanta, 2018; Gemaque et al., 2019) and invertebrates (Conradi and Depledge, 1999; Cadmus et al., 2018; Hernández-Flores et al., 2020) have been extensively studied. Moreover, the toxicity threshold of Fe and Zn differs with species. For example, 4.1 mg/L of Fe causes abnormal morphological development in the marine abalone *Haliotis rubra* larvae (Gorski and Nugegoda, 2006). Whereas low concentrations of 0.14–0.33 mg/L Fe suppresses the survival in minute marine rotifer *Proales similis* (Rebolledo et al., 2021). Furthermore, Fe and Zn have been reported to involve in spermatogenesis, affecting the sexual reproduction of species (Aitken et al., 1993; Perera et al., 2002; Desouky, 2009; Yamaguchi et al., 2009). A certain amount of Fe and Zn is essential for germination, normal sperm function, and fertilization; however, high levels inhibit sperm viability, which has been reported in Japanese eel *Anguilla japonica* (Yamaguchi et al., 2009), marine mytilid *Brachydontes variaibilis* (Desouky, 2009), and sea urchin *Lytechinus pictus* (Clapper et al., 1985). A recent study observed Zn sex-specific reproductive sensitivity based on antioxidant and detoxification systems in female and male water flea *Daphnia magna* (Lee et al., 2021). Nevertheless, whether or how Fe and Zn long-term/overdose exposure regulates the reproductive patterns in rotifer species, which remains unknown and deserves investigation.

Another restrictive factor of rotifer production is a high level of ammonia, the primary nitrogenous product of protein catabolism (Yoshimura et al., 1996; Timmons et al., 2002). It is released from the excreta of various species (Alcaraz et al. 1994) and the mineralization of organic detritus, such as unconsumed feed and feces (Rain-Franco et al., 2014). The total ammonia-nitrogen consists of toxic un-ionized NH_3-N and nontoxic ionized NH_4^+-N

(Thurston et al., 1981), and their equilibrium is dependent on temperature, pH, and salinity (Emerson et al., 1975). NH_3-N levels increase as the population of an aquatic species expands. High NH_3-N concentrations can disrupt the normal metabolism of aquatic vertebrates and invertebrates, resulting in growth retardation, neurotoxicity, and respiratory impairment by inducing oxidative stress (Eddy, 2005; Timmons et al., 2002; Ching et al., 2009; Yang et al., 2012; Xiao et al., 2019; Zhang et al., 2020). Therefore, determining the thresholds at which NH_3-N levels affect the reproduction of aquatic species and how to manage its levels during cultivation may help to improve aquaculture productivity. Several studies have examined ammonia toxicity in rotifer species (Schlüter and Groeneweg, 1985; Yu and Hirayama, 1986; Snell and Boyer, 1988; De Araujo et al., 2000, 2001; Gao et al., 2021). Kim et al. (2020) described an escape behavior with vigorous swimming in rotifer *Brachionus plicatilis* in response to NH_3-N (5–20 mg/L) short-term (48 h) exposure. However, how ammonia effects on rotifer reproduction and locomotion are poorly understood.

My study employed two marine rotifer sibling species in the *B. plicatilis* species complex, the *B. plicatilis* and *Brachionus rotundiformis*, which are originated from temperate (water temperatures of 15–30°C) and tropical (20–35°C) habitats, respectively. Moreover, these two species have been used extensively in establishing feeding regimes for fish larvae, due to their different size distributions. *B. plicatilis* has a large lorica size (L-type) of 275 μm, while the super-small (SS) type *B. rotundiformis* has a lorica size of 179 μm (Hagiwara et al., 1995b, 2001; Jung et al., 1997). They exhibit different growth characteristics. *B. rotundiformis* grows faster than *B. plicatilis* in either their respective optimal conditions (Hagiwara et al., 1993; Yoshimura et al., 1996; Balompapueng et al., 1997) or in settings with similar culture temperature and salinity, such as 25°C and 22 parts per thousand (ppt) (Ito et al., 1981). During this process, they may be subjected to different levels of stressors, such as NH_3-N. For example, 1.0–8.0 mg/L and 0.2–1.2 mg/L of NH_3-N are recorded when the maximum density of *B. rotundiformis* and *B. plicatilis* reached 12,000 individuals/mL and 680 individuals/mL, respectively (Yoshimura et al., 1996; Balompapueng et al., 1997), which may reflect different NH_3-N tolerance. It was reported that the stress tolerance of aquatic species was size-dependent. Generally, smaller animals were less tolerant to toxicants than that of larger species (Jiang et al., 2012; Vesela and

Vijverberg, 2007). This pattern was also observed in the *Brachionus* rotifers when exposed to the metal cadmium (Kang et al., 2019). Nevertheless, Tanaka et al. (2009) observed that the SS-type *B. rotundiformis* exhibited higher tolerance in survival than the L-type *B. plicatilis* in response to juglone compound-induced oxidative stress.

Environmental factors affect the stress responses of aquatic organisms. For example, temperature governs metabolisms of species through affecting oxygen consumption rates (Childress, 1976). There is a positive correlation between the metabolic rate of organisms and the temperatures, within the temperature ranges of 0 to 40°C (Gillooly et al., 2001). However, a high metabolic rate not only promotes the absorption of toxicants by species but also enhances the generation of reactive oxygen species (ROS), inducing oxidative stress (Childress, 1976; Li et al., 2014). Hence, the species from varied habitats with distinct temperatures may be subjected to different levels of oxidative stress. A biological phenomenon of "environmental hormesis" is recognized recently, in which previous exposure to low levels of given environmental stress has the effect of priming defenses against a subsequent heightened level of stress, thereby enabling organisms to recover/re-establish homeostasis (resilience) more effectively (Agathokleous, 2018; Agathokleous et al., 2018; Erofeeva, 2022). Furthermore, researchers have identified local adaptations in relation to habitat experience in the rotifer *B. plicatilis* species complex. Those rotifers coming from higher salinity habitats show better tolerance to high salinity (Campillo et al., 2009, 2011), and the populations from the least saline lake are adapted to low salinity (Alcantara-Rodriguez et al., 2012). Therefore, it is expected that habitat adaptation affects rotifer reproductive patterns in response to stressful conditions, finally regulating their population fitness.

Based on these considerations, this study aims (1) to compare the reproductive flexibility of two sibling species of marine rotifers from distinct habitats in response to different concentrations of trace metals (Fe and Zn) or ammonia, all of which play an important role in regulating marine ecosystem productivity. (2) to ascertain the underlying metabolisms in regulating rotifer stress-reproductive responses. The investigations are conducted via multiple endpoints, including survival, reproduction at both the individual and population levels, behavior (female and male swimming speed), as well as genetic and biochemical (intracellular reactive oxygen species [ROS] production, antioxidant

defensive activity, lipid status, and sperm viability) modifications. Chapter II investigates two rotifer species' reproductive and behavioral flexibility following exposure to low to high amounts of ferrous sulfate ($FeSO_4 \cdot 7H_2O$). The involved metabolisms are established by modulations in neutral lipid and ROS levels (Chapter II.1), as well as sperm viability and stress enzymatic activity (Chapter II.2). Chapter III determines the reproductive and behavioral responses of two rotifer species following short-term and long-term zinc chloride ($ZnCl_2$) exposure. Chapter IV investigates acute and chronic NH_3-N tolerance in the two rotifer species, and identifies species-specific antioxidant defenses associated with their reproductive sensitivity. Finally, a general summary of rotifer reproductive and behavioral responses, as well as a discussion of the potential effects of habitat-associated adaptations in regulating rotifer stress-reproductive patterns were presented in the Chapter V.

Chapter II

Iron responses in two rotifer sibling species: Reproduction, behavior, and stress defensive metabolisms

II-1. Species-specific effects of iron on temperate and tropical marine rotifers in reproduction, lipid, and ROS metabolisms

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1. Introduction

The essential trace metal iron (Fe) has conflicting effects on the physiological activity of all living organisms. Iron is an essential nutrient for aquatic biology and an important component of several enzymes (e.g., superoxide dismutase [SOD], catalase [CAT], and cytochrome P450s [CYPs]) and metal proteins (e.g., hemoglobin and myoglobin), which are involved in the process of oxygen transport, redox reactions, immune regulation, and the metabolism of spermatozoa (Paoli et al., 2002; Tvrda et al., 2015). However, iron inhibits metabolism, and in excessive amounts it can induce oxidative stress, resulting in respiratory disorders and adversely affecting the growth, metabolism, and reproduction of organisms (Papanikolaou and Pantopoulos, 2005). In microalgae (e.g., *Chlorella vulgaris* and *Botryococcus* spp.), iron overload inhibits the cell division rate, impairs photosynthesis, and damages the cell membrane of algae because of the generation of reactive oxygen species (ROS) (Barhoumi and Dewez, 2013; Xavier et al., 2016; Yeesang and Cheirsilp, 2011). Moreover, in some freshwater species (e.g., Oligochaete worm *Lumbriculus* sp., knife fish *Notopterus* sp., and boreal toad tadpoles *Bufo boreas*), inhibited reproduction and development have been observed because of an excessive amount of iron, resulting in organisms that are significantly smaller in size than average (Cadmus et al., 2018; Mohanty and Samanta, 2018).

Multiple sources of iron in the ocean include atmospheric deposits, sea ice, hydrothermal fluids, and anthropogenic factors (Boyd and Ellwood, 2010; Duce and Tindale, 1991). A site study recorded higher levels (> 13 μg/mL) of iron in marine waters

due to human activities (Jonathan et al., 2011). Iron is found in two oxidation states within the hydrosphere: reduced ferrous iron (Fe II) and oxidized ferric iron (Fe III). Soluble ferrous iron, which is toxic, rapidly oxidizes to ferric iron under oxygenated conditions. Ferric iron, the predominant form of iron in seawater, is insoluble and rapidly precipitates into hydroxides and oxy-hydroxides at pH > 6.5 (Cadmus et al., 2018; Emerson, 2000; Hermann and Neumann-Mahlkau, 1985). The low concentration of dissolved iron present in natural systems (0.56–17 × 10^{-5} μg/mL in seawater and 2.8–140 × 10^{-3} μg/mL in freshwater) limits its bioavailability (Hirst et al., 2017; Johnson et al., 1997; Martin and Fitzwater, 1988; Martin et al., 1990). Previous studies have indicated that iron-fortification (e.g., improving the concentration of dissolved iron 100-fold) in iron-poor areas of the ocean promoted the growth of phytoplankton (Tsuda et al., 2003) and induced a short-term drawdown of CO_2 (Watson et al., 2000). In the microalga *Chlorella vulgaris*, supplementation with 2 μg/mL $FeCl_3$ enhanced the biomass productivity of *C. vulgaris* by 57% and produced an accumulation of neutral lipids (Liu et al., 2008). In the green microalga *Scenedesmus dimorphus*, the highest biomass and lipid accumulation was achieved with 45 μg/mL iron enrichment (Ruangsomboon et al., 2013). Iron is also required for the development of muscles in fishes. The dietary requirement of iron was estimated to 83.9 μg/g (dry weight) for marine medaka *Oryzias melastigma* larvae (Wang and Wang, 2016), 60–100 μg/g for Atlantic salmon *Salmo salar* L. (Andersen et al., 1996), and 285–444 μg/g for red drum *Sciaenops ocellatus* (Zhou et al., 2009). Typically, 1.3–10 μg/mL of iron is added to the microalgae culture medium, which is used in the cultivation of zooplankton, such as rotifers and fish larvae (Borowitzka, 1997; Brown, 2002; Gopinathan, 1982; Xavier et al., 2016). In this procedure, iron accumulation in the digestive and sexual organs of rotifers was confirmed by X-ray fluorescence analysis (Ezoe et al., 2002). In the aquaculture facilities, rotifers are generally fed on iron-fortified microalgal and accumulated iron (Wang et al., 2019) by proper concentrations induced positive growth-performance and immune responses of fish larvae (Lim and Klesius, 1997; Rigos et al., 2010). Based on these issues, it was expected that iron contributes to improving ocean productivity (Bjerrum and Canfield, 2002; Schmidt et al., 2016).

Rotifers are small aquatic invertebrates that feature rapid population growth rate with ease of culture and sensitive to a vast number of exogenous substances (Gómez, 2005;

Snell, 1998; Snell and Marcial, 2017; Wallace, 2002). Such attributes make them a useful model for *in vivo* toxicological and lifespan studies (Dahms et al., 2011; Snell and Janssen, 1995). The monogonont rotifer *Brachionus plicatilis* sp. complex is the most extensively studied rotifer group and is composed of 15 genetically different species (Mills et al., 2017) with the 3 morphotypes—L, S, and SS—based on their lorica size of 275 μm (mean value), 212 μm, and 179 μm, respectively (Hagiwara et al., 1995, 2001, 2007). Of these, *B. plicatilis* (NH17L strain), which has a larger lorica than other morphotypes of the species, is found in the temperate regions with water temperatures < 25°C, whereas the smaller *B. rotundiformis* (Kochi strain) is common in tropical or subtropical regions at temperatures > 30°C (Hagiwara et al., 2001; Kotani and Hagiwara, 2003). The two rotifer species have adapted to their respective environments and thus show interspecific differences in response to temperature, salinity, pH, and chemical substances (Fielder et al., 2000; Kang et al., 2019; Marcial et al., 2005; Radix et al., 2002; Snell and Joaquim-Justo, 2007). In general, the toxicity of chemical substances is dependent on temperature (Gordon, 2003; Khan et al., 2006). However, few studies have investigated the chemical effects on animals that have adapted to different temperature conditions or studied the mechanisms of such adaptions. In this study, two rotifer species from the *B. plicatilis* sp. complex found in temperate and tropical climates were investigated in response to iron, which has positive (essential nutrients) and negative (metabolism inhibition) roles depending on their abundance. We also hypothesized that the temperate and tropical rotifer species are likely to adopt different response patterns for iron exposure at 25°C, at which the 2 species can co-occur. To test this hypothesis while examining the mechanisms involved, three experiments were conducted: (1) sexual (mixis, fecundity, and resting egg production) and asexual (population growth rate) reproduction parameters in response to different concentrations of iron over 7 days in a batch culture; (2) the lifetable demography (lifespan, age-specific survivorship, fecundity of amictic and mictic females, and intrinsic growth rate) of rotifers under different iron concentrations in an individual culture; and (3) the role of iron in the generation of ROS and lipid metabolism. Intracellular ROS levels were measured to estimate the oxidative status of rotifers in response to iron, whereas the quantity of neutral lipids was assessed using Nile red staining. Finally, the mechanisms of the observed phenomena were confirmed with the mRNA expression on the following

functions: oxidative/antioxidant response, lipid metabolism, and rotifer reproduction in two *Brachionus* rotifers distributed in different climatic zones, temperate and tropical estuaries.

2. Materials and methods

2.1. Rotifer preparation

The rotifer *B. plicatilis* (NH17L strain, L-type) and *B. rotundiformis* (Kochi strain, SS-type) (Hagiwara et al., 2001) have been maintained at the Aquaculture Biology Laboratory (Nagasaki University, Japan) for over two decades. The rotifers were cultured in filter/autoclaved artificial seawater (ASW) at 17 parts per thousand (Marine Art Hi, Tomita Pharmaceutical, Tokushima, Japan) and fed with the microalga *Nannochloropsis oculata* (7.0×10^6 cells/mL) at 25°C in complete darkness. The neonates (< 2 h) that hatched from the amictic eggs were subjected to the same experimental conditions. To obtain the neonate, approximately 15,000 female rotifers carrying amictic eggs of the stock culture were inoculated 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 (Marcial et al., 2005) at 25°C. The eggs were observed every 30 min, and neonates (< 2 h) were obtained.

2.2. Iron concentrations

Ferrous sulfate ($FeSO_4 \cdot 7H_2O$, purity 99.0–102.0%; Wako, Osaka, Japan) was used in this study. A stock solution was prepared by dissolving 10 mg $FeSO_4 \cdot 7H_2O$ in 1 mL of distilled water, followed by magnetic stirring for 30 min. The nominal test solution was obtained by diluting the stock solution with ASW. The actual iron concentrations shown in **Table 1-1** were measured using Palintest Iron MR according to the standard laboratory procedure used for iron detection (Photometer 8000, Palintest Ltd, Gateshead, England). The samples were preserved with high-purity nitric acid at pH < 2 before measurement to avoid metal hydrolytic precipitation and adsorption (Cadmus et al., 2018).

2.3. Reproductive parameters

Based on the preliminary test, 6 concentrations of $FeSO_4 \cdot 7H_2O$, which did not induce mortality within 48 h, were used to investigate the effects of iron on the rotifer. They are 0, 6, 12, 24, 48, and 96 μg/mL for *B. plicatilis* NH17L and 0, 1.5, 3, 6, 12, and 24 μg/mL for B. *rotundiformis* Kochi. The rotifers were inoculated into 100 mL screw-capped bottles with 80 mL working volume at 1 individual/mL, then fed daily with *N. oculata* (7.0×10^6 cells/mL) and incubated in complete darkness at 25°C for 7 days. 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) (Satuito and Hirayama, 1990). 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 eggs (RF) were daily counted. These numbers were used to calculate the population growth rate (*r*), mixis induction (%), and fertilization (%). Half of the culture medium was renewed daily (Hagiwara et al., 1988; Marcial et al., 2005; Hu et al., 2020). All experiments were performed in triplicate. The reproductive parameters were calculated as follows:

Population growth rate (*r*): $\ln (Nt/N_0)/t$
where *t* represents the culture day, *Nt* is the number of female rotifers at 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

2.4. Life history parameters of the females

Twelve neonates (< 2 h) were individually inoculated into a well of a 24-well microplate containing 1 mL test solution and 7.0×10^6 cells/mL of *N. oculata.* The concentrations of $FeSO_4 \cdot 7H_2O$ and the culture conditions applied to the individual cultures were the same as those used for the batch cultures of the two rotifer species. After a period of 20 h, the plates were checked every hour to monitor (1) the time taken to bear the first

egg, and (2) the time taken to produce the first offspring (generation time). Thereafter, the rotifers were inspected every 12 h to assess the survival of the maternal rotifers and the number of neonates. The parental rotifer was transferred to a new well containing fresh food and test solution every 24 h. This process was continued until the maternal rotifer died. The generation time, lifespan, age-specific survival rate, and fecundity were determined based on the data collected. The intrinsic growth rate (*r*) was calculated according to the Lotka equation (Lotka, 1907):

$$\sum lxmx\ e^{-rx}=1$$

where *lx* is the probability of surviving rotifers at age *x* and *mx* represents the number of offspring produced by one individual at age *x*.

The resting egg production of one fertilized mictic female under each iron concentration was estimated. 30 female neonates (< 2 h) that hatched from amictic eggs and 20 newborn males (< 2 h) that hatched from mictic eggs were cultured together in a well on a 6-well microplate containing 5 mL food suspension (Gómez and Serra, 1996) and the iron solution, as described above. The plates were checked every 12 h until the resting egg-bearing female (RF) appeared. Ten RFs at each concentration were individually transferred into a 24-well microplate, and the rotifers were checked every 12 h to record the number of resting eggs produced. Each day, the maternal female was transferred to a new well-containing food and iron solutions. This procedure was continued until the maternal rotifer died. The mean and standard deviation of 10 individuals were then used to determine the total number of resting eggs produced at each iron concentration.

2.5. Reactive oxygen species level detection

The mechanism by which iron affects rotifer reproduction and lifetable parameters was determined based on fatty acid metabolism, oxidative stress, and antioxidant activity. Rotifer neonates (< 2 h) obtained using the same methods as those described in the previous sections were exposed to two iron concentrations (6 and 12 μg/mL), which effectively induced different responses in sexual and asexual reproduction of two rotifer species in the first experiment. After 7 days of culture, the following parameters were measured: (1)

intracellular ROS level, (2) lipid droplet content in sexual organs, (3) gene expression related to oxidative stress, lipid metabolism, and the sexual and asexual reproduction of the rotifers.

Based on the sexual and asexual reproductive characteristics of batch-cultured rotifers, approximately 6000 rotifers were obtained from the 7 days batch cultures at each iron concentration. These rotifers were homogenized in a buffer solution containing 0.32 M sucrose, 20 mM HEPES, 1 mM $MgCl_2$, and 0.5 mM PMSF (pH 7.4, regulated by NaOH and HCl) by sonication (Branson Sonifier 150, EMERSON, St. Louis, MO, USA). The homogenate was centrifuged at 10,000 × g for 20 min at 4°C, and the supernatant was transferred to new tubes for detection. A cell-permeable fluorogenic probe 2',7'-dichlorofluorescin diacetate (H_2DCFDA, Sigma-Aldrich, St. Louis, MO, USA), which can be oxidized to produce fluorescent 2',7'-dichlorofluorescein (DCF) via the use of intracellular ROS, was used to estimate the ROS level. The reactant consisted of 170 μL phosphate-buffered saline, 20 μL H_2DCFDA probe, and 10 μL sample. It was incubated at 25°C for 30 min in a 96-well black microplate (Nunc A/S, Thermo Scientific™, Waltham, MA, USA). Fluorescence was detected using a multimode plate reader (BioTek Cytation™ 3, Winooski, VT, USA) with excitation and emission wavelengths of 485 and 520 nm, respectively. The data was normalized to total protein and expressed relative to the control (100%). Total protein was measured using the Bradford assay (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA, USA) (Jeong et al., 2019). All tests were repeated thrice to ensure biological replicates.

2.6. Neutral lipid accumulation

Thirty rotifers (amictic females) per treatment were stained with Nile red to detect neutral lipid droplets. Nile red (Sigma-Aldrich, St. Louis, MO, USA) was prepared as a stock solution by dissolving it in acetone at a concentration of 1 mg/mL. Rotifers were transferred to 6-well microplates containing 5 mL ASW per well and stained with Nile red (to a final concentration of 2.5 μg/mL) for 5 min at 25°C, then fixed with 10% formalin (Lee et al., 2019). Stained and fixed rotifers were observed under a fluorescence microscope (Ts2-FL, Nikon, Tokyo, Japan) at an excitation wavelength of 550 nm and an

emission wavelength of 630 nm. The area of neutral lipids was measured using the image analysis software NIS-Elements F version 4.60. The measured area was expressed as a percentage relative to the control.

2.7. Gene expression analysis

Rotifers (approximately 10,000 individuals) from each treatment were used for the extraction of RNA. The 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, 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 260 nm and the ratios of A260/280 and A230/260 using a spectrophotometer (NanoDrop™ 2000, Thermo Scientific™).

cDNA was used to detect the genetic mechanism of the observed phenomenon using real-time quantitative PCR, with 1 μL cDNA template with 0.5 μL forward and reverse primers (10 μM) for each gene and 10 μL TB Green Premix Ex Taq (2×) (Takara Bio) in a total volume of 20 μL. The analyzed genes are listed with the primer sequences in **Table A1**. The primer sequences used in this study were designed using Primer Premier 6.0 (PREMIER Biosoft, San Francisco, CA, USA), unless and otherwise mentioned. 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. The housekeeping gene elongation factor 1-αwas used as a reference gene to normalize the transcript levels. The $2^{-\Delta\Delta CT}$ method was used to calculate the transcriptional level. All experiments were performed in triplicate.

2.8. Statistical analyses

Data analysis was conducted using R 3.6.3. Bartlett's test was used to evaluate the homogeneity of the variance. Significant differences were analyzed via one-way analysis of variance followed by Tukey's honest significant difference (HSD) test. For individual cultures, data that could not meet the normal distribution were analyzed using the nonparametric Kruskal–Wallis test followed by the Wilcoxon rank sum test (Bonferroni adjustment).

3. Results

3.1. Reproductive parameters

In *B. plicatilis*, both sexual and asexual reproduction was significantly inhibited ($p < 0.05$) by treatment with 96 μg/mL iron, resulting in the lowest population growth rate and resting egg production. However, no significant difference was observed for rotifer population density in the 0–48 μg/mL group. A slight reduction in the percentage of mixis was observed under 24 and 48 μg/mL iron treatments. Although rotifer fertilization was not inhibited by exposure to iron, the resting egg production significantly reduced ($p < 0.05$) as the concentration of iron increased **(Fig. 1-1 and Table 1-2)**.

A positive stimulation of both asexual and sexual reproduction of *B. rotundiformis* was induced in response to iron, with a significant dose-dependent increase ($p < 0.05$) in the rotifer population growth rate. The highest population density was observed in the 12 μg/mL iron group, with a significantly higher ($p < 0.05$) growth rate of 0.65±0.02. An increase in the number of male-producing mictic females was observed on day 6 of iron exposure. *B. rotundiforum* that were exposed to a 6 μg/mL iron culture medium showed the highest resting egg production at a significant level ($p < 0.05$) reaching 4235.6±632.8 eggs/mL **(Fig. 1-1 and Table 1-3)**.

3.2. Life history parameters

B. plicatilis individuals that were exposed to 0–48 μg/mL iron solution exhibited no significant difference in age-specific survival rate and asexual fecundity during their

lifetime compared to the control (**Fig. 1-2**). The cumulative asexual fecundity, lifespan, and intrinsic growth rate (*r*) of the control rotifers were 32.3±19.7 offspring/female, 11.3±4.9 d, and 0.37±0.17, respectively, showing no significant differences compared to those under iron treatment. In contrast, inhibition of individual resting egg production by one fertilized mictic female was observed in the iron treatment. The number of resting eggs produced in the control was 3.1±1.2 eggs/female; under 6–96 μg/mL of iron treatment, the production changed from 2.4±0.8 to 0.9±0.7 eggs/female. Treatment with 96 μg/mL iron led to significant inhibition ($p < 0.05$) in the fecundity (16.8±3.9 offspring/female) and intrinsic growth rate (*r*) (0.19±0.05) of the females, but extended the lifespan (16.3±3.5 d) ($p < 0.05$) **(Table 1-4)**.

The age-specific survival rate of *B. rotundiformis* showed a different pattern in terms of iron concentration **(Fig. 1-2)**. From day 3 to 6, the survival (%) of the control rotifers decreased from 83.3% to 33.3%, whereas it changed from 100% to 54.5% in the 1.5–6 μg/mL iron group; higher concentrations of iron (12 and 24 μg/mL) resulted in 90.9% and 88.9% of the survival rate, respectively. Regarding the lifetable parameters, fecundity, lifespan, and intrinsic growth rate (*r*), *B. rotundiformis* showed an insignificant increase with iron treatment. The lifespan and fecundity of the control rotifers were 6.12±3.31 d and 19.4±14.4 offspring/female, respectively. As the iron concentration increased to 24 μg/mL, the lifespan and fecundity of the rotifers increased to 7.82±3.20 d and 30.4±17.4 offspring/female, respectively. Moreover, the reproductive period increased because of iron treatment, between 4.7 and 5.1 d for the 6–24 μg/mL iron treatment group compared to the control (3.5 d). The individual resting egg production of *B. rotundiformis*, compared to the control, was not affected by iron exposure **(Table 1-5)**.

3.3. Reactive oxygen species level detection

Intracellular ROS levels were significantly increased ($p < 0.05$) in the iron-treated groups (at 6 and 12 μg/mL) after 7 days of exposure **(Fig. 1-3)**. The ROS levels in *B. plicatilis* showed a 2.6- and 2.4-fold increase in response to 6 and 12 μg/mL of iron, respectively. However, *B. rotundiformis* under the same iron concentration resulted in

higher ROS levels, reaching 5.8- and 6.0-fold increases, respectively, compared to the control.

3.4. Neutral lipid accumulation

A neutral lipid area stained with Nile red was generally observed in the sexual organs (vitellarium/ovary) with variation in both rotifer species exposed to different iron concentrations **(Fig. 1-4 A, *B. plicatilis* and 1-4 B, *B. rotundiformis*)**. A significant 1.9- and 1.7-fold reduction ($p < 0.05$) in the amount of neutral lipids was observed in *B. plicatilis* in response to 6 and 12 µg/mL iron, compared to the control. In *B. rotundiformis*, a significant increase of 1.3- and 2.1-fold ($p < 0.05$) was observed with the same treatment **(Fig. 1-4 C)**.

3.5. Gene expression analysis

The transcript levels of iron oxidase were upregulated 1.5- and 1.2-fold in *B. plicatilis* in response to 6 and 12 µg/mL iron, respectively, whereas in *B. rotundiformis* it was upregulated 3.5- and 7.8-fold, respectively, under the same conditions. Fe-S mRNA levels in *B. plicatilis* were significantly upregulated 1.3-fold ($p < 0.05$) in response to 12 µg/mL iron only, whereas in *B. rotundiformis*, Fe-S was significantly upregulated 4.2- and 13.1-fold ($p < 0.05$) under both 6 and 12 µg/mL treatments, respectively. Fe-S cluster mRNA levels were both upregulated in *B. plicatilis* (1.4- and 2.0-fold) and *B. rotundiformis* (2.1- and 2.6-fold) that were exposed to 6 and 12 µg/mL iron, respectively, compared to the control **(Fig. 1-5 A)**.

Oxidative stress and antioxidant status were determined based on the expression of NADPH oxidase (*Nox*), *CYP*, heat shock protein 70 (*Hsp70*; *Hsc70 like 1*), copper/zinc superoxide dismutase (*CuZnSOD*), manganese superoxide dismutase (*MnSOD*), catalase (*CAT*), and glutathione S transferase (*GST*) **(Fig. 1-5 B)**. The mRNA expression of *Nox* was downregulated 2.3-fold in *B. plicatilis* exposed to 6 µg/mL iron but upregulated 1.3-fold ($p < 0.05$) in the 12 µg/mL group at a significant level, compared to the control. The upregulation ($p < 0.05$) in *B. rotundiformis* reached 67.1- and 244.7-fold in response to 6

and 12 µg/mL iron, respectively. The transcript level of *CYP3048A2*, which belongs to clan 2 in *B. plicatilis*, was downregulated 1.5-fold under the 6 µg/mL treatment. A different *CYP* gene (*CYP3045A2*) belonging to clan 3 was downregulated 1.3-fold under 6 µg/mL exposure and upregulated 1.5-fold in the 12 µg/mL groups. In *B. rotundiformis*, the level of *CYP3043A1* mRNA (clan 2) was upregulated 20.4- and 49.0-fold after exposure to 6 and 12 µg/mL of iron, respectively. The level of *CYP3045C10* (clan 3) was upregulated 6.1- and 15.8-fold under the same conditions. The mRNA level of the *Hsc70 like 1* gene was downregulated 14.7- and 3.9-fold in *B. plicatilis* and *B. rotundiformis* exposed to 6 µg/mL iron treatment, respectively, whereas a 2-fold increase was observed in *B. rotundiformis* under 12 µg/mL treatments.

For the antioxidant responses, *B. plicatilis* showed a significant downregulation ($p < 0.05$) of *CuZnSOD*, 1.5- and 1.4-fold decrease in response to 6 and 12 µg/mL treatments, respectively. A 1.2-fold upregulation of *CAT* and 2.3-fold upregulation of *GSTs2* were observed under 6 and 12 µg/mL of iron, respectively. Compared to *B. plicatilis*, *B. rotundiformis* showed relatively higher antioxidant responses, with 6 and 12 µg/mL iron causing 1.7- and 1.5- fold upregulation of *CuZnSOD*, respectively. In addition, *CAT* was significantly upregulated (1.5- and 3.2-fold) ($p < 0.05$), whereas *GSTo1* was also upregulated (5.7- and 3.1-fold) in response to 6 and 12 µg/mL, respectively. No difference was observed in *MnSOD* expression compared with the control for both *B. plicatilis* and *B. rotundiformis* in response to 6 µg/mL iron solution. However, a 2.2-fold increase was observed for *B. rotundiformis* at 12 µg/mL.

Regarding lipid metabolism, no significant difference was observed between the transcript levels of citrate lyase in the control and iron-treated *B. plicatilis*. The mRNA expression of acetyl-CoA carboxylase (*ACC*) was downregulated 7.6-fold after treatment with 6 µg/mL. The mitochondrial *CYP* gene (*CYP3335A1*) was downregulated 1.4-fold after treatment with 6 µg/mL, but upregulated 1.6-fold in the 12 µg/mL group. In *B. rotundiformis*, the transcript level of citrate lyase was upregulated 7.4- and 3.4-fold under treatment with 6 and 12 µg/mL of iron, respectively, whereas the *ACC* level was downregulated 2.5-fold under 12 µg/mL treatments. The expression of mitochondrial *CYP* (*CYP3047B1*) was upregulated 2.1- and 1.9-fold in the presence of 6 and 12 µg/mL of iron, respectively. No significant difference was observed between the control and iron-treated

groups in terms of the mRNA levels of calmodulin (*CaM*) in *B. plicatilis*. However, a significant increase (2.6- and 2.3-fold) was observed ($p < 0.05$) in *B. rotundiformis* exposed to 6 and 12 μg/mL iron, respectively **(Fig. 1-6 A)**.

The gene expression related to sexual and asexual reproduction in response to different concentrations of iron is shown in **Fig. 1-6 B**. In *B. plicatilis*, the mRNA levels of p300/CREB-binding protein (*p300/CBP*) and the Rho-family GTPase *cdc42p* did not change at 6 μg/mL iron but were upregulated 1.5- and 1.4-fold, respectively, in response to 12 μg/mL treatments. The expression of *histone H2A* mRNA decreased by 1.6-fold when exposed to 6 μg/mL iron. In *B. rotundiformis*, the transcript levels of *p300/CBP* were upregulated by 5.7- and 14.2-fold at 6 and 12 μg/mL, respectively. The expression of *cdc42p* increased 3.5- and 7.8-fold under 6 and 12 μg/mL iron exposure, respectively, whereas the level of *histone H2A* was upregulated 2.0- and 3.2-fold, respectively, under the same exposure conditions. No significant difference was observed in the transcript levels of the asexual-related gene *cathepsin L* in *B. plicatilis* under both conditions. However, it was upregulated 4.0- and 6.8-fold in *B. rotundiformis*. The same pattern of 1.7- and 4.6-fold upregulation was observed in another asexual-specific gene, *septin-2*, in *B. rotundiformis* at 6 and 12 μg/mL, respectively. The expression of the sex-specific gene *vasa* showed a 1.2-fold upregulation in *B. plicatilis* exposed to 12 μg/mL iron solution. A more significant 5.8- and 5.1-fold upregulation was observed in *B. rotundiformis* under 6 and 12 μg/mL iron treatments, respectively. No significant difference was observed between the control and experimental groups in the mRNA expression of the rotifer lifespan related gene, *sirtuin-2* in *B. plicatilis*, whereas this gene was significantly upregulated 3.7- and 6.4-fold ($p < 0.05$) in *B. rotundiformis* under treatment with 6 and 12 μg/mL iron, respectively.

4. Discussion

This study determined the different response patterns in sexual and asexual reproduction, as well as life history parameters associated with iron ($FeSO_4 \cdot 7H_2O$) concentrations in the two climate rotifer species. Strong negative effects of iron on the resting egg production observed as low fecundity of a temperate *B. plicatilis* female was

caused by an expected factor of poorer quality of male spermatozoa at concentrations > 6 μg/mL. Sperm viability is significantly related to the iron concentration in mammals. However, high concentrations have negative effects (Rotem et al., 1990; Upadhyaya et al., 1998). For example, the amount of iron required for sperm viability and function should be less than 17.4 μg/mL of $FeSO_4.7H_2O$ in humans (Kasperczyk et al., 2016). Regarding the molecular responses in the sexual and asexual reproduction of *B. plicatilis*, two iron levels (6 and 12 μg/mL) did not induce active reactions in the transcript levels of the *septin-2* and *cathepsin-L* genes, which occur during asexual female development and yolk processing (Gilbert, 2004; Hanson et al., 2013; Pagliuso et al., 2016); *p300/CBP*, *cdc42p*, and *histone H2A* participate in gametogenesis or chromosome segregation (Hanson et al., 2013; Johnson, 1999; Shi and Mello, 1998), and the conserved germ cell-specific gene *vasa* is involved in germ cell formation and is required for gametogenesis and embryogenesis (Extavour et al., 2005; Smith et al., 2010; Zhang et al., 2016). Significant inhibitory effects on sexual and asexual reproduction, as well as the extension of lifespan under 96 μg/mL, were observed. *B. plicatilis* exposed to high iron stress may adopt a trade-off strategy to maintain the basal metabolic rate to achieve homeostasis, thereby inducing lower fecundity in both sexual and asexual reproduction (Han and Lee, 2020; Kooijman and Troost, 2007; Yoshinaga et al., 2000).

The positive effects of iron on both reproductive phases were observed with 0–24 μg/mL of iron in the tropical species *B. rotundiformis* because of the extended lifespan and increased fecundity. The population growth of *B. rotundiformis* was more stable in the presence of iron, inducing a higher survival rate and an increase in fecundity (4–6 offspring/d) over long periods (5 d at 12 μg/mL iron) than in the control. In the culture of rotifers, the age of 3–6 d is important, as it is the phase of exponential population growth. When the production of a large number of offspring by single amictic females occurs, the high rate of survival and fecundity at this stage directly affects the entire population distribution of rotifers (Yoshinaga et al., 2003). Molecular expressions related to rotifer reproduction were significantly upregulated under iron treatment. In addition, the aging-related gene *sirtuin-2* (Lee et al., 2018; Tsuchiya et al., 2006) showed no modulation in its expression in *B. plicatilis* but was upregulated in *B. rotundiformis* in all iron-treated groups. In comparison, for *B. plicatilis*, the tested iron concentrations induced active molecular

responses in both sexual and asexual reproduction and the lifespan of the tropic *B. rotundiformis*. This phenomenon suggests that the different strategies and tolerances related to iron concentrations in *B. plicatilis* species complex in a habitat-dependent manner.

For the biological metabolism of organisms, the importance of the balance between ROS generation and clearance has been widely recognized (Garg and Manchanda, 2009; Mittler et al., 2004). The antioxidant defense system (SOD, CAT, CYP, glutathione [GSH], and glutathione peroxidase [GPx]) has been shown to effectively eliminate ROS (Covarrubias et al., 2008; Kim et al., 2017; Yang et al., 2013). Iron is directly involved in the process of ROS production via the "Fenton reaction," in which labile ferrous iron (Fe^{2+}) catalyzes the breakdown of H_2O_2 to yield hydroxyl radicals (Nakamura et al., 2019). In our study, iron exposure triggered iron utilization in the two rotifer species and induced higher levels of ROS production, which was demonstrated by the identified molecular biomarkers of iron homeostasis-maintaining genes (e.g., iron oxidase, Fe-S protein, and Fe-S cluster) and stimulus stresses such as Nox and HSPs (Hartl, 1996; Lambeth, 2004; Kalmar and Greensmith, 2009). However, substantial downregulation of *CuZnSOD* and two *cytochromes* (*CYP3048A2* and *CYP3045A2*) under iron exposure was observed, suggesting that the tested *B. plicatilis* species may not activate an effective antioxidant defense system to alleviate the elevated ROS levels induced by the presence of iron. A previous study indicated that ROS-triggered oxidative stress could inactivate the enzymatic antioxidant activity in the rotifer *B. plicatilis*, further aggravating the overproduction of ROS (Wang et al., 2015). Moreover, the Fe-S cluster, as a redox sensor of glutaredoxin2, involved in antioxidant defense mechanisms in humans and zebrafish, can be damaged by ROS (Bräutigam et al., 2013; Mitra and Elliott, 2009). Agarwal et al. (2020) indicated that oxidative stress levels could be an important index in the assessment of sperm abnormalities. In human males, a higher level of oxidative stress disrupts the mitochondrial membrane potential, impairs male fertility associated with the electron transport chain, and affects the quality of sperm (Agarwal et al., 2014; Uribe et al., 2015). In *B. plicatilis*, it is likely that excessive ROS accumulation affects the fecundity of fertilized mictic females or the fertility of males, thus leading to a decrease in resting egg production.

The negative effects of iron on neutral lipids in the reproductive organs (e.g., vitellarium/ovary) of *B. plicatilis* were observed. As a reservoir of carbon and energy, neutral lipids such as triacylglycerols and wax esters have important roles in the development and reproduction of marine species (Hakanson et al., 1987; Ohman et al., 1987). Resting eggs, produced by the fertilization of male and mictic females, are essential to ensuring the persistence of the rotifer population under temporally harsh environmental conditions, requiring sufficient resources to maintain their viability during diapause (García-Roger and Ortells, 2018). The storage of lipids as an energetic resource, typically in the form of lipid droplets, has been suggested as playing an important role in the diapause of many zooplankton species (Lee et al., 2006). Recent studies have noted that lipid droplets are the central managers in lipid metabolism and function as safeguards in response to various types of cellular stress. They also maintain energy and redox homeostasis while protecting cells against lipotoxicity by sequestering toxic lipids into their neutral lipid core (Petan, 2018; Welte and Gould, 2017). As summarized in **Fig. 1-7**, iron is involved in lipid metabolism through the Krebs cycle. Ferritin, an iron storage protein, contains fatty acid binding sites that can modulate iron uptake and release (Bu et al., 2012) and can be transported to the mitochondria for the synthesis of iron–sulfur (Fe-S) clusters. It plays a key role in both the Krebs cycle and electron transport chain (Martin and Matyushov, 2017; Rouault, 2015). The Fe-S cluster regulates the generation of citrate, which feeds into the biosynthesis of cholesterol or fatty acids to promote the formation of lipid droplets (Berteau, 2018; Crooks et al., 2018; Rockfield et al., 2018) through binding with the enzyme aconitase. Citrate lyase and acetyl-CoA carboxylase (ACC) are essential enzymes in lipid biosynthesis (Barber et al., 2005; Jones et al., 2017; Munday, 2002; Sun et al., 2010), and the mitochondrial *CYP* gene has been shown to be involved in steroidogenesis, cholesterol, and steroid metabolism (Han et al., 2019a, 2019b; Midzak and Papadopoulos, 2016). The observed nonactivated (e.g., *citrate lyase*) and downregulated genes (e.g., *ACC* and MT *CYP*) suggested that iron exposure may inhibit the process of lipid metabolism in *B. plicatilis*, although the expression of *ACC* and MT *CYP* genes were upregulated at 12 μg/mL of iron. This may be caused by the plasticity of *B. plicatilis* in coping with higher stresses. A previous study found that *B. plicatilis* reacted to metal (Cd)

toxicity stimulates the expression of stress protein kinase (e.g., p-ERK) at an early stage, which subsequently decreases to basal levels (Kang et al., 2019).

Nevertheless, in *B. rotundiformis*, the ROS-scavenging systems were effectively stimulated so that all the antioxidant genes (*SOD, CAT, GST,* and *CaM*) showed substantial upregulation in response to iron. In addition, the area containing neutral lipids was significantly expanded, whereas the transcript levels of *citrate lyase* and mitochondrial *CYP* were significantly upregulated following iron exposure. They suggested that iron affects *B. rotundifotmis* by stimulating both the ROS and lipid metabolic pathways and promoting the synthesis of lipid droplets. The effects of iron on oxidative stress and lipid accumulation have been documented in different species (**Table 1-6**). For example, in the culture of the green microalga *Botryococcus braunii*, the lipid content was significantly increased from 22% to 35% when the initial iron concentration was increased from 9 to 27 μg/mL (Ruangsomboon, 2012). For the aquatic invertebrate *Hydropsyche contubernalis* L. (Trichoptera), iron exposure can alter the value of the cholesterol-to-phospholipid molar ratio, and the cholesterol content increased under lower iron concentrations (3 mg/L) (Regerand et al., 2005). In rats, iron-deficient diets induced significantly lower cholesterol concentrations in the liver and serum lipoproteins than in controls (Stangl and Kirchgessner, 1998), whereas excess iron loading was associated with insulin resistance, increased mitochondrial dysfunction, and oxidative stress (Choi et al., 2013). These studies have documented the effects of iron on lipid metabolism; however, their molecular mechanisms remain underexplored.

Overall, these findings suggest that the reproduction of the two marine rotifer species was affected differently by iron concentrations. The bioavailability and toxicity of iron for aquatic species are regulated by multiple factors, such as pH, temperature, amount of organic matter, and dissolved oxygen in the water. Fe (II) is considered more toxic to aquatic animals than Fe (III), which is abundant in oxygenated conditions and can be reduced to Fe (II) through the photoreduction of iron chelates (Faust and Zepp, 1993). In oxygenated seawater, the overall ratio of Fe (II)/Fe (III) is predicted to be 10^{-10}, and it then shifts to 10^{-7} in mildly anoxic seawater (Shaked, 2008; Waite, 2001). In addition, the oxygen content in aquatic waters decreased as the temperature increased; for example, saturated water retains approximately 10.92 mg/L oxygen at 4°C and 8.68 mg/L at 21°C

(Cairns et al., 1975; Wetzel, 2001). Higher temperatures lead to significantly higher oxygen consumption rates and an increase in the metabolic rates of organisms, thereby enhancing the production of ROS (Childress, 1976; Li et al., 2014). Hence, I suggest that *B. plicatilis*, which naturally inhabits temperate regions, coped well with low levels of oxidative stress, and did not need to evolve resistance against stressful conditions. On the other hand, tropical *B. rotundiformis* has evolved an effective mechanism to adapt to ambient stresses and showed higher tolerance to toxicants (e.g., juglone) inducing oxidative stress (Tanaka et al., 2009). A beneficial acclimation strategy for harsh environments may be adopted by *B. rotundiformis* to increase its fitness. Previous studies have shown that epigenetic modification and phenotypic plasticity can be used by monogonont rotifers to handle environmental stress (Kim et al., 2014; Serra and Carmona, 1993). In particular, the rotifer *B. koreanus* conferred adaptability in response to low pH stress and generated multigenerational plasticity through transcriptomic and histone modifications (Lee et al., 2020a). On the other hand, the different tolerances in the two *Brachionus* species may be derived from the interaction of substances with environmental factors. As a consequence of climate change, warmer waters interact with harmful compounds (Gordon, 2003; Noyes et al., 2009), affecting aquatic organisms such as earthworms (Velki and Ecimovic, 2015), whereas higher temperatures tend to cause an increase in contaminant toxicity in freshwater fish (Patra et al., 2015). The experimental temperature (25°C) used in this study may strengthen the toxicity of iron in *B. plicatilis*. However, its effect on *B. rotundiformis*, which is adapted to higher temperatures, is less. In a chemical toxicity test for *B. plicatilis*, temperatures of 25, 30, and 35°C yielded high sensitivity to toxicants at lower LC_{50} than 20°C (Snell et al., 1991b). A similar pattern was also observed in *B. koreanus* in response to copper exposure (Li et al., 2014). As for several zooplankton species, including *B. plicatilis*, higher temperatures accelerate the metabolic rate of rotifers (Gillooly et al., 2001; Rebolledo et al., 2018) and can promote toxicant absorption, thus showing greater susceptibility to toxicity. Moreover, the presence of chronic iron stress may influence the thermal resistance of rotifer *B. plicatilis*, which further increases the risk of temperature stress.

5. Conclusions

The current study demonstrated the habitat adaptation of two climatic rotifer species associated with iron concentrations. Tropical species may have evolved effective strategies to cope with iron-induced oxidative stress and, thus, exhibited higher iron resilience than temperate species. For the temperate species, iron had no effect on asexual reproduction under the tested concentrations. However, negative effects on sexual reproduction in terms of the fecundity of a fertilized female were observed. These circumstances suggest the potential deterrent effects of iron on the quality of rotifer sperm.

Table 1-1. The nominal and actual concentrations of iron applied in the investigations.

	Iron concentration (μg/mL)							
Nominal	0	1.5	3	6	12	24	48	96
Actual	0.0	1.5±0.1	3.3±0.1	8.6±0.4	14.7±0.8	27.6±0.3	54.8±4.2	104.5±1.4

Values are mean ± standard deviation of two replicates. The data given was the calculated results indicating the conversion of Fe and $FeSO_4 \cdot 7H_2O$.

Table 1-2. Sexual and asexual reproduction parameters of *Brachionus plicatilis* (NH17L strain) when exposed to different concentrations of $FeSO_4 \cdot 7H_2O$ during 7 days batch culture.

Treatment (μg/mL)	Population growth rate (*r*)	Mixis (%)	Fertilization (%)	Resting eggs /mL
0	0.60 ± 0.01^{a}	22.3±0.4	15.6 ± 5.7^{ab}	834.4 ± 96.2^{a}
6	0.62 ± 0.00^{a}	27.9±2.9	19.6 ± 3.5^{ab}	665.6 ± 78.1^{ab}
12	0.64 ± 0.01^{a}	22.5±4.6	26.6 ± 4.5^{ab}	642.2 ± 87.0^{b}
24	0.64 ± 0.02^{a}	21.6±1.2	30.7 ± 17.4^{ab}	563.3 ± 24.0^{b}
48	0.63 ± 0.01^{a}	20.9±0.3	36.0 ± 2.6^{a}	506.7 ± 5.8^{b}
96	0.35 ± 0.04^{b}	15.9±12.2	8.3 ± 14.4^{b}	87.8 ± 48.6^{c}

Data are mean ± standard deviation. The letters indicate significant differences among treatments (a > b > c, Tukey HSD test, $p < 0.05$, n = 3).

Table 1-3. Sexual and asexual reproduction parameters of *Brachionus rotundiformis* (Kochi strain) when exposed to different concentrations of $FeSO_4 \cdot 7H_2O$ during 7 days batch culture.

Treatment (μg/mL)	Population growth rate (*r*)	Mixis (%)	Fertilization (%)	Resting eggs /mL
0	0.48 ± 0.02^{c}	38.5±9.3	27.5 ± 1.4^{b}	2364.4 ± 679.1^{b}
1.5	0.56 ± 0.04^{b}	36.7±9.2	41.1 ± 7.0^{ab}	3462.2 ± 511.6^{ab}
3	0.61 ± 0.01^{ab}	37.1±5.5	47.6 ± 8.4^{a}	3737.8 ± 763.7^{ab}
6	0.64 ± 0.04^{a}	33.0±2.8	44.8 ± 7.1^{ab}	4235.6 ± 632.8^{a}
12	0.65 ± 0.02^{a}	31.7±2.7	38.0 ± 8.1^{ab}	3111.1 ± 355.1^{ab}
24	0.60 ± 0.02^{ab}	25.4±6.1	43.2 ± 7.0^{ab}	2877.8 ± 495.9^{ab}

Data are mean ± standard deviation. The letters indicate significant differences among treatments (a > b > c, Tukey HSD test, $p < 0.05$, n = 3).

Table 1-4. Life history parameters of female rotifer *Brachionus plicatilis* (NH17L strain) when exposed to different concentrations of $FeSO_4 \cdot 7H_2O$.

Treatment (μg/mL)	Time (h) of bearing the first egg	Time (h) to release the first neonate	Fecundity		Intrinsic growth rate (*r*)
			Asexual (offspring/female)	Sexual (eggs/female)	
0	27.7 ± 1.6^{B}	44.5 ± 0.5^{B}	32.3 ± 19.7^{AB}	3.1 ± 1.2^{a}	0.37 ± 0.17^{A}
6	26.3 ± 0.7^{B}	43.2 ± 1.0^{C}	35.1 ± 14.2^{A}	2.4 ± 0.8^{ab}	0.34 ± 0.10^{A}
12	28.9 ± 2.7^{AB}	46.7 ± 4.3^{AB}	32.5 ± 16.6^{AB}	1.9 ± 1.0^{abc}	0.36 ± 0.05^{A}
24	28.4 ± 2.4^{B}	46.0 ± 3.0^{B}	35.0 ± 18.4^{AB}	2.0 ± 0.9^{abc}	0.37 ± 0.15^{A}
48	28.1 ± 2.9^{B}	45.5 ± 1.4^{B}	27.5 ± 15.6^{AB}	1.8 ± 1.1^{bc}	0.33 ± 0.11^{A}
96	32.8 ± 2.9^{A}	49.9 ± 3.2^{A}	16.8 ± 3.9^{B}	0.9 ± 0.7^{c}	0.19 ± 0.05^{B}

Data are mean ± standard deviation. The letters indicate significant differences among treatments (a > b > c, Tukey HSD test, $p < 0.05$, n = 10–12; A > B > C, Wilcoxon rank sum test, $p < 0.05$, n = 10–12).

Table 1-5. Life history parameters of female rotifer *Brachionus rotundiformis* (Kochi strain) when exposed to different concentrations of $FeSO_4 \cdot 7H_2O$.

Treatment (μg/mL)	Time (h) of bearing the first egg	Time (h) to release the first neonate	Fecundity		Intrinsic growth rate (*r*)
			Asexual (offspring/female)	Sexual (eggs/female)	
0	24.3±1.6	39.4±2.0^{b}	19.4±14.4	4.3±1.5	0.40±0.30
1.5	25.3±1.3	40.4±2.2^{ab}	27.9±25.6	4.2±1.3	0.40±0.12
3	25.2±1.2	42.3±1.8^{a}	28.7±20.7	4.2±1.8	0.53±0.21
6	25.9±1.3	42.3±1.8^{a}	28.3±21.1	4.3±1.0	0.52±0.21
12	24.8±1.0	39.3±1.5^{b}	30.1±12.7	4.2±1.6	0.46±0.09
24	25.1±1.3	40.8±2.0^{ab}	30.4±17.4	4.4±0.9	0.44±0.12

Data are mean ± standard deviation. The letters indicate significant differences among treatments (a > b, Tukey HSD test, $p < 0.05$, n = 10–12).

Table 1-6. Effects of iron compounds on various species and the metabolic processes involved.

Iron	Species	Results summary	References
Ferrous sulfate (663.5, 6354.4 μg/g, dry diet)	African catfish (*Clarias gariqinus*)	-Higher dietary iron ingestion suppressed the growth of fish and stimulated the generation of malondialdehyde (MDA) in fish liver and heart, suggesting the elevated oxidative stress following iron exposure.	Baker et al., 1997
Ferric chloride (0.75 μg/mL)	Indian knife fish (*Notopterus Notopterus*)	-The highest iron accumulation in fish liver (36.32–50.54 μg/mL) and gill (13.50–18.48 μg/mL) after 7 to 28 days iron exposure, induced the oxidative stress in fish tissues, and inhibited the activities of antioxidant defense (e.g., SOD, CAT, GST, and GPx).	Mohanty and Samanta, 2018
Ferrous and Ferric ions (1–30 μg/mL)	Tropical Fish (*Leporinus friderici*)	-Two forms of iron ions (Fe^{2+} and Fe^{3+}) induced absolute lethality at the level of 30 μg/mL; Under the lower concentrations (< 15 μg/mL), iron suppressed the glutathione (GSH) levels, enhanced the contents of hemoglobin and methaemoglobin.	Gemaque et al., 2019
Iron polluted water (87 μg/mL)	Common carp	-Iron deposits on fish gills cause tissue necrosis and damage the excretory function of gills, resulted in a higher level of ammonia in the blood plasma.	Slaninova et al., 2014
Ferric chloride (0–8.0 μg/mL)	Boreal toad tadpoles Oligochaete worm	-Long-term (35 days) iron exposure delayed the growth and development of *Bufo boreas*, and inhibited the reproduction of *Lumbriculus*.	Cadmus et al., 2018
Fe	Euryhaline rotifer (*Brachionus plicatilis*)	-Rotifer incubated in microalgae enriched "green water" would change the gut filling patterns. Through enriching with microalgae, iron concentration in L-type rotifer significantly increased by 20%. -Rotifer feeding with *chlorella* diet accumulated iron in their digestive and sexual organ.	Ezoe et al., 2002 Wang et al., 2019

Fe (0.1–4.0 μg/mL)	Freshwater rotifer (*Euchlanis dilatata*)	-The concentration of 2.49 μg/mL iron inhibited the intrinsic growth rate of rotifer. Iron was accumulated in the organ of mastax, lateral retractors of the corona and trophic.	Hernández-Flores et al., 2020
Ferric chloride hexahydrate (0–3.2 μg/mL)	Marine microalgae (*Chlorella vulgaris*)	-Supplementation of 3.2 μg/mL iron enhanced the biomass productivity of algae by 57% and produced an accumulation of neutral lipids.	Liu et al., 2008
		-In the cultivation of *C. vulgaris*, the cell density and the contents of specific neutral lipid are correlated to the intracellular ROS levels under stress conditions.	Menon et al., 2013
		-Stressful conditions result in lipid accumulation during photosynthesis by an up-regulation of Acetyl CoA carboxylase (ACC) and possibly stimulate the fatty acids synthesis in photosynthetic organisms including *C. vulgaris*.	Plank et al., 2001
Ferrous sulfate (9–45 μg/mL)	Green microalgae (*Botryococcus braunii, Scenedesmus dimorphus*)	-The lipids contents increased by 35% in *B. braunii* at 27 μg/mL iron fortification.	Ruangsomboon, 2012
		-For *S. dimorphus*, the highest biomass and lipid accumulation was achieved with 45 μg/mL of iron enrichment.	Ruangsomboon et al., 2013
Ferrous sulfate (0–683.6 μg/mL)	Mixed microalgae (Synechocystis PCC-6803, *Chlorella sorokiniana*, *Diphylleia rotans*)	-Iron supplementation (e.g., 470.9 μg/mL) increased the contents of total protein by 60% and stimulated the highest biomass of algae and the amounts of chlorophyll.	Ermis et al., 2020

Ferric chloride hexahydrate (10–200 μg/mL)	Green microalgae (*Botryococcus* spp.)	-The optimum iron concentration for the growth of algae SK and PSU strain were around 100 and 10 μg/mL, in respective. -Higher level of iron (200 μg/mL) induced a negative effect on the growth of KB strain. -The combination of high iron concentration and nitrogen deficiency negatively effect on the biomass of microalgae, but enhanced the lipid contents.	Yeesang and Cheirsilp, 2011

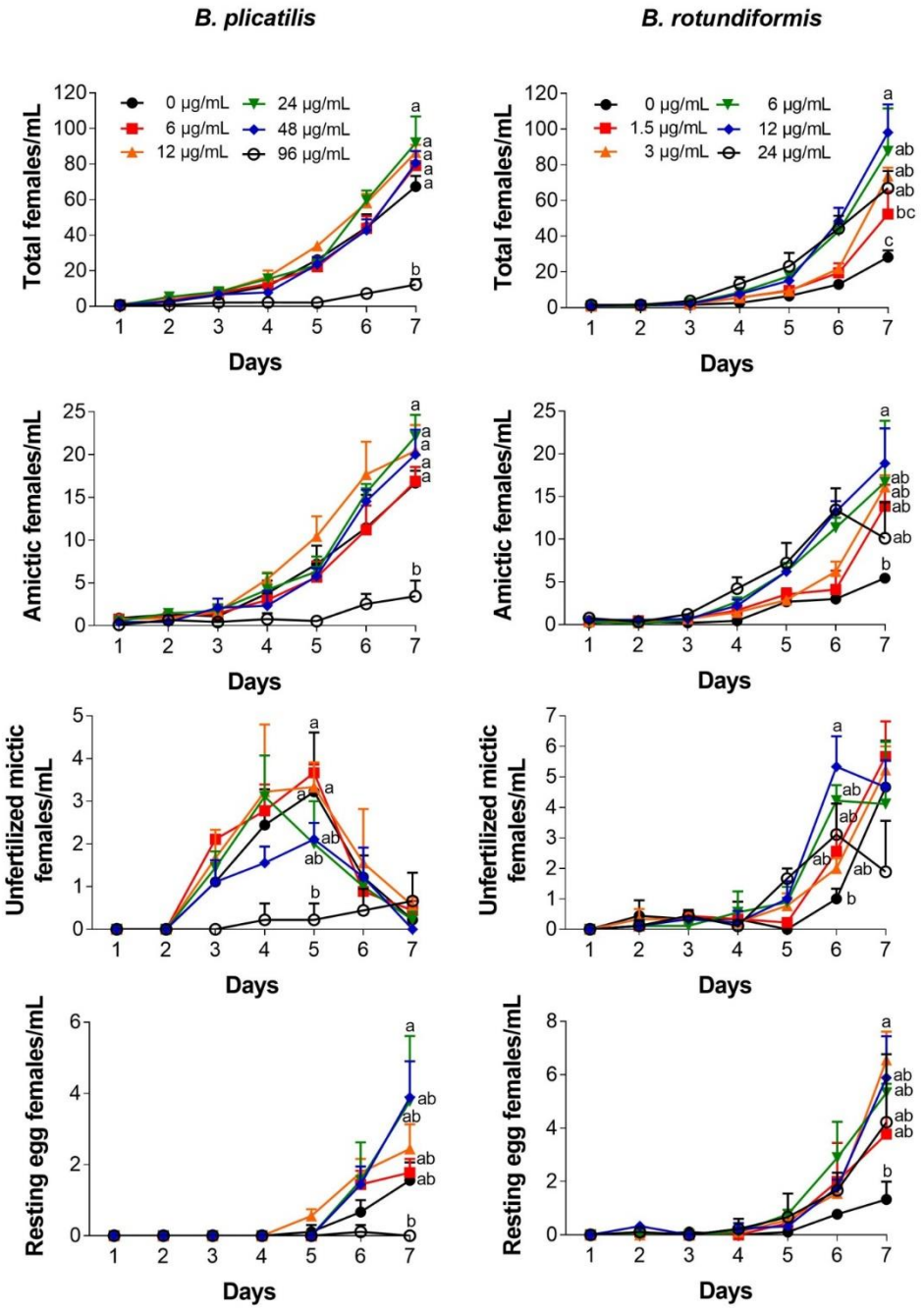

Fig. 1-1. Population growth of total females, the amictic female, and two types of mictic females (unfertilized and resting egg carrying females) of *Brachionus plicatilis* and *Brachionus rotundiformis* exposed to the different concentrations of $FeSO_4 \cdot 7H_2O$ during 7 days batch culture (a > b > c, Tukey HSD test, $p < 0.05$, n = 3).

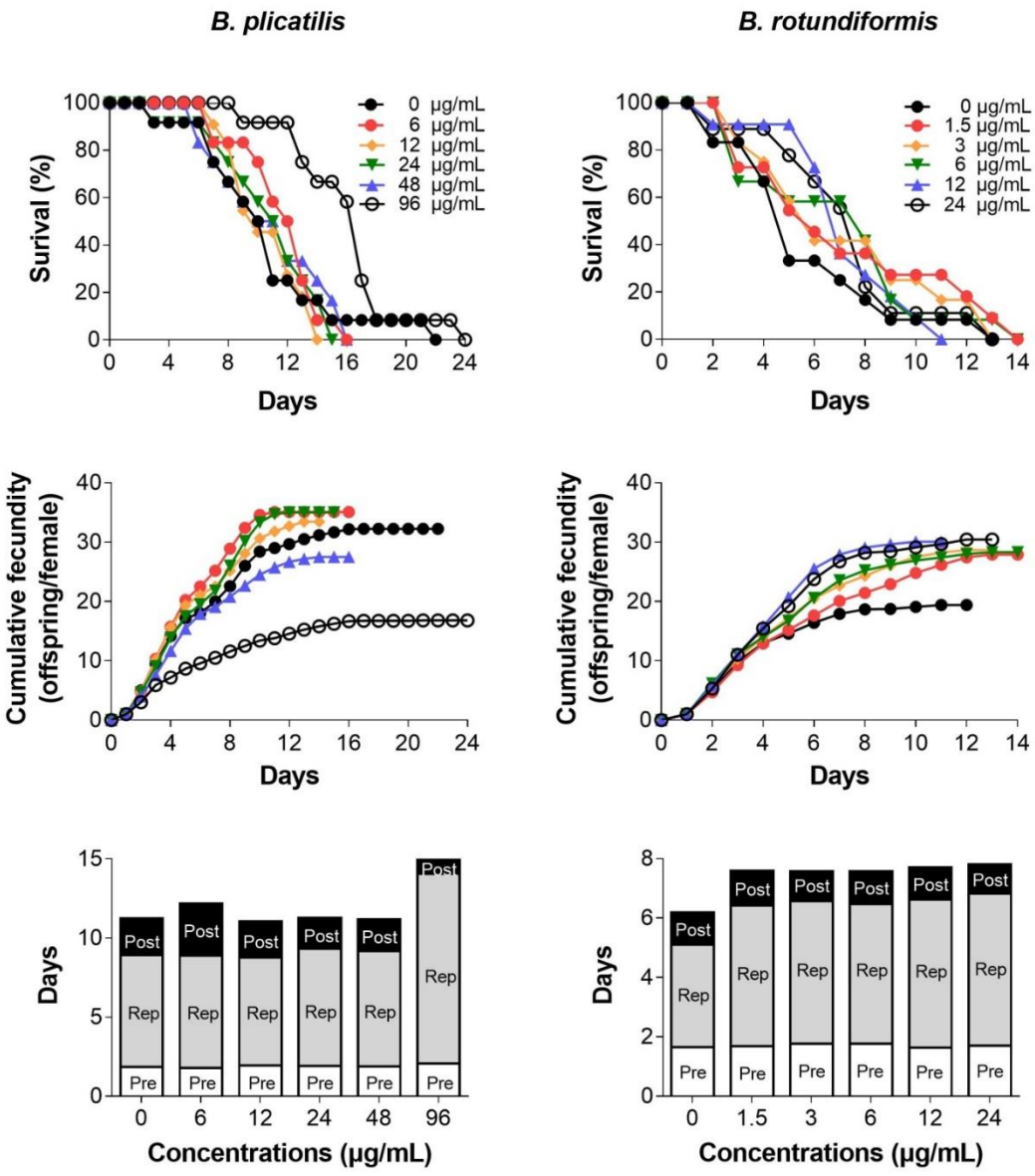

Fig. 1-2. Age-specific survival rate (%), fecundity, and reproductive period (pre for pre-reproductive period; rep for reproductive period; post for post-reproductive period) of rotifer *Brachionus plicatilis* and *Brachionus rotundiformis* exposed to different concentrations of $FeSO_4 \cdot 7H_2O$. n = 10–12.

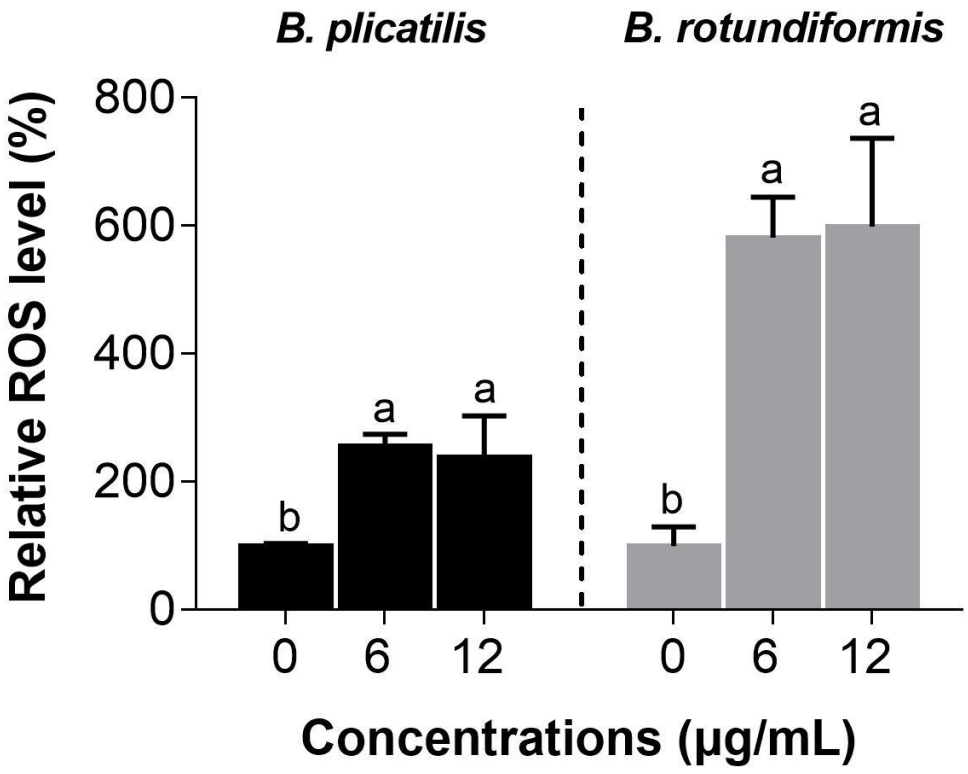

Fig. 1-3. Effects of $FeSO_4 \cdot 7H_2O$ (0 [control], 6, and 12 μg/mL) on the reactive oxygen species level in the two rotifer species (black bar for *Brachionus plicatilis* and grey bar for *Brachionus rotundiformis*) during 7 days exposure (a > b, Tukey HSD test, $p < 0.05$, n = 3).

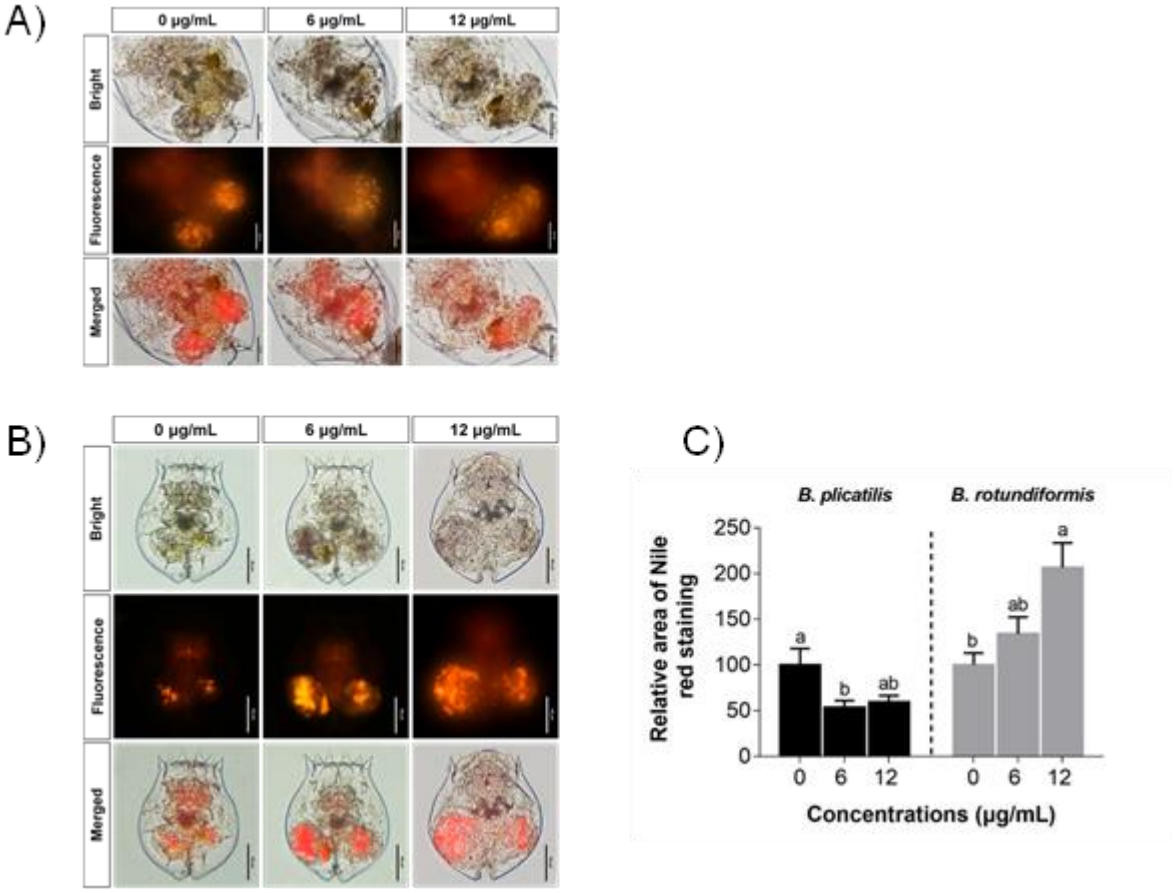

Fig. 1-4. Lipid accumulations in *Brachionus plicatilis* (A) and *Brachionus rotundiformis* (B) exposed to different concentrations of $FeSO_4 \cdot 7H_2O$ (0 [control], 6, and 12 µg/mL) for 7 days. Red fluorescence indicates accumulated neutral lipid droplets in cells. (C) The area of neutral lipid droplets in the two rotifer species (black bar for*Brachionus plicatilis* and grey bar for *Brachionus rotundiformis*) exposed to different concentrations of $FeSO_4 \cdot 7H_2O$ (0 [control], 6, and 12 µg/mL) was represented as a percentage (%) of the relative area (a > b, Tukey HSD test, $p < 0.05$, n = 30).

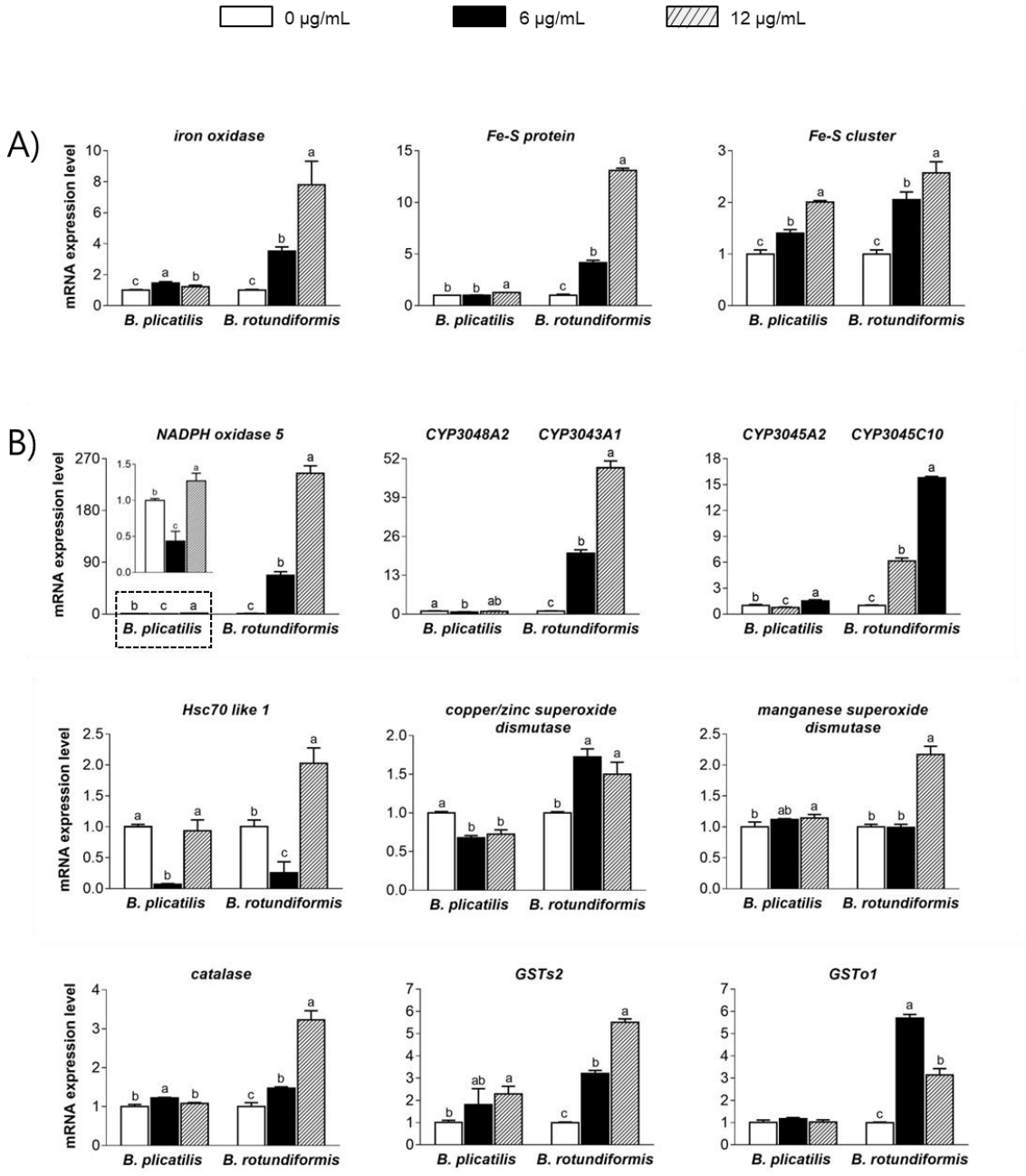

Fig. 1-5. Transcript expression of the genes related to A) iron metabolism, B) oxidant and antioxidant metabolism in *Brachionus plicatilis* and *Brachionus rotundiformis* under different iron treatments (0 [control], 6, and 12 µg/mL $FeSO_4 \cdot 7H_2O$). Data are mean ± standard deviation. Alphabet letters indicate significant differences among treatments (a> b > c, Tukey HSD test, $p < 0.05$, n = 3).

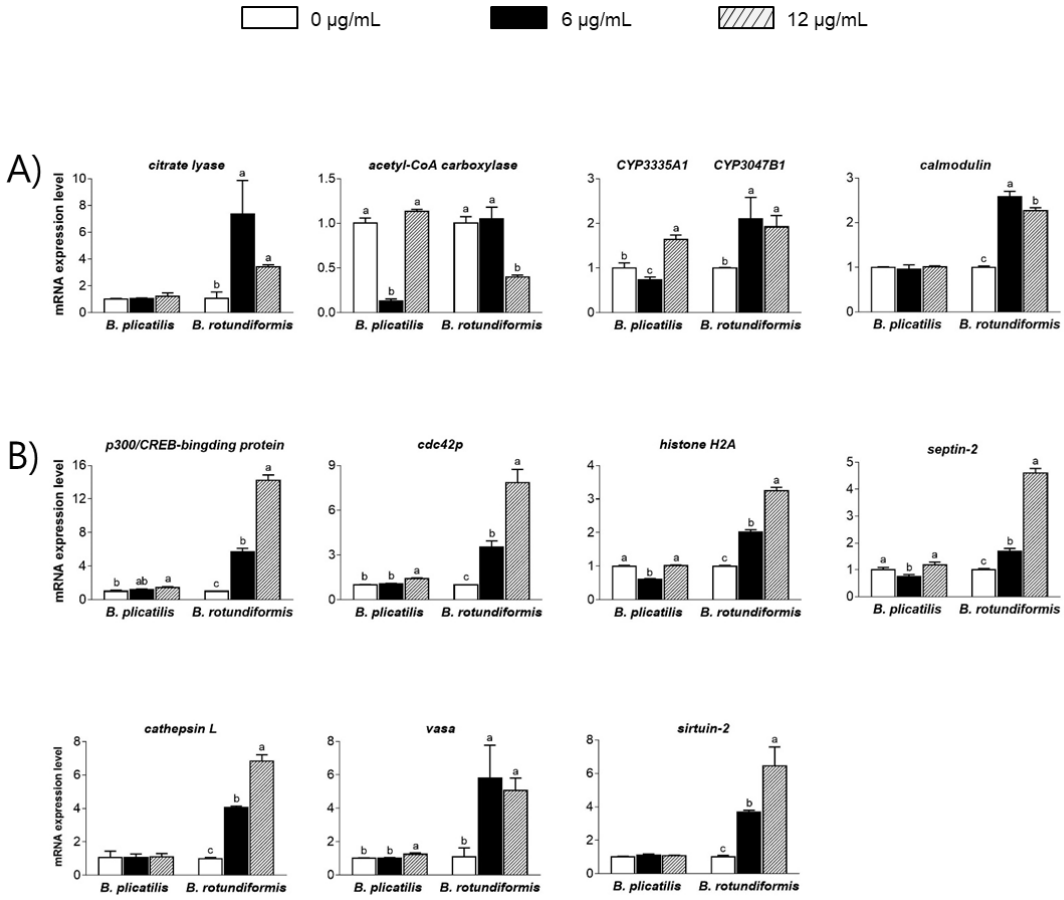

Fig. 1-6. Transcript expression of the genes related to A) lipid metabolism, B) sexual and asexual reproduction in *Brachionus plicatilis* and *Brachionus rotundiformis* under different iron treatments (0 [control], 6, and 12 µg/mL $FeSO_4 \cdot 7H_2O$). Data are mean ± standard deviation. Alphabet letters indicate significant differences among treatments (a > b > c, Tukey HSD test, $p < 0.05$, n = 3).

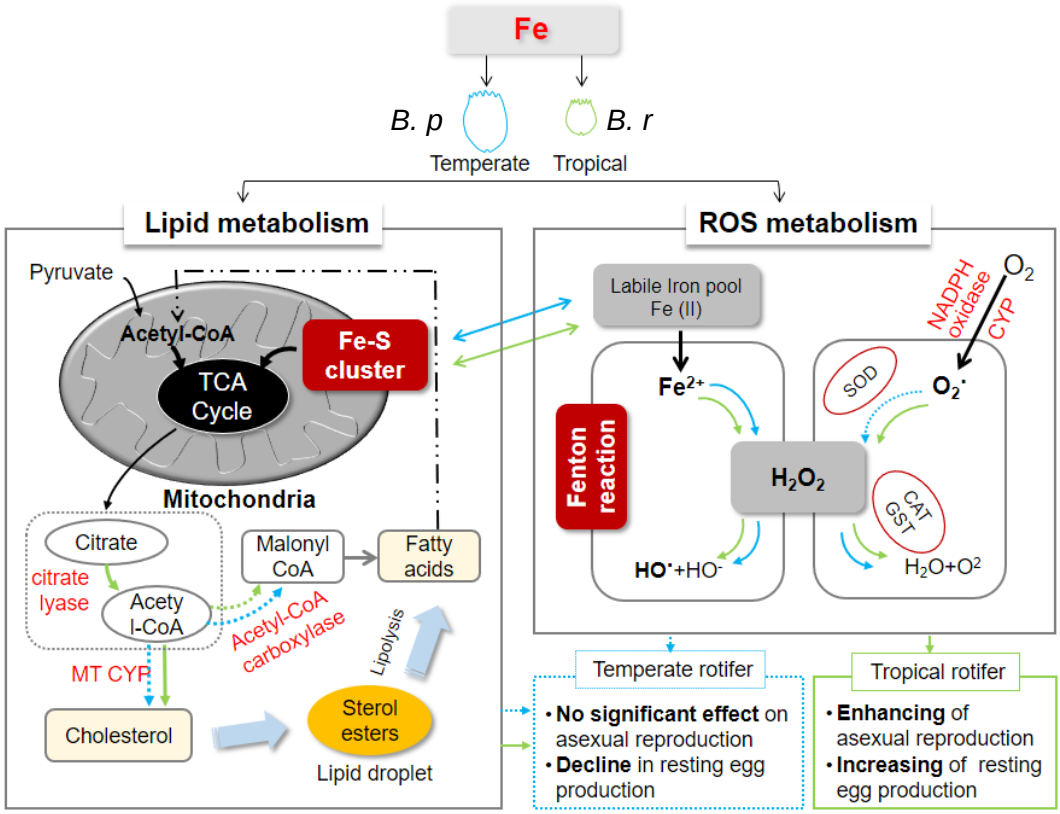

Fig. 1-7. Schematic of the potential function of iron in the metabolism of lipid and reactive oxygen species (ROS) in two rotifer species. Blue represents *Brachionus plicatilis* (*B. p*), Green represents *Brachionus rotundiformis* (*B. r*). The solid arrow line indicates gene up-regulation. The dotted arrow line indicates gene down-regulation.

II-2. Iron reproductive toxicity of marine rotifer sibling species: Adaptation to temperate and tropical habitats

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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). My previous study 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, it is 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 viability (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 (**Table 1-7**) 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.

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 **Table A1.** 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.

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. The number of normal REs, which have regular oval shape with an extra-embryonic space and a light brown embryo, as well as abnormal REs, which are less compact and have irregular shape and dark brown embryos, were counted individually. 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 1-9**).

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. 1-8 A**). The ROS levels showed a 1.3-fold 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 significant difference from the control.

Different response patterns in the oxidative/antioxidant enzymatic activities were observed in the two rotifer species in response to acute iron exposure. The relative MDA levels in the iron-exposed group were significantly suppressed (a 1.3-fold reduction, $p < 0.05$) in *B. plicatilis*, while a 1.3-fold increase was observed ($p < 0.05$) in *B. rotundiformis* compared with the control. The AChE enzymatic activity was significantly inhibited by 1.5- and 1.3-fold ($p < 0.05$) by iron in *B. plicatilis* and *B. rotundiformis*, respectively (**Fig. 1-8 B**). Meanwhile, the antioxidant SOD enzymatic activity was inhibited 1.0-fold in *B. plicatilis*, but it stimulated 1.1-fold in *B. rotundiformis*. CAT activity showed no variation in *B. plicatilis*, but was significantly upregulated (a 1.4-fold increase, $p < 0.05$) in *B. rotundiformis* in response to iron exposure (**Fig. 1-8 C**).

3.3. Molecular biomarkers analysis

The mRNA expression of iron metabolism-related genes, *iron oxidase*, and *Fe-S protein* were significantly downregulated by 1.8- and 1.9-fold ($p < 0.05$) in *B. plicatilis* in response to iron, respectively. For *B. rotundiformis*, the *Fe-S protein* and *Fe-S cluster* were significantly upregulated 1.4- and 7.1-fold, respectively ($p < 0.05$) (**Fig. 1-9 A**). The oxidative stress biomarker *Hsc70 like 1* was significantly upregulated 1.7- and 1.3-fold ($p < 0.05$) in *B. plicatilis* and *B. rotundiformis*, respectively. The *NADPH oxidase 5* was significantly upregulated in both two species, 1.7-fold in *B. plicatilis* and 14.5-fold in *B. rotundiformis* ($p < 0.05$). The expression of *AChE* was significantly downregulated by 1.4- and 1.8-fold ($p < 0.05$) in *B. plicatilis* and *B. rotundiformis*, respectively (**Fig. 1-9 B**). The transcript levels of antioxidant responses related *to CuZnSOD* and *CYP* were downregulated 1.34- and 2.15-fold ($p < 0.05$) in *B. plicatilis*, while they were significantly upregulated 1.73- and 3.56-fold in *B. rotundiformis*, respectively, after iron incubation. There was no difference in the transcript level of *CAT* after iron exposure in *B. plicatilis*, compared to the control, while it was significantly upregulated 2.57-fold ($p < 0.05$) in *B. rotundiformis* (**Fig. 1-9 C**).

3.4. Lifespan and fecundity of sexual female and male

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. 1-10 A**). 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. 1-10 B**). 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. 1-10 C**) 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. 1-10 D**).

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. 1-10 E**), and from 2.19 ± 0.18 to 1.02 ± 0.06 d in *B. rotundiformis* (**Fig. 1-10 F**).

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 1-8**).

3.6. Swimming speed analysis

Iron induced different swimming responses in male and female rotifers (**Fig. 1-11**). 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 1-9**.

3.7. Viability of male spermatozoa

Spermatozoa with high viability showed comparatively strong green fluorescence, while those with low vitality showed weak signals (**Fig. 1-12**). 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 **Figs. 1-13 A** 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-fold) increase ($p < 0.05$) in the abnormal resting egg rate at 60 and 75 μg/mL of iron (**Fig. 1-13 C**).

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, e.g., sperm vitality, and rotifer swimming behavior 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 weights between the two species, *B. plicatilis* (495 ng/ind), and *B. rotundiformis* (220 ng/ind) (Yúfera et al., 1997), I 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 1-9**). 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-fold 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., 1991a). 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. It fluctuates significantly over daily and seasonal timeframes (Tucker and D'Abramo, 2008), while for marine waters the pH 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 proven to be involved in behavioral regulation (e.g., 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 inhabit condition of 20°C for *B. plicatilis*. Higher temperatures may improve their metabolic rates, making them more active (Li et al., 2014).

Furthermore, as hypothesized that higher iron exposure affected the quality of male spermatozoa. SYBR-14 and PI are two fluorescent nucleic stains that are used to evaluate the vitality of sperm cells by staining the nuclei of living and dead sperm, respectively (Garner and Johnson, 1995). The SYBR fluorescent signals gradually decreased in *B.*

plicatilis under both iron treatments, and in *B. rotundiformis* after iron concentrations exceeding 30 μg/mL, indicating deteriorated vitality or quantity of sperm cells. No PI-fluorescent signal was detected in either treatment, which may be due to the use of youngest (< 30 min) males, as sperm cell mortality and low quality increased with age (Snell and Hoff, 1987). Moreover, as the iron concentration increased, the number of abnormal resting eggs increased. The poor sperm quality and a higher frequency of abnormal resting eggs, contribute to the deleterious effects of iron on the sexual reproductivity of rotifers.

5. Conclusions

As summarised in **Fig. 1-14**, the present study defined the variation in iron ($FeSO_4 \cdot 7H_2O$) oxidative stress triggered oxidative/antioxidant enzymatic responses, such as MDA, AChE, SOD, and CAT between the two marine *Brachionus* sibling rotifers in different climatic regions. These findings could help to better understand rotifer reproductive adaptations under stressful conditions: vulnerability of male performances such as shortened lifespan, reduced sperm viability, and swimming speed.

Table 1-7. The nominal and actual concentrations of iron applied in investigations.

	Iron concentration (μg/mL)					
Nominal	0	20	30	45	60	75
Actual	0.0	19.5±0.2	30.8±0.3	43.5±0.4	58.8±0.3	72.1±0.4

Values are mean ± standard error of two replicates. The data given was the calculated results indicating the conversion of Fe and $FeSO_4 \cdot 7H_2O$.

Table 1-8. Iron ($FeSO_4 \cdot 7H_2O$) chronic effects on the reproductive patterns of temperate rotifer *Brachionus plicatilis* and tropical *Brachionus rotundiformis*.

		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. rotundiformis	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}

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

Table 1-9. 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 *Brachionus 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}	469.6 (438.3–501.0)	363.6 (323.4–406.0)
LC_{50}/ng DW	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

Values in parentheses are 95% confidence intervals.

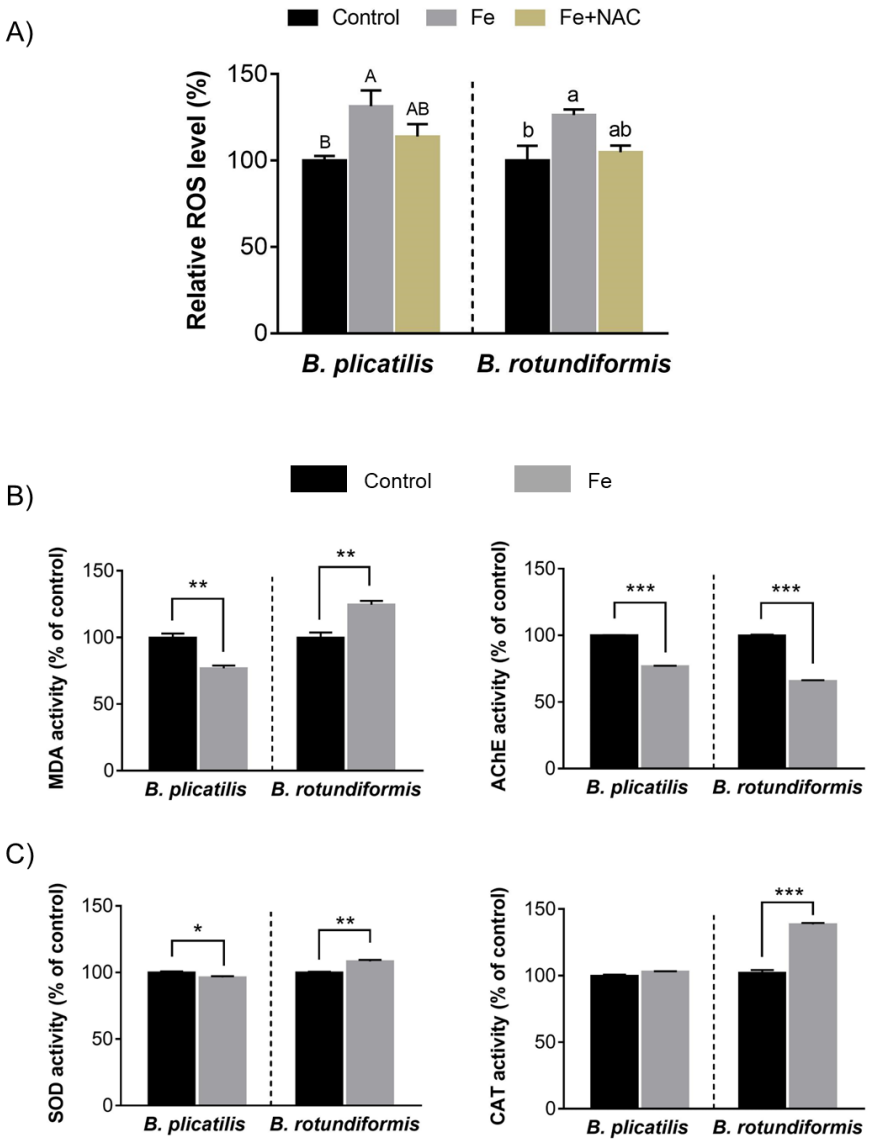

Fig. 1-8. Effects of iron ($FeSO_4 \cdot 7H_2O$) on (A) intracellular reactive oxygen species (ROS) levels with and without N-acetyl-L-cysteine (NAC) treatment, and the enzyme activities related to (B) oxidative stress and (C) antioxidant metabolisms in the temperate *Brachionus plicatilis* and tropical *Brachionus rotundiformis* after 12 h iron ($FeSO_4 \cdot 7H_2O$) exposure (A > B, a > b, Tukey HSD test, $p < 0.05$; *, **, *** indicate $p < 0.05$, 0.01, 0.001 respectively, Student's t-test, n = 3).

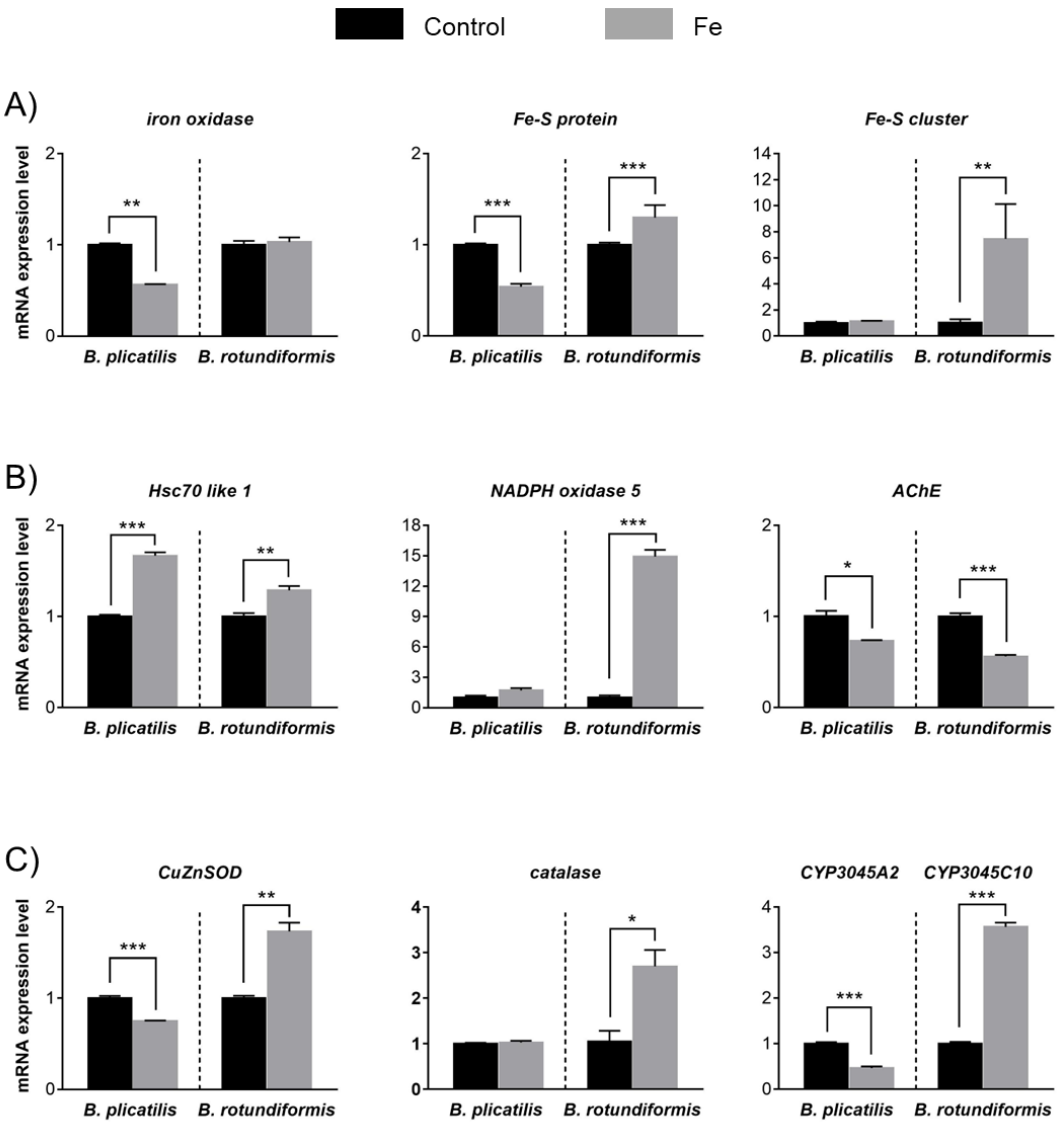

Fig. 1-9. Transcriptional levels of genes involved in (A) iron metabolism, (B) oxidative stress, and (C) antioxidant responses in temperate *Brachionus plicatilis* and tropical *Brachionus rotundiformis* after 12 h iron ($FeSO_4 \cdot 7H_2O$) exposure (Student's t-test, *, **, *** indicate $p < 0.05$, 0.01, 0.001 respectively, n = 3).

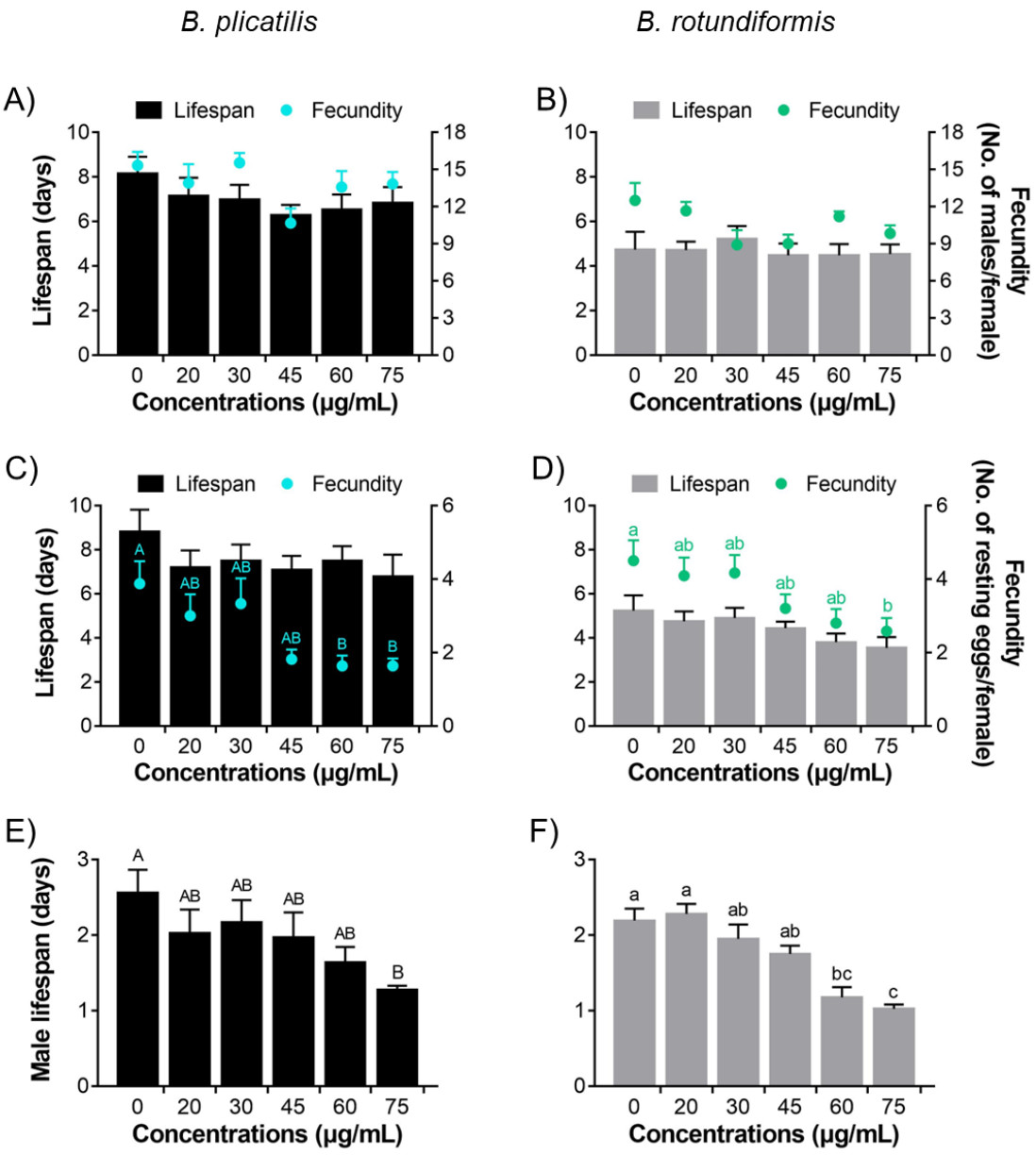

Fig. 1-10. Patterns of lifespan and fecundity of (A–B) unfertilized and (C–D) fertilized mictic females, and (E–F) male lifespan in temperate *Brachionus plicatilis* and tropical *Brachionus rotundiformis* under the different iron ($FeSO_4 \cdot 7H_2O$) concentrations (A > B, a > b > c, Tukey HSD test, $p < 0.05$, n = 12).

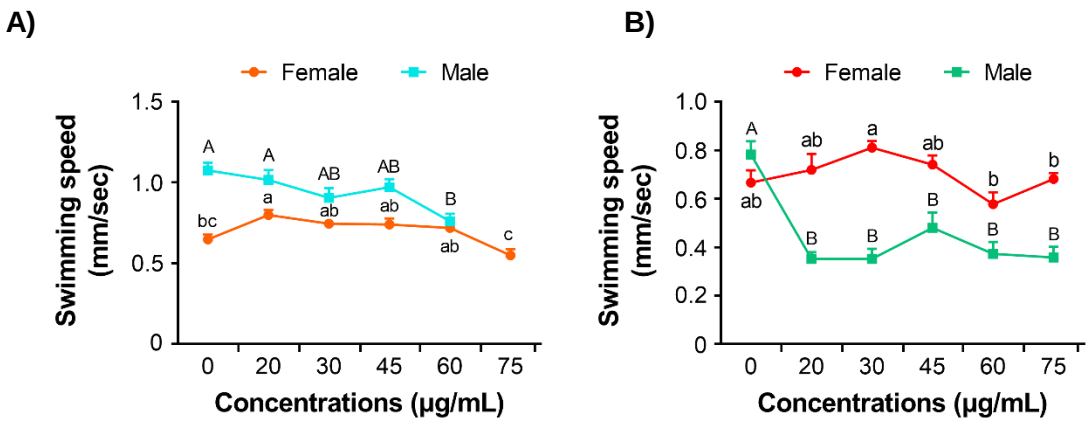

Fig. 1-11. The swimming speed of females and males in the (A) temperate *Brachionus plicatilis* and (B) tropical *Brachionus rotundiformis* under the different iron ($FeSO_4 \cdot 7H_2O$) concentrations (A > B, a > b > c, Tukey HSD test, $p < 0.05$, n = 20).

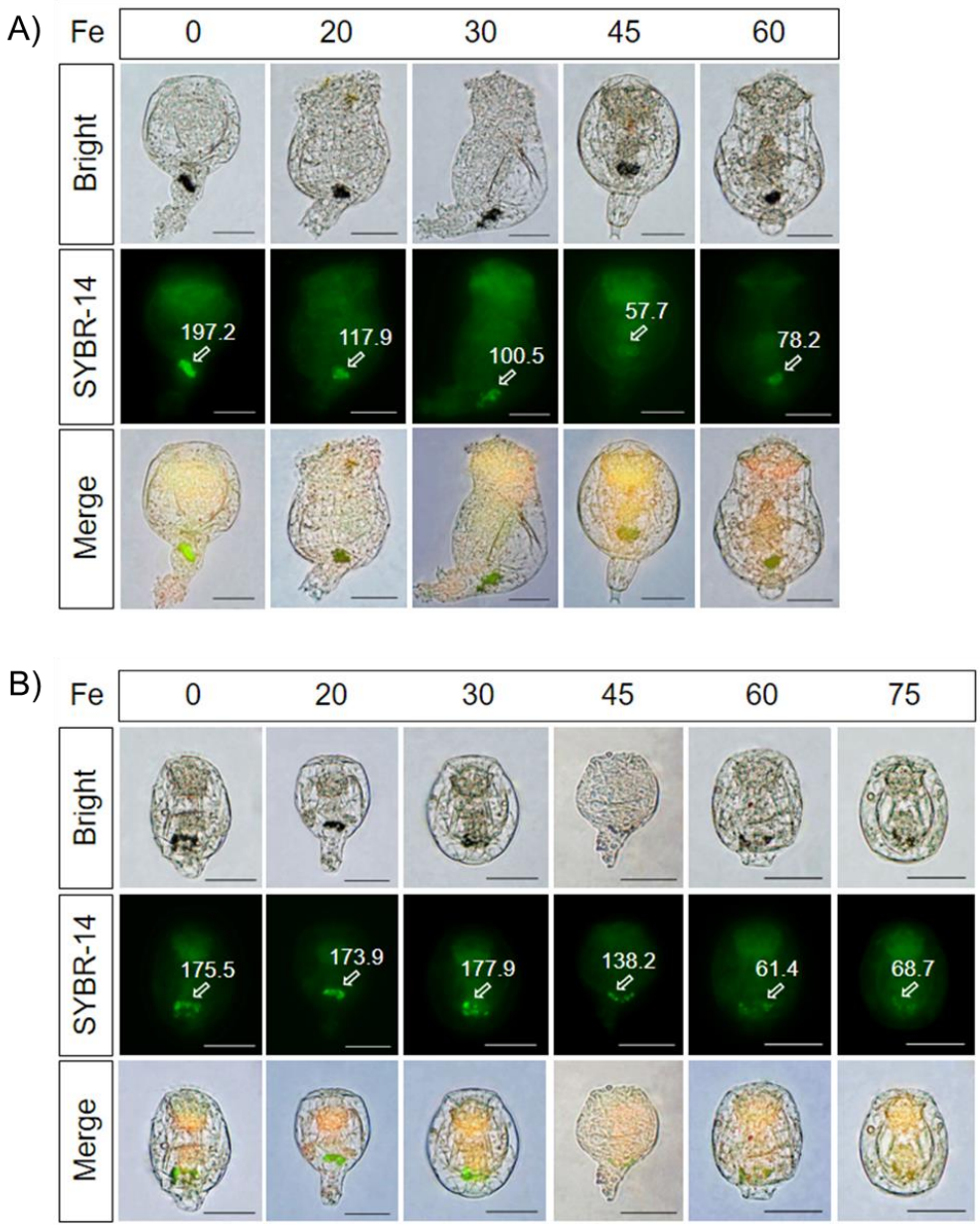

Fig. 1-12. The male and stained living spermatozoa of rotifer (A) temperate *Brachionus plicatilis* and (B) tropical *Brachionus rotundiformis* under the different iron ($FeSO_4 \cdot 7H_2O$) concentrations. The merged image contains the fluorescence of SYBR-14 (green) and PI (propidium iodide, red) staining. Arrow indicated data are the quantified fluorescence intensity. All scale bars represent 50 μm.

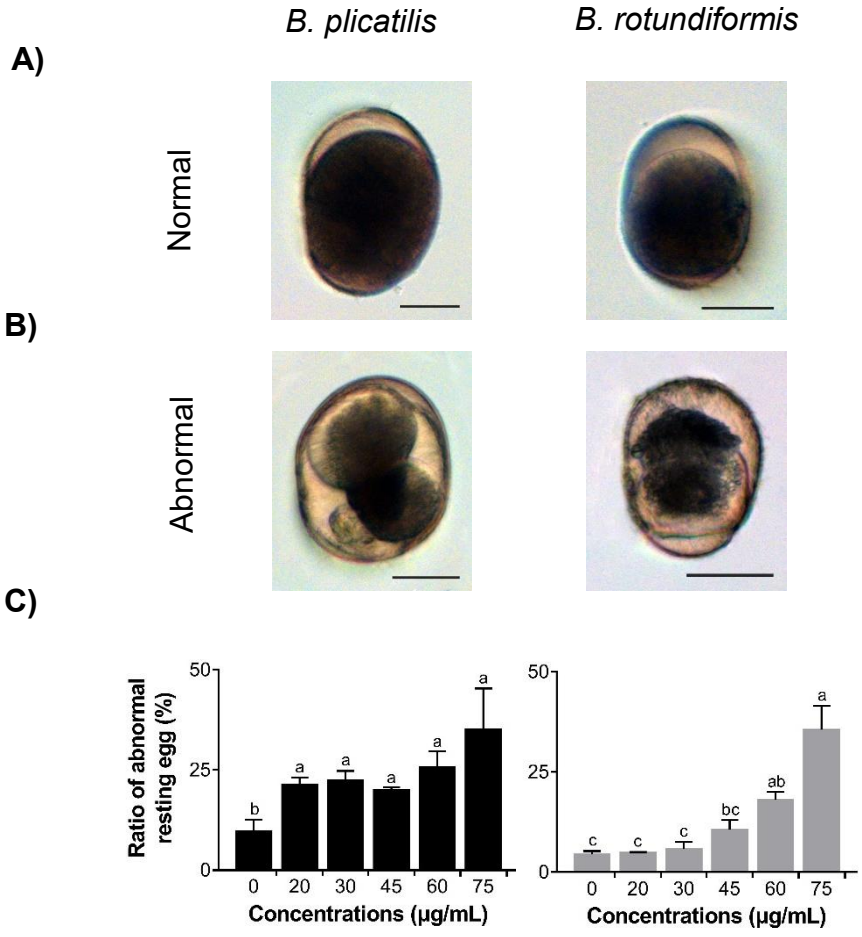

Fig. 1-13. The (A) normal and (B) abnormal resting egg of rotifer temperate *Brachionus plicatilis* and tropical *Brachionus rotundiformis.* All scale bars represent 50 µm. (C) The ratio (%) of abnormal resting egg under the different iron ($FeSO_4 \cdot 7H_2O$) concentrations in the two rotifers (black bar for *Brachionus plicatilis* and grey bar for *Brachionus rotundiformis*) (a > b > c, Tukey HSD test, $p < 0.05$, n = 3).

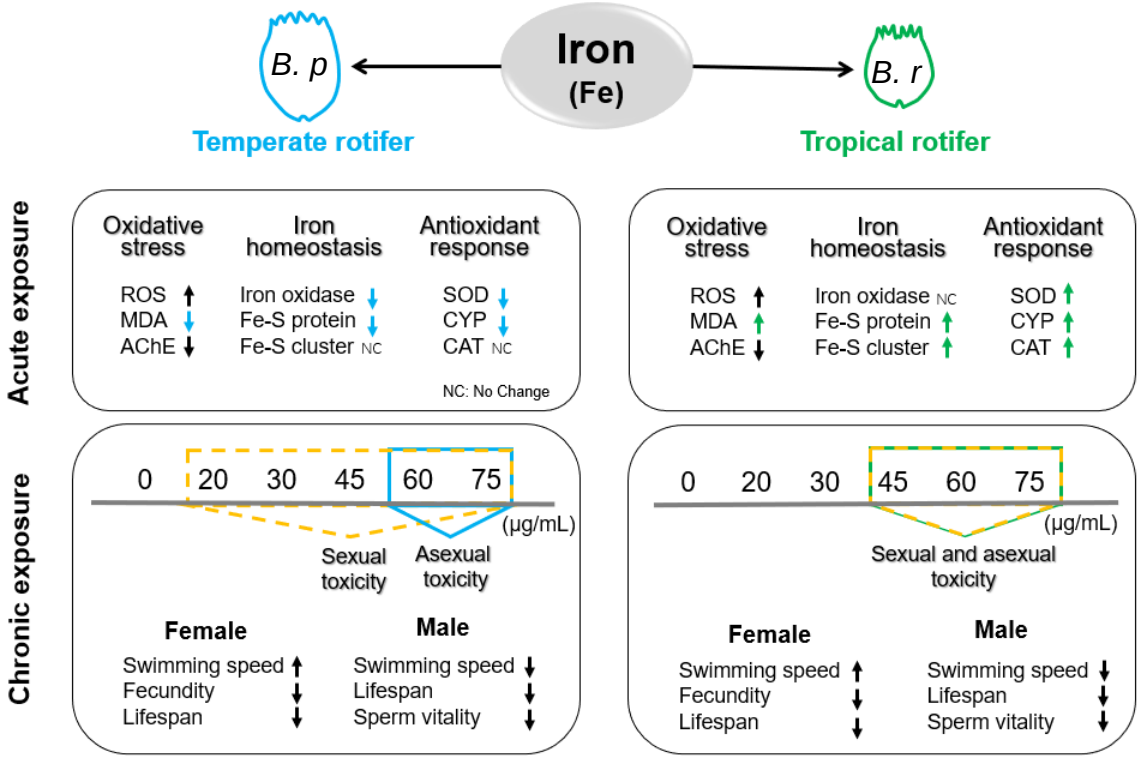

Fig. 1-14. Summary of iron acute and chronic toxicity responses in the two different climatic rotifer species. Blue and green represent the temperate rotifer *Brachionus plicatilis* (*B. p*) and the tropical rotifer *Brachionus rotundiformis* (*B. r*), respectively.

Chapter III

Differential modes of zinc chloride response in the two marine rotifers *Brachionus plicatilis* and *Brachionus rotundiformis*: Reproductive flexibility and stress-defensive activity

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1. Introduction

Zinc (Zn) is a trace element essential for diverse metabolic activities in a wide range of aquatic organisms, including biomass enhancement in algal cells (Sunda, 2006) and the promotion of survival, growth, immune abilities, and skeletal development in fish species (Watanabe et al., 1997). The beneficial effects of Zn are largely attributable to its role as a specific cofactor of various enzymes, including free radical scavenge-functioned copper-zinc superoxide dismutase and metallothionein, which play important roles in maintaining the balance of physiological metabolism (Formigari et al., 2007). While the natural sources of Zn are typically low, such as 0.3–2 nM in marine water (Ellwood and Van den Berg, 2000) and 0.3–19.2 μM in freshwater (Fu et al., 2016). Insufficient trace nutrients in aquafeed inhibit aquaculture productivity. In aquaculture operations, 0.023–2.2 mg/L of inorganic Zn is commonly applied as a dietary supplement for the stock and mass culture of marine algae (Guillard, 1975). The algae suspension without filtration is supplied in the cultural tanks of zooplankton (e.g., rotifer) and fish larvae, which is termed the "green water" technique (Naas and Harboe, 1992; Neori, 2011). Many studies have investigated Zn effects on the reproductive performances of aquatic animals. The results suggest that Zn is required in a concentration-specific manner depending on the feed period, species features, and Zn source (Dawood et al., 2022). Certain amounts of Zn enhance the growth performance, total antioxidant capacity, and muscle protein contents of fish species, such as the Nile tilapia *Oreochromis niloticus* (Kishawy et al., 2020). Moreover, Zn accumulation in the mitochondria of spermatozoa is closely associated with the regulation of sperm viability in the sea urchin *Lytechinus pictus* (Clapper et al., 1985) and Japanese eel *Anguilla japonica* (Yamaguchi et al., 2009). However, high levels of Zn exceeding

certain thresholds are detrimental. For example, chronic Zn (≥ 0.6 mg/L) exposure impairs the reproduction of marine amphipod *Corophium volutator*, resulting in a shortened life expectancy and retarded growth and maturation (Conradi and Depledge, 1999). Furthermore, a recent study observes Zn sex-specific effects on the reproduction of water flea *Daphnia magna*. 1.5 mg/L Zn inhibits the diet ingestion rate and antennae movement in males, whilst the fecundity of females is suppressed when the concentrations are ≥ 0.75 mg/L (Lee et al., 2021). Hence, the determination of Zn thresholds and the modes of Zn actions in various aquatic species is important for improving aquatic productivity and promoting the sustainable development of aquaculture.

Rotifers are significant secondary consumers in hydrosphere food webs, consuming microalgae and serving as essential sources of live food for the mass rearing of fish larvae (Hagiwara et al., 2007). Because of the size-selective predation of larvae, marine rotifers of *Brachionus plicatilis* and *Brachionus rotundiformis*, with large (mean lorica length 275 μm) and super-small (179 μm) size distributions, are used extensively in establishing feeding regimes for fish larvae (Hagiwara et al., 1995). On the other hand, the two species originate from temperate and tropical habitats, respectively, with water temperatures of 15–25°C and 20–35°C (Hagiwara et al., 2001; Gabaldon et al., 2017). Environmental factors, such as temperature, influence not only the physiological responses of aquatic species but also toxicant thresholds. For example, temperature governs the metabolisms of species by affecting oxygen consumption rates. A higher metabolic rate under high temperatures promotes the absorption of toxicants, including metals, by species, and potentially contributes to the generation of excessive levels of reactive oxygen species (ROS), resulting in oxidative stress (Wang, 1987; Gillooly et al., 2001). Thus, the species from varied habitats with distinct temperatures may be subjected to different levels of stress, and they may have evolved different reproductive strategies in coping with such situations. Because it has been described that the type and degree of stress experienced by organisms could affect their reproductive adaptability to other threats (Agathokleous, 2018). Hence, it is hypothesized that the ecological experiences of temperate and tropical rotifers may reflect different Zn thresholds and reproductive flexibility in response to chronic Zn exposure.

In this study, a multilevel approach was adopted to investigate the effects of zinc chloride ($ZnCl_2$) on the survival, growth, reproduction, and behavior of the temperate rotifer *B. plicatilis* and the tropical rotifer *B. rotundiformis*. Furthermore, to determine the potential metabolisms in regulating rotifer reproductive flexibility in response to $ZnCl_2$ exposure, the mitochondrial-mediated stress defenses, such as intracellular reactive species (ROS) levels and antioxidant genetic/enzymatic activities, and energy sources, such as lipid storage and adenosine triphosphate (ATP) production were evaluated.

2. Materials and methods

2.1. Reagent preparation

Zinc chloride ($ZnCl_2$, purity ≥ 98.0%; Nacalai Tesque, Kyoto, Japan) was used in this study. A 100 mg/L stock solution of $ZnCl_2$ was prepared by dissolving 2 mg $ZnCl_2$ in 20 mL of distilled water, followed by magnetic stirring for more than 30 min. Nominal test solutions were obtained by diluting the stock solution with artificial seawater (ASW) at a salinity of 17 parts per thousand (ppt) (Marine Art Hi, Tomita Pharmaceutical, Tokushima, Japan).

2.2. Experimental species

The rotifers used in this study were derived from populations of *B. plicatilis* (NH17L strain, mean lorica length 275 μm) (Suga et al., 2011) and *B. rotundiformis* (Kochi strain, mean lorica length 179 μm) (Kim et al., 2014) that have been maintained at the Aquaculture Biology Laboratory, Nagasaki University, Japan, for more than two decades. The stock cultures of each species were maintained in 17 ppt ASW at 25°C in total darkness and fed 7.0×10^6 cells/mL of the microalga *Nannochloropsis oculata* daily. All experiments were performed with neonates (< 2 h) hatched from amictic eggs, which were collected according to the following procedure. Approximately 10,000 stock culture female rotifers bearing amictic eggs were introduced into screw-capped vials containing 10 mL of culture medium and agitated to detach the eggs. Having repeated this process several times, the

detached eggs were incubated in the wells of a six-well plate containing 5 mL of seawater at 25°C and observed at hourly intervals for hatching.

2.3. Acute exposure test

Ten neonates (< 2 h) of each of the two species were placed in the wells of a six-well plate containing 5 mL of working solutions of $ZnCl_2$ at concentrations selected based on preliminary range assessments [0 (control), 6, 12, 24, 36, 48, 60, and 72 mg/L for *B. plicatilis*, and 0 (control), 1, 2, 4, 8, 16, 32, and 50 mg/L for *B. rotundiformis*], with each concentration being assessed in triplicate. The culture conditions were identical to those used to maintain the stock cultures, with the exception that no food was provided during the observation period. The surviving and dead rotifers were enumerated under a model SZ-STS stereomicroscope (Olympus, Tokyo, Japan) at ×60 magnification following exposure for 24 and 48 h. Confirmation of mortality was based on a lack of cilial or mastax movement for more than 30 s. Lethal concentrations (LCs) and 95% confidence intervals (CIs) were determined based on probit analysis.

2.4. Life history parameters

Based on preliminary assessments, we selected $ZnCl_2$ concentrations of 0 (control), 0.0125, 0.025, 0.05, 0.1, 1, and 2 mg/L) to investigate the life history-associated responses of rotifers. Briefly, 12 neonates (< 2 h) were placed individually in wells of a 24-well plate containing 1 mL test solution and 7.0×10^6 cells/mL of *N. oculata* and then incubated at 25°C in total darkness. After 15 h, neonates were inspected at hourly intervals to monitor the times for the first eggs and initial offspring production (generation time). The survival and number of offspring produced by maternal rotifers were assessed at 12 h intervals. The maternal rotifers were transferred daily to a fresh test solution. This process was repeated until all parent rotifers had died. Based on these observations, the age-specific survival rate was estimated by the following equation defined by Maia et al. (2000):

$$\text{Age-specific survival rate (\%)} = \left[\frac{\text{Numbers surviving at each age}}{\text{The initial number of rotifers}}\right] \times 100$$

The generation time (average time taken to produce the first offspring among all assessed individuals), lifespan (average survival time of individuals), and fecundity (average number of offspring produced by each female during its lifetime) were recorded. The intrinsic growth rate (*r*) was calculated using the Lotka equation (Lotka, 1907):

$$\sum lxmxe^{-rx} = 1,$$

where *lx* indicates the likelihood of rotifer survival at age *x*, and *mx* denotes the number of offspring produced by an individual at age *x*.

2.5. Reproductive response patterns

$ZnCl_2$ effects on the sexual and asexual reproductive characteristics of *B. plicatilis* and *B. rotundiformis* were assessed over 7 days of batch exposure, under three concentrations of $ZnCl_2$ [0 (control), 1, and 2 mg/L], which were found to influence the fecundity or intrinsic growth rate in both species in the life history parameter test. Eighty neonates (< 2 h) were introduced into 100 mL screw-capped bottles containing 80 mL of working solution at an initial density of 1 individual/mL and then incubated in total darkness at 25°C. They were fed daily with 7.0×10^6 cells/mL of *N. oculata*. Half of the culture medium was renewed daily with a fresh diet and $ZnCl_2$ to maintain the initial $ZnCl_2$ concentration in the solution. To minimize the precipitation of microalgae cells, the cultivations were gently agitated on a shaker (Triple Shaker NR-80, TAITEC, Saitama, Japan) with horizontal shaking at 85 ± 1 rpm. At daily intervals during the culture period, we recorded the numbers of each of four types of females: non-ovigerous females, amictic females carrying large female eggs (FF), unfertilized mictic females carrying numerous small male eggs (MF), and resting-egg females carrying yellowish eggs (RF) (Hagiwara et al., 1988). Feeding was terminated on the final day of cultivation, and resting eggs (RE) were harvested after 3 days of starvation. All experiments were conducted in triplicate, for which the following reproductive parameters were calculated based on the recorded data:

Population growth rate (*r*): $\ln(Nt/N0)/t$

where *t* is 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.

Calculations of mixis and fertilization rate were based on analyses of the area under growth curves constructed for each type of sexual and asexual female during the 7 days of culture using the following equations:

$$\text{Mixis (\%)} = \left[\frac{(\text{MF+RF})\text{area}}{(\text{FF+MF+RF})\text{area}}\right] \times 100$$

$$\text{Fertilization (\%)} = \left[\frac{(\text{RF})\text{area}}{(\text{MF+RF})\text{area}}\right] \times 100$$

For subsequent metabolic analyses, we established a series of 600 mL rotifer cultures in a 1000 mL screw-capped bottle,which include 600 rotifer neonates (< 2 h) of each species and were exposed to one of three $ZnCl_2$ levels [0 (control), 1, and 2 mg/L]. After 7 days of cultivation, rotifer samples were collected for further analysis. The experimental conditions, including diet and incubation settings, were identical to those employed in the aforementioned reproductive patterns investigation.

2.6. Swimming activity and sperm viability

For analysis of swimming behavior, 10 female rotifers bearing a single amictic egg and 10 male rotifers (< 2 h) were collected from 5 days cultures (at which time numerous mictic females had emerged) exposed to one of the three assessed $ZnCl_2$ treatments (0, 1, and 2 mg/L). To obtain new-born males, 50 unfertilized mictic females from each $ZnCl_2$ treatment were released into the wells of a six-well plate containing 5 mL of $ZnCl_2$ solution and incubated at 25°C, during which time, they were monitored at the 30 min interval, with new-born males being collected for analysis. The selected individuals were placed in a glass-bottomed dish (1.5 mm thick, 14 mm in diameter; Matsunami, Osaka, Japan) containing 200 µL of $ZnCl_2$ solution. Swimming activity was recorded for 20 s under a HAS-UZ stereomicroscope (Olympus) equipped with a HAS-X Viewer digital camera (Ver. 12.1201; DITECT, Tokyo, Japan), and the swimming speed (mm/s) of male and female rotifers was measured using Dipp Motion Pro Ver. 1.1.31 the tracking software (DITECT).

For assessments of sperm viability, 15 males (< 30 min old) were collected upon hatching from the aforementioned mictic eggs, and their viability was determined using a LIVE/DEAD® Sperm Viability Kit (Molecular Probes, Thermo Fisher Scientific, Waltham, MA, USA). Specifically, individual males suspended in a 10 μL volume of diluted (40×) *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES) buffer (pH 7.4) containing 10 mM HEPES, 150 mM NaCl, and 10% bovine serum albumin were placed on glass slides, to which was added 5 μL of diluted SYBR-14 (50×), followed by incubation at 25°C for 15 min. After adding 2.5 μL of propidium iodide (PI), the preparation was gently covered with a coverslip, followed by a further 15 min incubation at 25°C. The stained samples were examined using a Ts2-FL fluorescence microscope (Nikon) at excitation/emission of 488/516 nm for SYBR-14 and 535/617 nm for PI. The intensity of testes fluorescence was measured using NIS-Elements F version 4.60 image analysis software.

2.7. Neutral lipid contents

Variations in energy content (neutral lipid droplets) in the reproductive organs of *B. plicatilis* and *B. rotundiformis* in response to $ZnCl_2$ (0, 1, and 2 mg/L) exposure were determined based on Nile Red staining. The protocol was the same as in Han et al. (2021). A detailed description was shown in Supplementary Information.

2.8. Mitochondrial membrane potential and ATP/ATPase levels

Changes in rotifer mitochondrial membrane potential were estimated using MitoTracker Red CMXRos (Invitrogen, Carlsbad, CA, USA), of which we initially prepared a 1 mM stock solution by dissolving in a specific volume of dimethyl sulfoxide (DMSO). Fifteen female rotifers, each bearing an amictic egg, which had previously been subjected to one of the three $ZnCl_2$ treatments (0, 1, or 2 mg/L) were collected and rinsed in fresh ASW for 2 h before staining to clear the stomach contents and minimize the numbers of auto-fluorescing microalgae. These females were then transferred to the wells of a 24 well microplate containing 2 mL of a 5 μM solution of MitoTracker™ Red, followed by incubation in the dark at 25°C for 30 min (Snell et al., 2014), after which the

rotifers were rinsed with fresh ASW for 5 min, fixed in 4% formalin, and then rinsed a further time with fresh ASW. Images of the fluorescence signals emitted by stained samples were captured using a Ts2-FL fluorescence microscope (Nikon) with excitation/emission wavelengths of 579/599 nm. Fluorescence intensity analysis was performed using ImageJ 1.53e software (National Institutes of Health, USA), and the results are presented in terms of average pixel intensity.

ATP levels and ATPase activity in response to $ZnCl_2$ exposure were determined using a commercial ATP assay kit (Colorimetric/Fluorometric) (Abcam, Cambridge, UK) and ATPase assay kit (Colorimetric) (Abcam), respectively. The ATP assay used is based on the phosphorylation of glycerol that can be readily quantified colorimetrically at an optical density of 570 nm. The ATPase assay is based on the enzymic hydrolysis of ATP, which releases ADP and a free phosphate ion that can be detected at an optical density of 650 nm, with the signal intensity corresponding to the level of ATPase activity. The results thus obtained were normalized to total protein levels and are presented as a value relative to that of the control (100%). In addition, total protein concentrations were measured using the Bradford assay (Bio-Rad Protein Assay; Bio-Rad, Hercules, CA, USA). All analyses were performed in triplicate.

2.9. Intracellular reactive oxygen species levels and oxidative/antioxidant enzymatic activities

ROS were detected using 2′,7′-dichlorofluorescein diacetate (H_2DCFDA) (Sigma-Aldrich). Malondialdehyde (MDA) levels were detected based on the reaction with thiobarbituric acid using a commercial lipid peroxide (MDA) assay kit (Sigma-Aldrich). Acetylcholinesterase (AChE) activity was assessed using a commercial AChE assay kit (colorimetric) (Abcam). Superoxide dismutase (SOD) enzyme activity was quantified based on the SOD–superoxide anions–hydrogen peroxide (H_2O_2)–WST-1 (2-[4-Iodophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium, monosodium salt) reaction using a commercial SOD activity assay kit (colorimetric) (Abcam). Catalase (CAT) enzyme activity was measured based on a CAT–H_2O_2–probe reaction using a catalase

activity assay kit (colorimetric/fluorometric) (Abcam). Detailed descriptions of ROS and enzyme activity measurements are provided in the Supplementary Information.

2.10. Biomarker analysis

To identify sensitive biomarkers characterizing the response of rotifers to $ZnCl_2$ exposure, we examined the transcription levels of genes in three functional groups, namely, oxidative/antioxidant defenses, lipid synthesis, and mitochondrial respiration activities, the primer sequences for which are listed in **Table A1**. For RNA extraction, we used approximately 7,000 rotifers for each treatment. Extraction was performed using TRIzol® reagent (Invitrogen), with preparations being subsequently treated using a TURBO DNA-free™ kit (Ambion, Carlsbad, CA, USA) to remove genomic DNA. The quality and quantity of the isolated RNAs were determined spectrophotometrically at 230, 260, and 280 nm using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Aliquots of the purified RNA were then used for the synthesis of complementary DNA (cDNA) performed using a PrimeScript™ II 1st strand cDNA Synthesis Kit (TaKaRa Bio, Shiga, Japan). All the procedures were conducted based on standard laboratory protocols. Real-time quantitative reverse transcription-polymerase chain reaction (real-time qRT-PCR) was performed using 20 μL reaction mixtures containing 1 μL of cDNA template, 0.5 μL of each of the forward and reverse primers (10 μM), and 10 μL TB Green Premix Ex Taq (2×) (TaKaRa Bio) under the following thermal cycling conditions: initial denaturation 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. Amplification was performed using a LightCycler® 96 real-time PCR system (Roche Life Science, Basel, Switzerland) and melting curve cycles (95°C for 10 s, 55°C for 1 min, and 80 cycles at 55°C for 10 s, with a 0.5°C increase per cycle) were performed to confirm the amplification of specific products. The housekeeping gene elongation factor 1-α (*EF1-α*) was selected as an internal control since its consistent expression between and within the experimental groups, and the $2^{-\Delta\Delta CT}$ method was used to determine transcription levels. All assays were performed using three biological replicates.

2.11. Statistical analysis

All statistical analyses were conducted using R version 3.6.3 software (https://cran.r-project.org/bin/windows/base/old/3.6.3/). Results are presented as the means ± standard deviation. The normality of the data was assessed using Bartlett's variance homogeneity test and Student's *t*-test was used to determine significant differences in the acute toxicity test. For analysis of survival rates, we used a log-rank test, and for life-history parameters, we used a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. A one-way ANOVA followed by Tukey's honest significant difference (HSD) test was used to determine significant differences among reproductive parameters and biological metabolic activity at a $p < 0.05$ level of significant difference.

3. Results

3.1. Median lethal concentrations

For the two rotifer species *B. plicatilis* and *B. rotundiformis*, we obtained 24 h and 48 h $ZnCl_2$ LC_{50} values of 29.9 and 9.0 mg/L and 13.3 and 3.6 mg/L, respectively (**Table 2-1)**.

3.2. Lifetable parameters

At the assessed concentrations, higher concentrations of $ZnCl_2$ affected the life history patterns of both species **(Fig. 2-1)**. Specifically, in *B. plicatilis*, rotifer exposed to 2 mg/L $ZnCl_2$ reduced the generation time (pre-reproductive period), whereas there were no significant effects on the survival or fecundity of individuals. Compared with the controls, a 1.2-fold ($p < 0.05$) increase in intrinsic growth rate (r) was detected at 2 mg/L $ZnCl_2$. Furthermore, at $ZnCl_2$ concentrations ≥ 0.1 mg/L, we detected a shortening of lifespan from 8.6 ± 3.7 days (control) to 6.3 ± 0.8 days at 0.1 mg/L $ZnCl_2$ ($p < 0.05$). Comparatively, in *B. rotundiformis*, lifespan was reduced in response to $ZnCl_2$ at concentrations ≥ 1 mg/L, from 6.9 ± 1.6 days (control) to 5.3 ± 1.0 days (1 mg/L, $p < 0.05$) and 5.0 ± 1.0 days (2

mg/L, $p < 0.01$). High $ZnCl_2$ concentrations (2 mg/L) were also found to inhibit rotifer survival and fecundity, with the highest mortality of 45% being detected on day 5, and fecundity declined from 20.8 ± 1.7 offspring/female (control) to 16.9 ± 4.9 in individuals exposed to 2 mg/L $ZnCl_2$.

3.3. Reproductive patterns

Rotifer sexual and asexual reproductive parameters in response to exposure to 0 (control), 1, and 2 mg/L $ZnCl_2$ are presented in **Table 2-2**. For *B. plicatilis*, $ZnCl_2$ at 2 mg/L inhibited rotifer sexual mixis and fertilization, thereby resulting in an 11.6-fold reduction in resting egg production. Although the 1 mg/L $ZnCl_2$ treatment was found to induce a more rapid rate of population growth, the rate of fertilization was significantly inhibited ($p < 0.05$), resulting in lower resting egg numbers compared with those produced by control rotifers. Contrastingly, in *B. rotundiformis*, $ZnCl_2$ at ≥ 1 mg/L was found to stimulate rotifer mixis, with a higher occurrence of mictic females compared with that in control rotifers **(Fig. 2-2)**, and a 1.4-fold increase in resting egg production ($p < 0.05$) was detected at 1 mg/L $ZnCl_2$.

3.4. Variation in the swimming speeds of male and female rotifers

Under the detected concentrations, $ZnCl_2$ exposure stimulated the swimming speed of both the males and females in the two species **(Fig. 2-3)**. In response to 1 and 2 mg/L $ZnCl_2$, the swimming speed of female and male rotifers in *B. plicatilis* increased by 1.4-fold and 1.4–1.5-fold (both $p < 0.05$), respectively. A similar pattern was observed in *B. rotundiformis*, for which 1.3–1.8-fold and 1.4–1.5-fold higher speed for females and males (both $p < 0.05$), respectively, in response to 1 and 2 mg/L of $ZnCl_2$, compared with the controls.

3.5. Neutral lipid droplet contents

The patterns of neutral lipid droplet staining in the reproductive organs (vitellarium/ovary) of the two rotifer species are shown in **Fig. 2-4**. In *B. plicatilis*, a 2.0-fold ($p < 0.05$) reduction in neutral lipid contents under 2 mg/L of $ZnCl_2$ was observed, compared with the control rotifers. In contrast, no significant differences were detected in *B. rotundiformis* exposed to either 1 or 2 mg/L $ZnCl_2$ **(Fig. 2-4 C)**.

3.6. Sperm viability

$ZnCl_2$ exposure had a differential influence on male sperm viability of the two rotifer species **(Fig. 2-5)**, as indicated by the difference in the SYBR (green) fluorescence intensity of stained spermatozoa (with a higher intensity corresponding to higher viability). In *B. plicatilis*, we observed a significant reduction in relative fluorescence intensity (1.4- and 2.4-fold; both $p < 0.05$) in response to 1 and 2 mg/L $ZnCl_2$, respectively. Whereas there were significant increases (2.7- and 2.9-fold) ($p < 0.05$) in *B. rotundiformis* exposed to 1 and 2 mg/L $ZnCl_2$, respectively **(Fig. 2-5 C)**.

3.7. Mitochondrial membrane potential and ATP/ATPase levels

$ZnCl_2$ exposure activated the mitochondrial membrane potentials in both rotifer species **(Fig. 2-6)**. In *B. plicatilis*, a significant 1.0-fold increase ($p < 0.05$) was detected at 2 mg/L $ZnCl_2$. In *B. rotundiformis*, significant 1.1-fold increases ($p < 0.05$) were detected in response to 1 and 2 mg/L $ZnCl_2$, respectively, compared with the controls **(Fig. 2-6 C)**.

Two rotifer species exhibited different patterns of ATP and ATPase activity following $ZnCl_2$ exposure, with 2 mg/L $ZnCl_2$ inducing a 1.4-fold increase ($p < 0.05$) in the ATPase activity of *B. plicatilis* but a 1.4-fold reduction ($p < 0.05$) in that of *B. rotundiformis* **(Fig. 2-6 D)**. Furthermore, 1.3-fold higher levels of ATP ($p < 0.05$) were observed in *B. rotundiformis* at 2 mg/L $ZnCl_2$, compared with the control rotifers **(Fig. 2-6 E)**.

3.8. Intracellular ROS levels and oxidative/antioxidant enzymatic activity

$ZnCl_2$ exposure induced different patterns of intracellular ROS production in the two rotifer species **(Fig. 2-7 A)**. In *B. plicatilis*, a significant concentration-dependent reduction (1.3- and 2.3-fold; both $p < 0.05$) was detected in response to 1 and 2 mg/L $ZnCl_2$, respectively, compared with the controls. Whereas a significant 2.1-fold increase ($p < 0.05$) was observed in *B. rotundiformis* at 2 mg/L $ZnCl_2$ treatment.

For the antioxidant enzymatic activity, we detected activated SOD activity in both rotifer species upon exposure to $ZnCl_2$ **(Fig. 2-7 B)**, with a 1.2-fold increase ($p < 0.05$) in *B. plicatilis* exposed to 1 mg/L, and a 1.1-fold increase ($p < 0.05$) in *B. rotundiformis* induced by 2 mg/L. For the CAT activity, a significant 1.3-fold increase ($p < 0.05$) was detected in *B. plicatilis* under 2 mg/L $ZnCl_2$, compared with the controls. Whereas there was no significant difference in *B. rotundiformis* in either 1 or 2 mg/L $ZnCl_2$ **(Fig. 2-7 C)**.

Levels of the lipid peroxidation marker MDA in rotifers following $ZnCl_2$ treatment are shown in **Fig. 2-8 A**. For *B. plicatilis*, significant 1.8- and 1.1-fold reductions ($p < 0.05$) were recorded in response to 1 and 2 mg/L $ZnCl_2$, respectively, whereas at the same $ZnCl_2$ concentrations, significant 1.2- and 1.4-fold increase ($p < 0.05$) were detected in *B. rotundiformis*.

$ZnCl_2$ exposure suppressed AChE activity in both species **(Fig. 2-8 B)**. Specifically, in *B. plicatilis*, 1.8- and 1.5-fold reduction ($p < 0.05$) were recorded at 1 and 2 mg/L $ZnCl_2$, respectively, and a 1.1-fold inhibition ($p < 0.05$) was observed in *B. rotundiformis* exposed to 1 mg/L $ZnCl_2$, compared with the controls.

3.9. Biomarker transcription levels

The transcription levels of oxidative- and antioxidant-associated biomarkers are presented in **Fig. 2-9 A**. Compared with the respective controls, the expression of *Hsc70 like 1* was upregulated 1.7-fold ($p < 0.05$) in *B. plicatilis* at 2 mg/L $ZnCl_2$, and 1.4- and 1.3-fold ($p < 0.05$) in *B. rotundiformis* in response to 1 and 2 mg/L $ZnCl_2$, respectively. For the *AChE* transcription, a 1.6-fold upregulation ($p < 0.05$) was detected in *B. plicatilis* exposed to 1 and 2 mg/L $ZnCl_2$, and a 1.2-fold upregulation ($p < 0.05$) was observed in *B. rotundiformis* at 2 mg/L $ZnCl_2$. The expression of *CuZnSOD*, *cat*, *glutathione* S-*transferase sigma 2* (*GSTs2*), and *cytochrome P450* (*CYP3048A2*, clan 2) in *B. plicatilis*

was upregulated by 1.7- and 1.9-fold, 1.3- and 1.7-fold, 1.6- and 3.1-fold, and 2.0- and 1.3-fold (all $p < 0.05$) in response to 1 and 2 mg/L $ZnCl_2$, respectively. In *B. rotundiformis*, the expression of *CuZnSOD* was upregulated by 1.1-fold ($p < 0.05$) at 1 mg/L $ZnCl_2$ exposure, and *GSTs2* was upregulated by 1.1-fold at 2 mg/L $ZnCl_2$. In contrast, there were no significant changes in the expression of *cat* and *CYP3043A1* (clan 2).

The expression levels of lipid synthesis pathway biomarkers were shown in **Fig. 2-9 B**. In *B. plicatilis*, the mRNA levels of *mitochondrial CYP* (*CYP3335A1*) were significantly upregulated 1.3- and 1.8-fold ($p < 0.05$) in response to 1 and 2 mg/L $ZnCl_2$, respectively. Substantial downregulation (1.4-fold) and upregulation (1.4-fold) (both $p < 0.05$) of *acetyl-CoA carboxylase* (*ACC*) expression were detected at 1 and 2 mg/L $ZnCl_2$, respectively. For *B. rotundiformis*, a 1.3-fold upregulation ($p < 0.05$) of *ACC* was detected at 2 mg/L treatment. However, there was no substantial effect of $ZnCl_2$ exposure in either species on the expression of the *citrate lyase* gene.

$ZnCl_2$ effects on the gene expression in mitochondrial electron transport (e.g., *cytochrome c oxidase*) and apoptosis (e.g., *caspase-2* and *caspase-7*) in the two rotifer species were shown in **Fig. 2-9 C**. Upon exposure to 1 and 2 mg/L $ZnCl_2$, the mRNA levels of *cytochrome c oxidase* were upregulated 1.3- and 1.8-fold in *B. plicatilis* and1.3- and 1.3-fold in *B. rotundiformis*, respectively (all $p < 0.05$). The expression of *caspase-2* was significantly upregulated 1.7- and 1.7-fold in *B. plicatilis* and 1.3- and 1.8-fold in *B. rotundiformis*, respectively (all $p < 0.05$). The transcript of *caspase-7* was upregulated 1.2-fold ($p < 0.05$) in *B. plicatilis* at 2 mg/L treatment, and upregulated 1.2- and 1.3-fold (both $p < 0.05$) in *B. rotundiformis* in response to 1 and 2 mg/L $ZnCl_2$, respectively.

4. Discussion

This study defined differential modes of $ZnCl_2$ response in reproduction, particularly sexual reproduction, and stress defensive activities, including antioxidant responses and energy status in the temperate *B. plicatilis*, and tropical *B. rotundiformis* rotifers. Concerning $ZnCl_2$ acute exposure, the two species of marine rotifers have notably higher Zn tolerances, compared with the freshwater rotifer *B. calyciflorus* (24 h LC_{50}: 1.3 mg/L) (Snell et al., 1991), freshwater *D. magna* (48 h LC_{50}: 0.32 mg/L) (Lari et al., 2017), and

the rotifer *Proales similis* (24 h LC_{50}: 0.56–0.81 mg/L) at different salinity conditions (Rebolledo et al., 2021). Compared with marine species, the higher susceptibility of freshwater rotifers to metal toxicity has been widely reported and this may be associated with the greater abundance of free metal ions under lower salinity conditions, which tend to be more toxic than metal chloride complexes formed at higher salinity (McLusky et al., 1986; Hall and Anderson, 1995). In addition to salinity effects on metal moieties, the temperature has a substantial influence on the bioavailability and toxicity of metals, including Zn (McLusky et al., 1986; Wang, 1987; Pereira et al., 2017). Therefore, in this study, to control for the potential influence of temperature on $ZnCl_2$ responses in the two rotifers species, we conducted all experiments at the same incubation temperature of 25°C, an overlapped temperature range for *B. plicatilis* and *B. rotundiformis*, which is moderate for the fecundity of both species (Hirayama and Rumengan, 1993).

Asexual reproduction has several advantages under stable environments, such as rapid and low-energy cost population evolution. However, the ecological significance of sexual reproduction involves the generation of genetic diversity and the improvement of population fitness under harsh/changing circumstances (Crow, 1992). In response to chronic exposure, $ZnCl_2$ within the concentrations of 0.0125–0.1 mg/L had no negative effects on the survival and fecundity in both species. However, higher levels of $ZnCl_2$ induced different reproductive sensitivity, especially the sexual reproduction between the two rotifers. *B. plicatilis* exposed to $ZnCl_2$ at concentrations $\geq$ 1 mg/L significantly inhibited the mixis induction and resting eggs generation, while showing no detrimental effects on asexual reproduction at both individual fecundity and population growth levels. Contrastingly, *B. rotundiformis* exhibited stimulated sexual reproductivity in response to 1 mg/L $ZnCl_2$. Although exposure to 2 mg/L $ZnCl_2$ inhibited *B. rotundiformis* survival and fecundity at the individual level, it had no apparent detrimental effects on population growth. Sexual reproduction in rotifers is easily regulated by environmental cues, including crowding, nutrients, and stressors (Marcial and Hagiwara, 2007; Gilbert, 2010). The obtained results may reflect different stress-coping strategies in the two rotifer species and the higher resilience of the tropical species, which is consistent with the reports by Tanaka et al. (2009) and Han et al. (2021, 2022a, 2022b). As aforementioned in the Introduction, the tropical rotifer *B. rotundiformis* may be subjected to higher amounts of habitat-sourced

stress than the temperate species *B. plicatilis*, and thus they may have evolved specific strategies to adapt to ambient environments. Moreover, this process improves their reproductive flexibility against other stressors, which is considered the "environmental hormesis effect" (Agathokleous, 2018). Consequently, the habitat evolutionary experience in the two rotifer species may regulate their reproductive responses to coping with stressful situations.

The metabolisms regulating the sexual reproductive sensitivity in the two rotifer species were determined by mitochondrial-mediated energy and antioxidant activities. In all multicellular organisms, the mitochondria play significant roles in maintaining normal physiological activities. It not only provides a source of ATP but also regulates lipid storage and utilization, as well as harbors oxidative/antioxidant metabolic networks, maintaining a viable balance between ROS generation and detoxification (Andreyev et al., 2005; Benador et al., 2019). In this study, we detected differences in the mitochondrial activities of the two assessed rotifer species upon exposure to $ZnCl_2$. Specifically, mitochondrial membrane potential was significantly enhanced in *B. plicatilis* following exposure to 2 mg/L $ZnCl_2$, along with activated respiratory chain complex, such as *cytochrome c oxidase* and ATPase. Generally, there exists a positive correlation between mitochondrial membrane potential and ROS production, whereby mitochondria generate higher amounts of ROS at higher membrane potentials (Korshunov et al., 1997). Whereas we detected significant reductions in ROS levels in *B. plicatilis* upon exposure to 1 and 2 mg/L $ZnCl_2$, which might be attributable to overcompensation of stress-defense responses, as indicated by enhanced levels of transcriptomic and enzymatic activities for mitochondrial MnSOD and cytosolic CuZnSOD, CAT, CYP, and GSTs. The consensus holds that excessive mitochondrial ROS generation, along with poor scavenging defenses, results in the accumulation of ROS to levels exceeding the physiological threshold, thereby inducing oxidative stress. In contrast, overcompensated antioxidant activity will promote reductive stress (Rajasekaran et al., 2007; Zorov et al., 2014). Both excessively high (oxidative stress) and low (reductive stress) levels of ROS are deleterious. Hence, higher $ZnCl_2$ may induce reductive stress in *B. plicatilis*, thereby contributing to mitochondrial disorders in conjunction with disrupted lipid metabolisms, such as reductions in neutral lipid contents, lower levels of MDA, a product of lipid peroxidation, and induction of cell apoptosis-associated caspase (*caspase-*

2 and *caspase-7*) signals (Ohara et al., 2004). The occurrence of rotifer sexual reproduction is an energy-intensive process that depends mostly on the availability of energy-rich sources such as lipid reserves (García-Roger and Ortells, 2018). The suppressed lipid contents and inhibited sexual reproductivity were also observed in *B. plicatilis* in response to iron and unionized-ammonia stress (Han et al., 2021; Han et al., 2022b). Additionally, the incubation temperature used in this study, 25°C, may have accelerated the metabolic rate of *B. plicatilis*, which could promote the loss of lipid under the $ZnCl_2$ stressful situation. Because the starved *B. plicatilis* was shown to increase the exhaustion of endogenous lipid for reproduction and respiration at higher temperatures (18°C), compared with at low temperatures (< 8°C) (Olsen et al., 1993). Contrastingly, in *B. rotundiformis*, we detected somewhat more moderate anti-oxidative defense responses to $ZnCl_2$ exposure, with an increment in SOD activity, despite no significant changes concerning *CAT* or *CYP3043A1* (clan 2) levels. Moreover, although exposure to 2 mg/L $ZnCl_2$ significantly induced ROS production in *B. rotundiformis*, we detected no apparent deleterious effects on reproduction (at the population level) or lipid metabolisms, thereby tending to indicate that reproduction in *B. rotundiformis* is more tolerant/resilient to the imposition of stress, which is consistent with previous findings (Tanaka et al., 2009). Furthermore, we detected an enhancement of ATP generation following exposure to $ZnCl_2$, which could provide energy consumption in *B. rotundiformis*. Additionally, using a bioinformatics tool of integrated biomarker response (IBR) analysis, we identified the biomarkers reflecting the sensitive $ZnCl_2$ responses of rotifers, which include the antioxidant-associated *GSTs2* and *MnSOD*, behavior-associated *AChE*, and lipid synthesis-associated *ACC* and *MTCYP*, as well as *cytochrome c oxidase* and *caspase-2/7* **(Fig. 2-10)**.

Furthermore, Zn is an essential element for the progression of spermatogenesis in both invertebrates and vertebrates (Clapper et al., 1985; Yamaguchi et al., 2009). Zn, functioning as a cofactor of the antioxidant enzyme CuZnSOD, contributes to protecting spermatozoa by scavenging superoxide anions, which readily attack the polyunsaturated fatty acids in sperm cells membranes (Kim and Parthasarathy, 1998). Consequently, the stress-defensive abilities of species may modify the influence of Zn on rotifer sperm viability. In this investigation, $ZnCl_2$ exposure induced inhibitory and stimulatory effects on the sperm viability of *B. plicatilis* and *B. rotundiformis*, respectively. Additionally,

chronic exposure to $ZnCl_2$ had a modulating effect on rotifer swimming behavior, with enhanced swimming speed of the males and females in both species, along with inhibited AChE enzymatic activity, a sensitive stress-behavioral indicating biomarker (Jin et al., 2019). AChE enzyme contributes to regulating the normal muscle movement and contraction of species via the muscular–acetylcholine system, and consequently, its inhibition tends to be associated with irregular behavior, such as hyperactivity (Roast et al., 2001). Given the collective observations, we can thus speculate that the enhanced swimming activity and sperm viability of *B. rotundiformis* in response to $ZnCl_2$ exposure would contribute to stimulating sexual reproductivity. The process of rotifer mating can be divided into three discrete stages, in which males initiate contact with the female, then attempt circulation, and if successful, engage in copulation and insemination (Gilbert, 1963; Hagiwara et al., 1988). Accordingly, more rapidly swimming males might have a greater likelihood of encountering females, whereas higher sperm viability would predictably enhance the probability of successful fertilization, both of which contribute to the generation of resting eggs. Conversely, the significantly suppressed sperm viability detected in *B. plicatilis* exposed to either 1 or 2 mg/L $ZnCl_2$ would presumably be reflected in inhibited sexual reproductivity.

5. Conclusions

This study employed multiple endpoints to investigate $ZnCl_2$ effects on the survival, reproduction, behavior, and mitochondrial-mediated metabolisms dynamics of two sibling species of marine rotifers and identified $ZnCl_2$ concentration-dependent reproductive sensitivity in the temperate *B. plicatilis* and tropical *B. rotundiformis* rotifers, as summarized in **Fig. 2-11**. It was established that exposure to $ZnCl_2$ affected the swimming activity in both male and female rotifers and the viability of male sperm, indirectly regulating rotifer reproductive fitness. Moreover, the inhibited/stimulated sexual reproductivity associated with reduced/constant (or enhanced) lipid storage in the two rotifer species provides evidence suggesting a mechanistic link between lipid metabolism and rotifer sexual reproduction, warranting further investigation. Finally, these findings

suggested that species-specific tolerance and response manners should be considered when applying trace elements in aquaculture operations.

Table 2-1. Median lethal concentrations (LC_{50}, mg/L) of $ZnCl_2$ to *Brachionus plicatilis* and *Brachionus rotundiformis*.

Species	24 h LC_{50}	48 h LC_{50}
B. plicatilis	29.9 (25.4–34.8)^{A}	9.0 (3.0–16.9)^{a}
B. rotundiformis	13.3 (10.5–16.4)^{B}	3.6 (2.7–4.6)^{b}

Values in parentheses are 95% confidence intervals (A > B; a > b, Student's *t*-test, $p < 0.05$, n = 30).

Table 2-2. Variations in the parameters of sexual and asexual reproduction in *Brachionus plicatilis* and *Brachionus rotundiformis* following exposure to different concentrations of $ZnCl_2$ during 7 days batch culture.

Species	Treatment (mg/L)	Population growth rate (*r*)	Mixis (%)	Fertilization (%)	Total resting eggs
B. plicatilis	0	0.68 ± 0.01^{B}	21.2 ± 3.3^{A}	12.4 ± 2.8^{A}	149.2 ± 53.7^{A}
	1	0.70 ± 0.00^{A}	24.8 ± 2.9^{A}	5.6 ± 1.9^{B}	80.9 ± 41.5^{AB}
	2	0.68 ± 0.00^{B}	11.9 ± 1.7^{B}	4.6 ± 0.4^{B}	12.9 ± 13.6^{B}
B. rotundiformis	0	0.81 ± 0.02	23.4 ± 2.1^{b}	26.8 ± 3.3	2564.2 ± 318.3^{b}
	1	0.81 ± 0.00	31.3 ± 2.7^{a}	26.3 ± 6.3	3543.8 ± 457.8^{a}
	2	0.80 ± 0.01	30.9 ± 1.3^{a}	23.3 ± 2.3	2694.7 ± 97.3^{b}

Data are presented as the means ± SD of three replicates. Different letters indicate significant differences among treatments (A > B, a > b, Tukey HSD test, $p < 0.05$, n = 3).

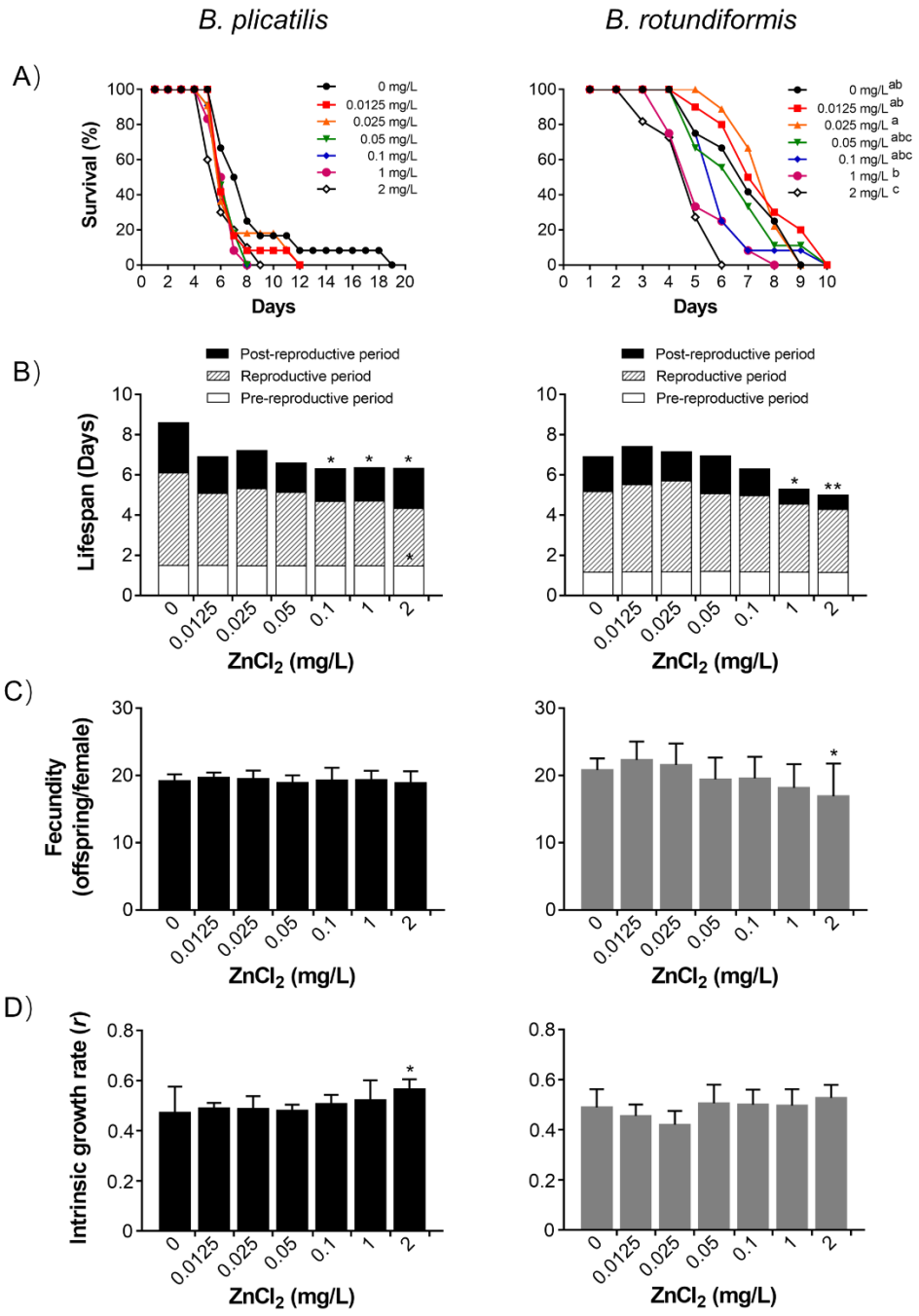

Fig. 2-1. (A) Age-specific survival rate (%), (B) lifespan (pre-reproductive, reproductive, and post-reproductive periods), (C) fecundity, and (D) intrinsic growth rate (*r*) of *Brachionus plicatilis* and *Brachionus rotundiformis* following exposure to different concentrations of $ZnCl_2$ (a > b > c, log-rank test, $p < 0.05$; *, ** indicate $p < 0.05$, 0.01, respectively, Dunnett's test, n = 12).

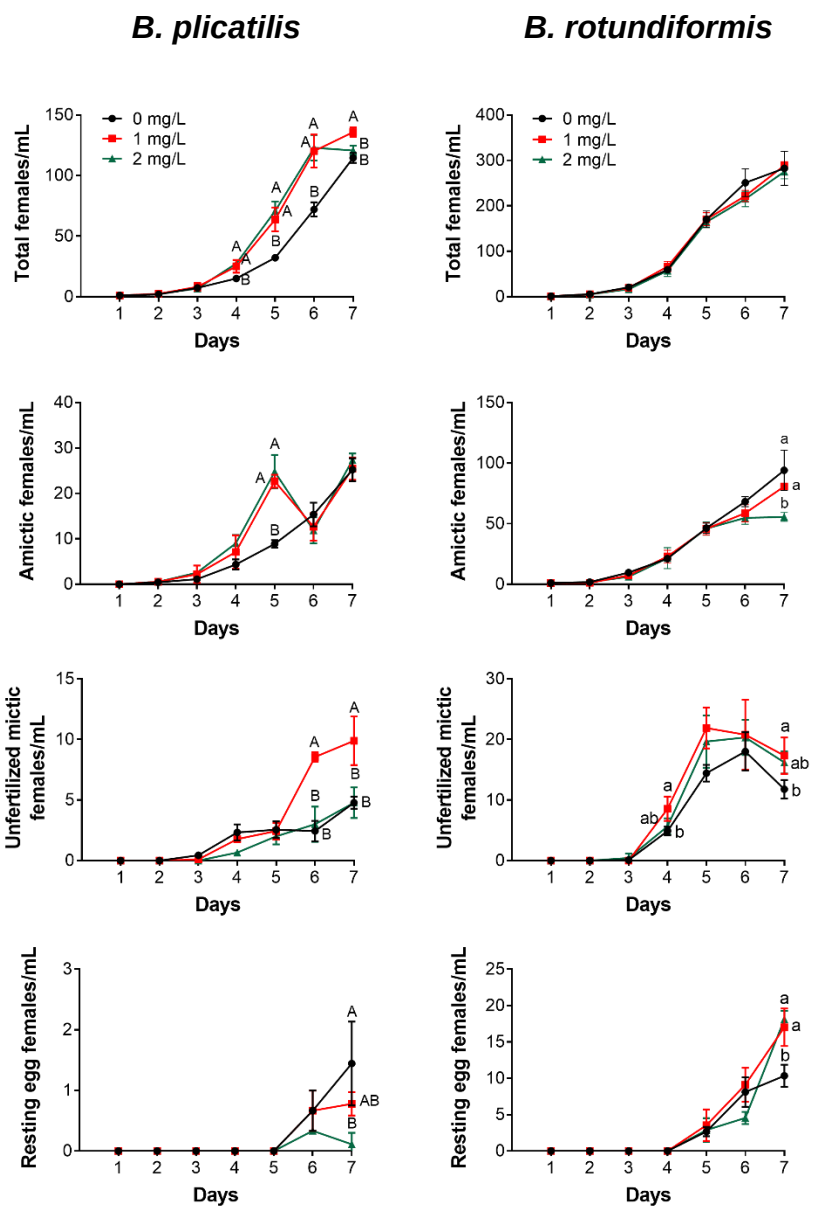

Fig. 2-2. Population growth of total females, the amictic female, and two types of mictic females (unfertilized and resting egg carrying females) of *Brachionus plicatilis* and *Brachionus rotundiformis* exposed to different concentrations of $ZnCl_2$ during 7 days batch culture (A > B > C, a > b > c, Tukey HSD test, $p < 0.05$, n = 3).

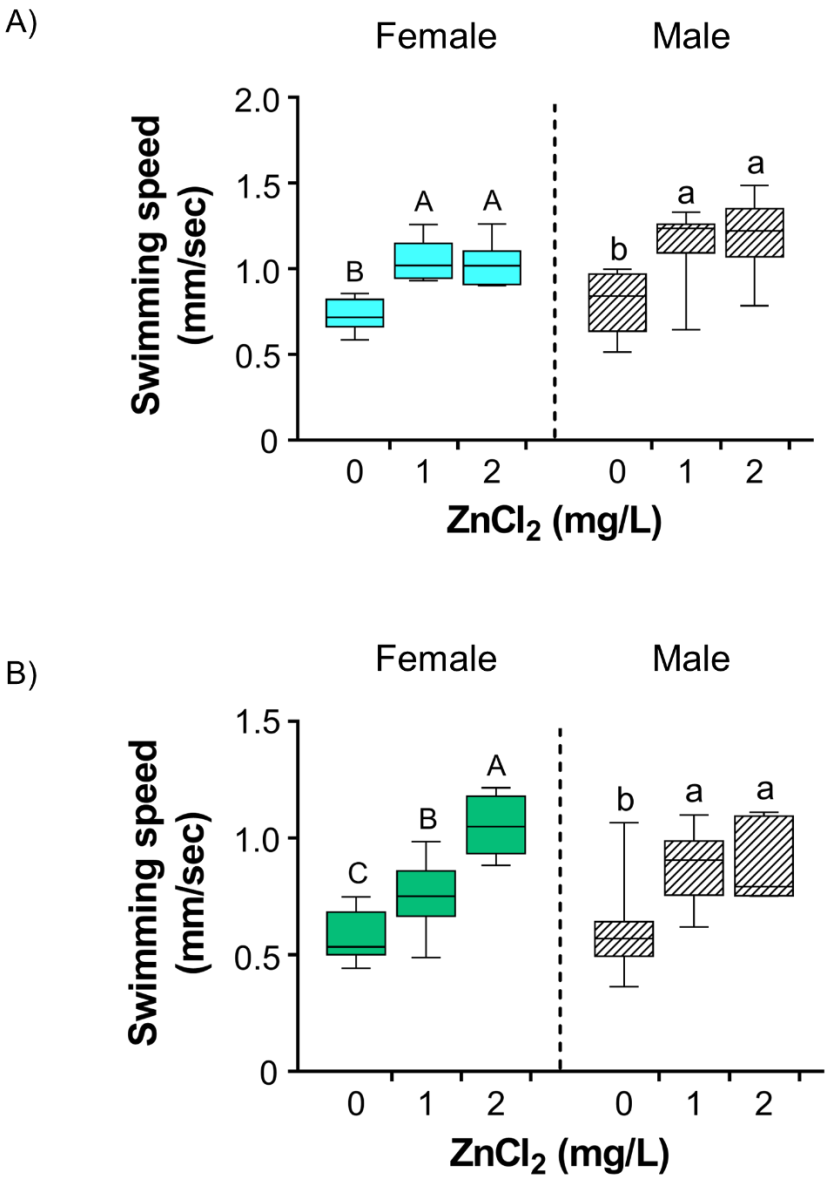

Fig. 2-3. Variation in the swimming speeds in male and female (A) *Brachionus plicatilis* and (B) *Brachionus rotundiformis* following exposure to different concentrations of $ZnCl_2$ [0 (control), 1, and 2 mg/L] (A > B, a > b > c, Tukey HSD test, $p < 0.05$, n = 6–10).

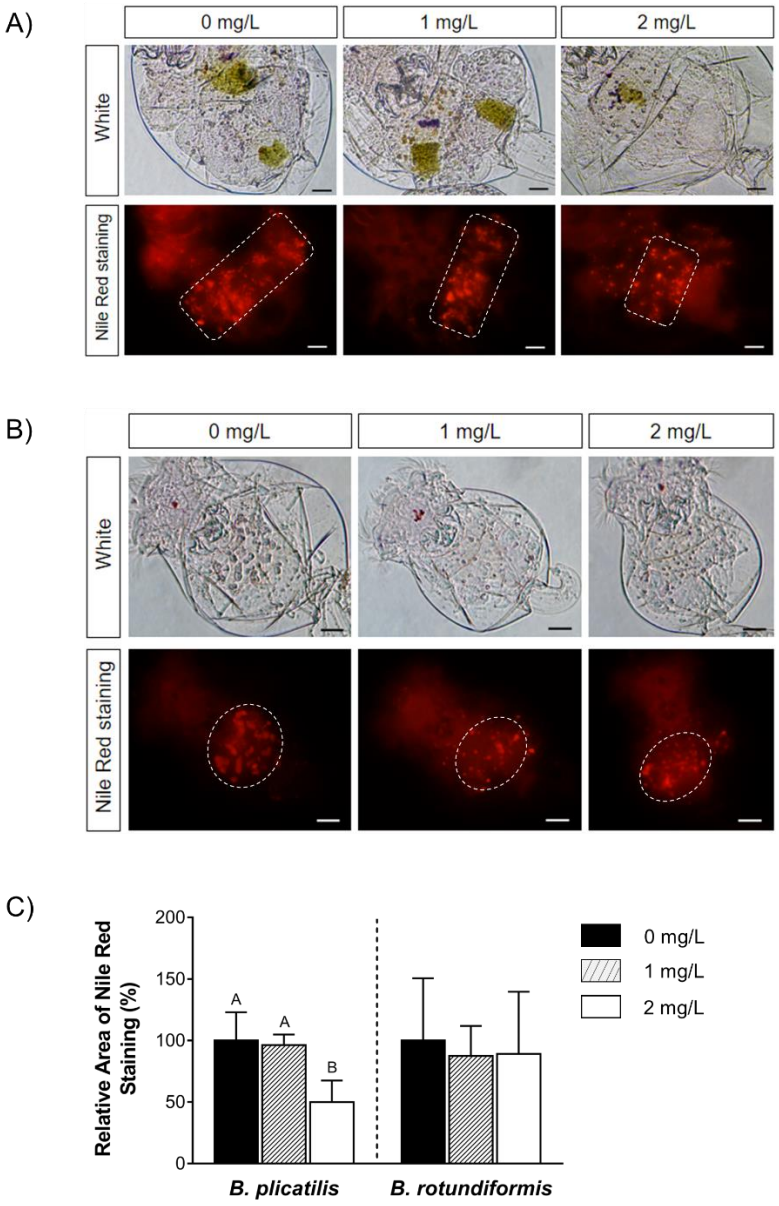

Fig. 2-4. Accumulation of lipid droplets (detected based on Nile Red staining) in the reproductive organs of (A) *Brachionus plicatilis* and (B) *Brachionus rotundiformis* following chronic exposure to $ZnCl_2$ [0 (control), 1, and 2 mg/L] during 7 days batch culture. All scale bars represent 25 μm. (C) Measurements are the relative area of lipid contents in the two rotifer species (A > B, Tukey HSD test, $p < 0.05$, n = 10–15).

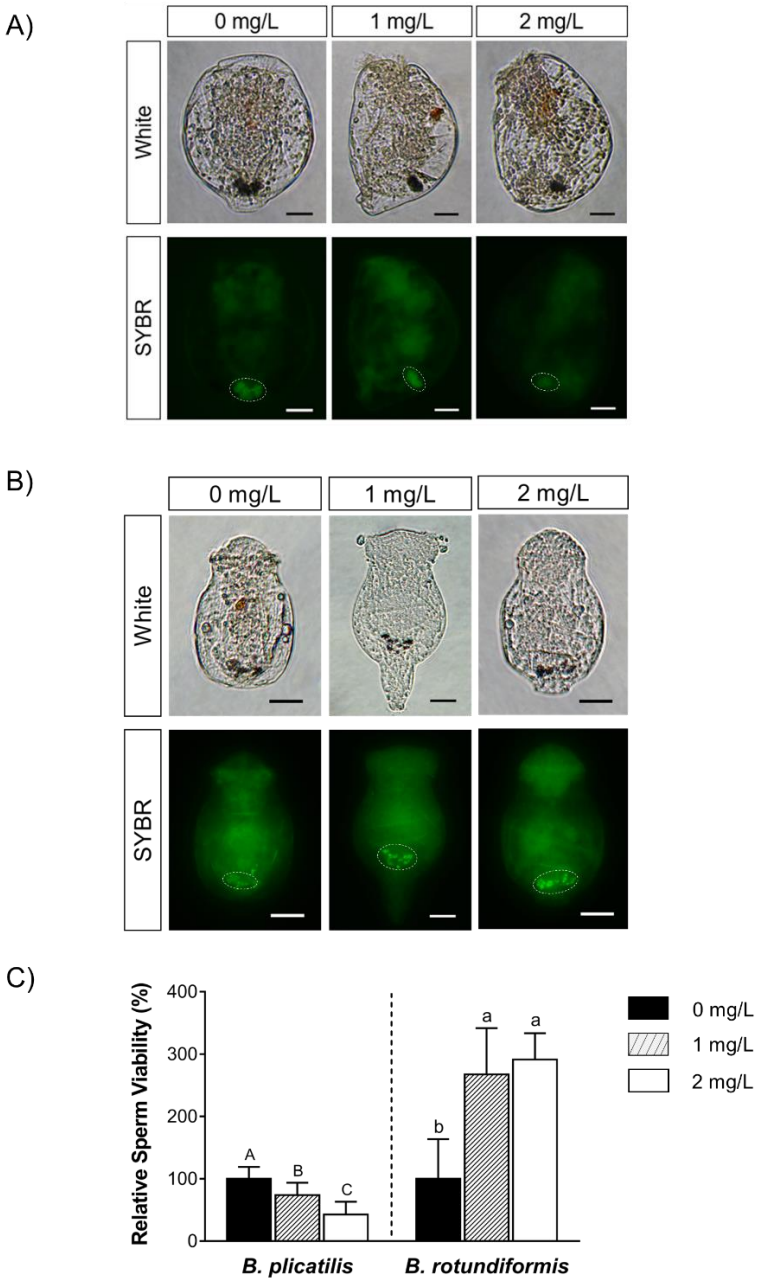

Fig. 2-5. Stained living spermatozoa of males of the rotifer species (A) *Brachionus plicatilis* and (B) *Brachionus rotundiformis* following exposure to different concentrations of $ZnCl_2$. (C) Differences in sperm viability were determined based on relative fluorescence intensity (A > B > C, a > b, Tukey HSD test, $p < 0.05$, n = 10–15).

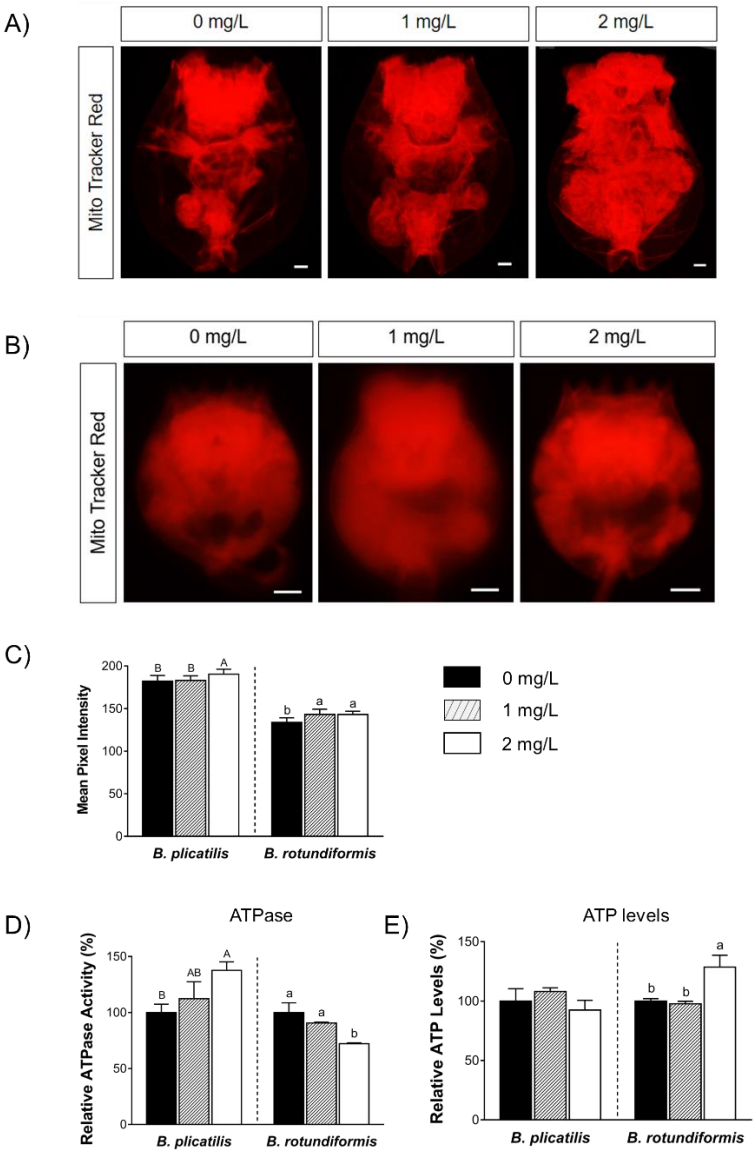

Fig. 2-6. Mitochondrial membrane potentials determined by MitoTracker Red staining in (A) *Brachionus plicatilis* and (B) *Brachionus rotundiformis* following chronic exposure to $ZnCl_2$ [0 (control), 1, and 2 mg/L] during 7 days batch culture. All scale bars represent 25 μm. (C) The average fluorescence intensity in the two rotifer species in response to the different $ZnCl_2$ treatments (A > B, Tukey HSD test, $p < 0.05$, n = 10). Changes in (D) ATPase enzymatic activity and (E) ATP levels in the two rotifer species in response to chronic exposure to $ZnCl_2$ [0 (control), 1, and 2 mg/L] during 7 days batch culture (A > B, a > b, Tukey HSD test, $p < 0.05$, n = 3).

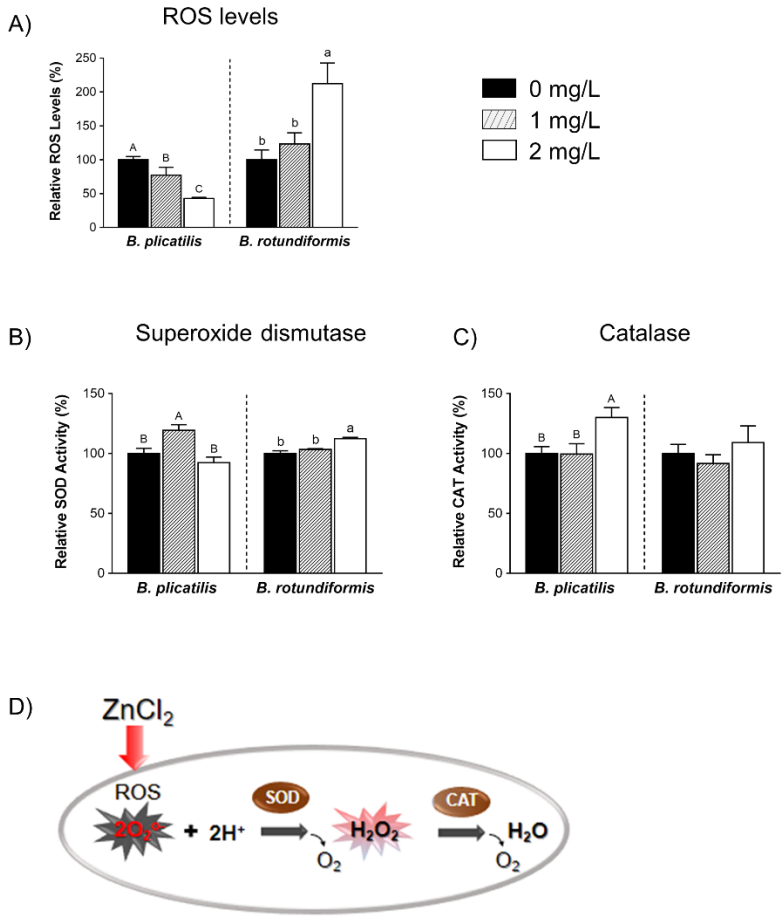

Fig. 2-7. (A) Intracellular reactive oxygen species (ROS) levels, and enzymatic activities of (B) superoxide dismutase and (C) catalase in *Brachionus plicatilis* and *Brachionus rotundiformis* following chronic exposure to $ZnCl_2$ [0 (control), 1, and 2 mg/L] during 7 days batch culture (A > B > C, a > b > c, Tukey HSD test, $p < 0.05$, n = 3). (D) A schematic diagram showing antioxidant defense systems and generation of ROS in rotifers in response to $ZnCl_2$ exposure.

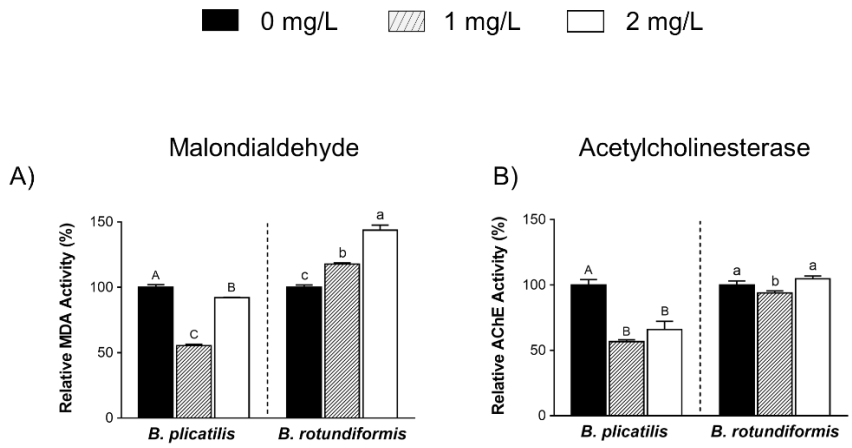

Fig. 2-8. (A) Levels of malondialdehyde (MDA) and (B) enzymic activity of acetylcholinesterase (AChE) in *Brachionus plicatilis* and *Brachionus rotundiformis* following chronic exposure to $ZnCl_2$ [0 (control), 1, and 2 mg/L] during 7 days batch culture (A > B > C, a > b > c, Tukey HSD test, $p < 0.05$, n = 3).

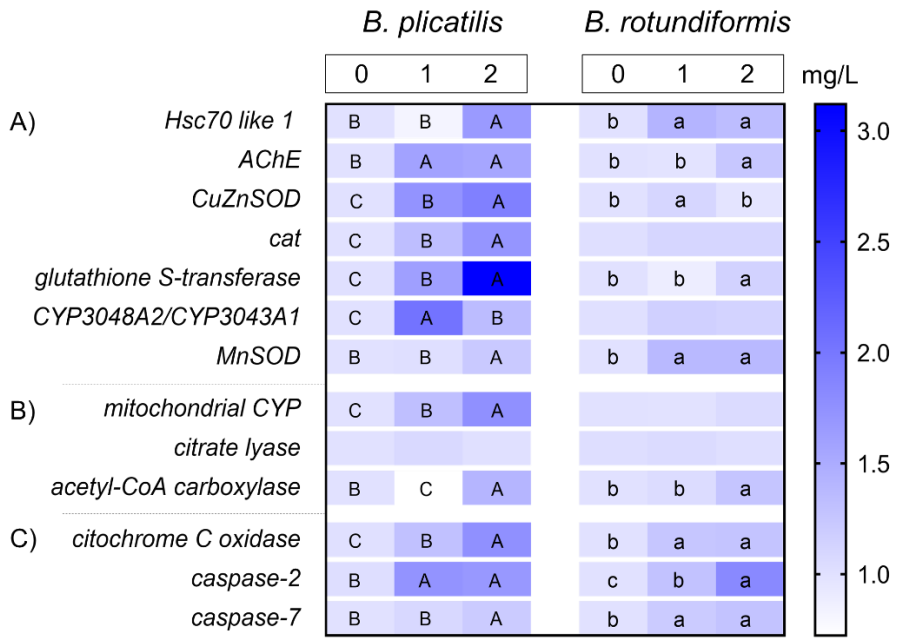

Fig. 2-9. mRNA expression of biomarkers associated with (A) stress responses, (B) lipid synthesis, and (C) mitochondrial respiratory activity in *Brachionus plicatilis* and *Brachionus rotundiformis* following chronic exposure to $ZnCl_2$ [0 (control), 1, and 2 mg/L] during 7 days batch culture (A > B > C, a > b > c, Tukey HSD test, $p < 0.05$, n = 3).

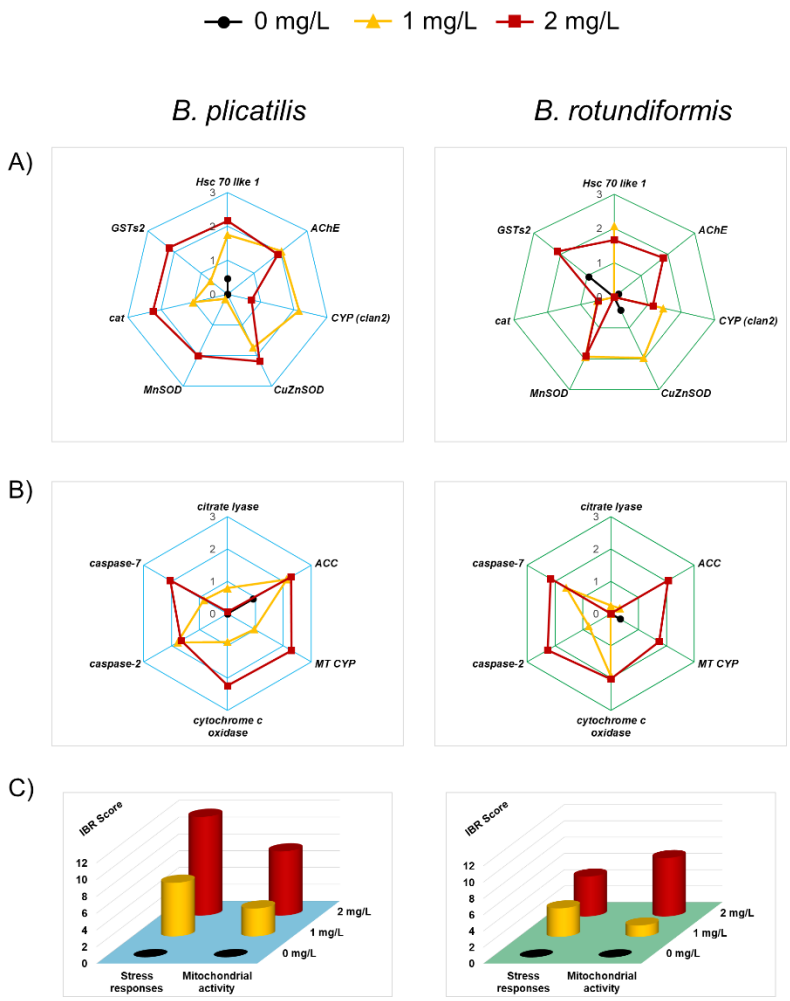

Fig. 2-10. Integrated biomarker response (IBR) star plot for (A) stress response-associated biomarkers, (B) mitochondrial activity-associated biomarkers, and (C) IBR scores of biomarkers in these two functional groups in *Brachionus plicatilis* and *Brachionus rotundiformis* following exposure to different concentrations of $ZnCl_2$ [0 (control), 1, and 2 mg/L].

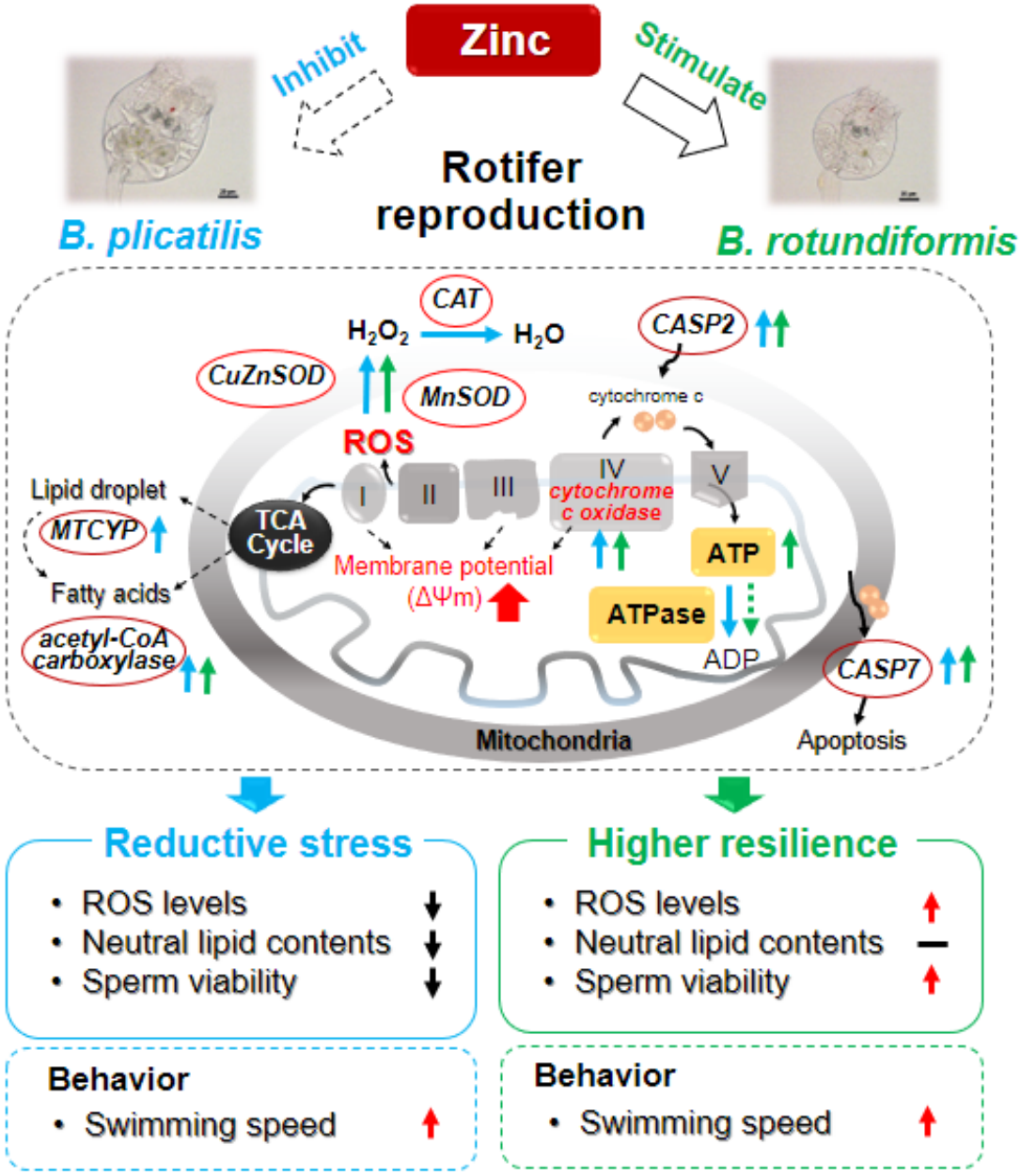

Fig. 2-11. Summary of the effects of $ZnCl_2$ on the reproduction and mitochondrial respiratory activities in two rotifer species. Blue and green represent *Brachionus plicatilis* and *Brachionus rotundiformis*, respectively. The solid and dotted (blue/green) arrowed lines denote upregulated and downregulated gene expression, respectively.

Chapter IV

Species-specific ammonia tolerance in the marine rotifers *Brachionus plicatilis* and *Brachionus rotundiformis*: reproductive characteristics and its mechanisms

Han et al., 2022. Aquaculture, 550, 737837

1. Introduction

Marine rotifer *Brachionus plicatilis* species complex is commonly used as a live food source for target fish larvae in aquaculture facilities. The feeding regime of fish larvae is established based on the different morphotypes of rotifers with the lorica size distribution of SS (super small), S (small), and L (large) types, because of the size-selective predation of larvae (Hagiwara et al., 1995b). In general, these three morphotypes exhibit different physiological characteristics with habitat adaptation. For example, L-type *B. plicatilis* is mainly distributed in temperate waters with higher performance at comparatively lower temperatures (< 20°C). The SS-type *B. rotundiformis* is found in tropical areas and performs actively at higher temperatures (> 30°C) (Gabaldón et al., 2017; Hagiwara et al., 2001). During a mass culture, tropical *B. rotundiformis* reaches a maximum density of 12000 ind./mL at 30°C, whereas the temperate species *B. plicatilis* is 680 ind./mL at 20°C (Balompapueng et al., 1997; Hagiwara et al., 1993; Yoshimura et al., 1996). Moreover, *B. rotundiformis* produces more biomass with an average of 65.96 mg of dry weight (DW)/mL/day than *B. plicatilis* (53.63 mg DW/mL/day) at the same culture temperature of 25°C (James and Abu-Rezeq, 1989).

The maximum number of rotifer individuals is significantly affected by multiple external factors in mass cultures. Among these factors, unionized ammonia (NH_3-N) is a major factor to regulate rotifer density (Timmons et al., 2002; Yu and Hirayama, 1986). Ammonia is a primary metabolite of nitrogen-containing substances, unconsumed feed, and feces in aquatic water (Prenter et al., 2004). Therefore, the concentration of ammonia increases with the growth of rotifer population. It was reported that NH_3-N concentrations were 1.0–8.0 mg/L and 0.2–1.2 mg/L when the mass culture of *B. rotundiformis* and *B. plicatilis* reached the above-mentioned maximum density, respectively (Balompapueng et

al., 1997; Yoshimura et al., 1996). The toxicity of ammonia has been attributed mostly to the unionized form, which shows high cell membrane permeability and occurs in equilibrium with nontoxic or appreciably less toxic ionized ammonia (NH_4^+-N) (Thurston et al., 1981). NH_3-N levels that exceed the tolerance limits of aquatic animals can induce physiological malfunctions, such as growth retardation, nervous disorder, and respiratory impairment (Eddy, 2005), and indirect damage via reactive oxygen species (ROS) stress (Ching et al., 2009). For example, high NH_3-N (> 2.3 mg/L) disrupts the immune defense system and stimulates the energy supply in the marine shrimp *Litopenaeus vannamei* (Xiao et al., 2019). Exposure to 0.5–1.3 mg/L NH_3-N induced oxidative stress in the freshwater clam *Corbicula fluminea*, resulting in DNA damage and cell apoptosis (Zhang et al., 2020). Several studies have examined ammonia toxicity in rotifer species (De Araujo et al., 2000, 2001; Gao et al., 2021; Schlüter and Groeneweg, 1985; Yu and Hirayama, 1986). NH_3-N levels ranging from 2.4 to 9.8 mg/L were reported to inhibit the reproduction of *B. plicatilis* and *B. rotundiformis*, leading to decreases in fecundity and glucosidase activity (De Araujo et al., 2000, 2001). *B. rotundiformis* batch cultured at 12.2 mg/L NH_3-N displayed oxidative damage that included a high level of lipid peroxidation and inhibited antioxidant activities (Gao et al., 2021). However, the mechanisms of ammonia effects on reproduction, and swimming behavior among rotifer sibling species dwelling in various habitats have not been clarified. NH_3-N toxicity is sensitive to temperature, and its toxicity increases with rising temperature (Kir et al., 2004; Miller et al., 1990). Thus, the tropical and temperate rotifers may experience different levels of habitat-resource NH_3-N stress, which may affect their reproduction adaptations. Based on these issues, it is hypothesized that tropical and temperate rotifer species may have different reproductive responses and adaptive strategies associated with NH_3-N levels.

To determine the ammonia endurance and the sensitive biomarkers of ammonia stress in the tropical and temperate rotifer species, the following three experiments were conducted with acute (lethal) and chronic (sub-lethal) doses of unionized ammonia exposure. (1) the first experiment assessed acute exposure by determining the 24 h median lethal concentration (LC_{50}) of NH_3-N for *B. plicatilis* and *B. rotundiformis*. (2) in the second experiment, the effects of chronic NH_3-N toxicity at individual levels (survival, lifespan, fecundity, and intrinsic growth rate) and population levels (population growth rate,

mixis, fecundity, and resting egg production) were examined. (3) in the third experiment, the mechanisms were explored at biochemical and behavioral levels by examining ROS, lipid metabolism, and swimming speed modifications. A suite of oxidative stress (e.g., *NADPH oxidase 5*, *AChE*, *superoxide dismutase*, *catalase*, and *cytochrome P450*), lipid synthesis (e.g., *citrate lyase*, *acetyl-CoA carboxylase*), and rotifer reproduction (e.g., *histone H_2A*, *vasa*) related biomarkers, as well as associated antioxidant enzymatic activities were measured. Finally, an integrated biomarker response (IBR) analysis (Beliaeff and Burgeot, 2002) was conducted to evaluate which functional group of biomarkers showed the strongest reactivity to NH_3-N exposure and to identify sensitive biomarkers in rotifers in response to NH_3-N stress.

2. Materials and methods

2.1. Chemicals

Ammonium chloride (NH_4Cl, purity > 99.5%; Wako, Osaka, Japan) was the source of ammonia. The stock ammonia solution of 1100 mg/L NH_4Cl was prepared by dissolving 2.1 g NH_4Cl in 500 mL of 17 parts per thousand (ppt) filtered and autoclaved seawater immediately before testing, which was further diluted in pre-prepared seawater to obtain a working solution at designed concentrations for testing. The pH of the whole test solution was adjusted to 8.0 using HCl and NaOH. The concentrations of total ammonia were measured using a model AT-2000 Quick Ammonia meter (Central Kagaku Corp., Tokyo, Japan) according to standard laboratory instructions (**Table 3-1**). Concentrations of the toxic NH_3-N fraction were derived from the nominal NH_4^+ values, pH, and temperature by the equilibrium constants (Emerson et al., 1975) as follows:

$$\text{NH3 ratio (\%)} = \frac{100}{\left[10^{(\text{pKa}-\text{pH})}+1\right]} \times 100$$

where, pKa=0.09018+2729.92/T, T=25°C, and pH=8.0 were used. All test results are described in the NH_3-N form.

2.2. Test species

The two rotifer species employed were temperate *B. plicatilis* (NH17L strain; mean lorica length 275 μm, mean dry weight 495 ng/ind.) (Suga et al., 2011) and tropical *B. rotundiformis* (Kochi strain; lorica length 179 μm, dry weight 220 ng/ind.) (Kim et al., 2014; Yúfera and Parra, 1997) have been maintained at the Aquaculture Biology Laboratory, Nagasaki University, Japan, for several decades. Rotifers were cultured in 17 ppt seawater at 25°C in complete darkness and were fed with the microalgae *Nannochloropsis oculata* (7.0×10^6 cells/mL). Neonates (< 2 h) hatched from amictic eggs were used for all tests. To obtain enough neonates, approximately 10,000 female rotifers carrying amictic eggs from stock culture were inoculated into a screw-capped vial containing 10 mL of culture medium and then agitated to shake off the eggs. Separated eggs were incubated in a well of a 6-well microplate with 5 mL seawater at 25°C and observed hourly.

2.3. Acute toxicity tests

The following concentrations of NH_3-N were selected based on the results of pilot range-finding tests: 0, 13.3, 26.6, 39.9, 53.2, and 79.8 mg/L for *B. plicatilis*, and 0, 13.3, 26.6, 39.9, and 53.2 mg/L for *B. rotundiformis*, respectively. Ten rotifer neonates were inoculated into a well of a 6-well microplate with 5 mL of working solution. Three replicates were performed for each concentration. The experimental conditions were identical to those employed in the stock culture, except that no food was provided during the observation period. After 24 h of exposure, the number of surviving and dead rotifers with no movement of cilia and mastax for more than 30 s were counted using a model SZ-STS stereomicroscope at 60× magnification (Olympus, Tokyo, Japan).

2.4. Chronic toxicity tests

2.4.1. Life history parameters

To compare ammonia responses between the two rotifer species, they were

individually cultured under the same NH_3-N concentrations: 0, 1.1, 2.1, 4.3, 8.5, 17.0, and 29.3 mg/L based on the preliminary tests. Twenty-four neonates (< 2 h) were individually inoculated into a well of a 24-well microplate containing 1 mL test solution with 7.0×10^6 cells/mL of *N. oculata* and then incubated at 25°C in total darkness. After 15 h, the plates were checked hourly to monitor life history parameters that included the time for bearing the first egg, and the time for producing the first offspring. These rotifers were further checked every 12 h to monitor the survival of the inoculated rotifers (maternal individuals) and the number of offspring. The maternal rotifers were transferred daily to a new test solution. This process was repeated until all maternal individuals died. With these observations, the age-specific survival rate which is the proportion surviving at each interval was estimated by the following equation defined in Maia et al. (2000).

Age-specific survival rate (%): $[\frac{\text{Number surviving at each age}}{\text{The initial total individuals}}] \times 100$

Moreover, the following lifetable parameters were recorded as described in Marcial and Hagiwara (2007): generation time is the time for producing the first offspring. Lifespan is the average survival time of all tested individuals. Fecundity is the average number of offspring produced by each female during its lifetime. The intrinsic growth rate (*r*) was calculated using the Lotka equation (Lotka, 1907):

$$\sum lxmxe^{-rx} = 1$$

where, *lx* denotes the likelihood of surviving rotifers at age *x* and m*x* denotes the number of offspring generated by a single parent at age *x*.

2.4.2. Reproductive parameters

The applied ammonia concentrations were the same as described above for the individual culture experiment. However, a low concentration of 0.5 mg/L NH_3-N was introduced, as the preliminary testing revealed that the sexual reproduction of *B. plicatilis* was highly sensitive to ammonia in batch cultures. Forty neonates (< 2 h) were inoculated into 50 mL screw-capped bottles with 40 mL test solution and incubated in complete darkness at 25°C for 7 days with daily feeding of *N. oculata* (7.0×10^6 cells/mL). Four

types of female rotifers (Hagiwara et al., 1988), non-ovigerous females (?F), amictic females bearing large female eggs (FF), unfertilized mictic females bearing small and numerous male eggs (MF), and resting egg females bearing yellowish eggs (RF) were recorded every 24 h during the culture period. The following reproductive parameters were estimated using the recorded data: rotifer population growth rate (r) for asexual reproduction, mixis rate (%), fertilization rate (%), and resting egg production for sexual reproduction. The whole test solution was renewed every 2 days and the ammonia concentration in each treatment was measured according to standard protocols before the replacement of the solution. All tests were performed in triplicate. Reproductive parameters were calculated using the following equations.

Population growth rate (r) was calculated as: $r = (ln\ Nt\text{-}N0)/t$
where, t represents the culture day, Nt is the number of female rotifers on day t, and $N0$ is the number of female rotifers on day 0.

Mixis (%): $\left[\frac{MF+RF}{FF+MF+RF}\right] \times 100$

Fertilization (%): $\left[\frac{RF}{MF+RF}\right] \times 100$

2.5. Assessments of mechanisms

Three levels of NH_3-N (0, 1.1, and 8.5 mg/L) were established. Six hundred milliliters of each concentration of culture medium were added to a 1000 mL screw-capped bottle. Neonates (< 2 h, n = 600) were inoculated into each bottle. These individuals were incubated under the same culture conditions as those used in the above reproductive tests. The samples were collected for further analysis after 7 days of culture.

2.5.1. Swimming behavior

Twenty female rotifers bearing one amictic egg each were sampled from each ammonia treatment on the last day of culture (day 7). The selected rotifers were transferred into a glass-bottom dish (14 mm diameter, 1.5 mm thick) (Matsunami, Osaka, Japan)

containing 200 μL of the test solution. Their swimming activity was recorded for 20 s using a model HAS-UZ stereomicroscope (Olympus) equipped with a digital camera (HAS-X Viewer Ver. 12.1201; DITECT, Tokyo, Japan). Dipp Motion Pro Ver. 1.1.31 tracking software (DITECT) was used to analyze swimming speed (mm/s).

2.5.2. Accumulation of neutral lipid droplets

Nile red (Sigma-Aldrich, St. Louis, MO, USA) staining was used to detect the content of neutral lipid droplets in the reproductive organs of rotifers after 7 days of ammonia exposure. A stock solution (1 mg/mL) was prepared by dissolving Nile red powder in acetone. Thirty female rotifers carrying one or two amictic eggs from each treatment were sampled and transferred to 6-well microplates containing 5 mL seawater and Nile red (final concentration 2.5 μg/mL) per well, stained for 5 min at 25°C, and then fixed with 10% formalin. The stained rotifers were examined using a model Ts2-FL fluorescence microscope (Nikon, Tokyo, Japan) at an excitation/emission wavelength of 550/630 nm. The fluorescent signal emitted by neutral lipids was quantified using NIS-Elements F version 4.60 image analysis software. The analyzed data were presented as the area (%) relative to the control.

2.5.3. Intracellular ROS levels and enzymatic/biomarkers activities

The intracellular ROS levels in rotifers exposed to ammonia were measured using a fluorogenic probe (2',7'-dichlorofluorescein diacetate, H2DCFDA; Sigma-Aldrich). This probe is oxidized by ROS to produce fluorescent 2',7'-dichlorofluorescein (DCF). Fluorescence was captured using a Cytation™ 3 multimode plate reader (BioTek Instruments, Winooski, VT, USA) with excitation and emission wavelengths of 485 and 520 nm, respectively.

Four enzymatic activities were detected after ammonia exposure. Malondialdehyde (MDA) enzyme activity was measured via a colorimetric signal produced by the reaction of MDA and thiobarbituric acid. Absorbance was determined at an optical density of 532 nm. AChE is involved in the process of acetylthiocholine hydrolysis to produce thiocholine.

The activity of the enzyme was quantified by 5,5-dithiobis (2-nitrobenzoic acid) at an absorbance of 410 nm. SOD catalyzes the dismutation of the superoxide anion into hydrogen peroxide (H_2O_2), which can react with WST-1 (2-[4-Iodophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium, monosodium salt) to produce a visible formazan dye evident at an absorbance of 440 nm. SOD activity and formazan quantity are inversely proportional; the higher the SOD activity, the less formazan is synthesized. CAT activity was measured by a CAT–H_2O_2–probe reaction in which CAT present in the samples reacted with H_2O_2 to form H_2O and O_2, whereas unconverted H_2O_2 reacted with the probe to produce a colorimetric signal at 570 nm. All enzyme activities were determined using a commercial enzyme kit according to standard laboratory procedures.

The associated molecular biomarkers involved in oxidative/antioxidant activity, lipid metabolism, and rotifer reproduction were measured. To extract RNA, approximately 6000 rotifers were sampled and TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) was used according to the manufacturer's instructions. The extracted RNA was treated with a TURBO DNA-free™ kit (Ambion, Carlsbad, CA, USA) to remove genomic DNA. The quality and quantity of RNA were checked spectrophotometrically at 230, 260, and 280 nm using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, complementary DNA (cDNA) was synthesized using the PrimeScript™ II 1st strand cDNA Synthesis Kit (TaKaRa Bio, Shiga, Japan) and was preserved at -20°C until use. Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) was performed using a 1 μL cDNA template, 0.5 μL forward and reverse primers (10 μM), and 10 μL TB Green Premix Ex Taq (2×) (TaKaRa Bio) in a total volume of 20 μL. The assay was performed using a LightCycler® 96 real-time PCR system (Roche Life Science, Basel, Switzerland) at 94°C for 4 min, followed by 43 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Melting curve cycles were performed under the following conditions to validate the amplification of specific products: 95°C for 10 s, 55°C for 1 min, and 80 cycles at 55°C for 10 s, with a 0.5°C increase per cycle. Two housekeeping genes (*elongation factor 1-α* [*EF1-α*] and *18S rRNA*) were examined in a pilot test. The *18S rRNA* gene was chosen as an internal control because of its high constant expression among and within the experimental groups. The transcriptional levels were

calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). All tests were performed in triplicate.

2.6. Integrated Biomarker Response (IBR)

IBR was determined as previously described (Beliaeff and Burgeot, 2002; Devin et al., 2013). Briefly, the general mean (m) and the standard deviation (s) of mRNA levels of target biomarkers in each functional group were computed. Each treatment was standardized to obtain Y, where $Y = \frac{X-m}{s}$, and X is the mean value for the biomarker at a given concentration. Then, Z was computed using $Z = -Y$ or $Z = Y$, which corresponds to a biological effect of inhibition or stimulation, respectively. The score (S) was determined by $S = Z + |Min|$, where $S \geq 0$ and $|Min|$ is the absolute value of the minimum value for all calculated Y of a given biomarker for all treatments. Star plots were then used to display score results (S) and to calculate the IBR score: $Ai = Si \times Si + 1 \times \sin(\frac{2\pi}{n})/2$, $IBR = \sum_{i=1}^{n} Ai$, where n denotes the number of biomarkers. IBR was calculated and plotted using Excel software (Office Professional Plus 2016; Microsoft, Redmond, WA, USA).

2.7. Statistical analyses

The Bartlett test of variance homogeneity was used to test the normality of the data. The log-rank test was used to compare survival curves. For the life history and reproductive parameters data, a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test was used for significant difference analysis. For the analysis of ROS levels, enzymatic/biomarker activity, swimming speed, and neutral lipid content, one-way ANOVA followed by Tukey's honest significant difference (HSD) test were used. Results are expressed as the mean ± standard error. All statistical computations were performed using R version 3.6.3 (https://cran.r-project.org/bin/windows/base/old/3.6.3/).

Probit analysis was applied to determine the lethal concentrations (LCs) and 95% confidence intervals by fitting a log dose-response curve to a line regression and estimating the models using maximum likelihood estimation (Sakuma, 1998).

The regression-based effective concentrations (EC_{10}, $EC_{20,}$ and EC_{50}) were used to summarize the chronic toxicity effects (Beasley et al., 2015), and were calculated using KaleidaGraph V4.1.1 software (Synergy Software Inc., Pennsylvania, USA) by sigmoid dose-response regression. For the acute to chronic ratio (ACR) parameter, LC_{50} was the acute endpoint, the EC_{10} (considered as no observed effect concentration, NOEC) and EC_{20} (the lowest observed effective concentration, LOEC) of the rotifer intrinsic growth rate (*r*) were the chronic endpoints. ACR was calculated as follows (US EPA, 1999):

$MATC = \sqrt{NOEC \times LOEC}$, (MATC: maximum acceptable toxicant concentration)

$$ACR = \frac{LC50}{MATC}$$

3. Results

3.1. Twenty-four-hour LCs and Acute to Chronic Ratio (ACR)

The computed acute toxicity parameters are listed in **Table 3-2**. The 24 h LC_{50} of NH_3-N (mg/L per ng of dry weight) was 0.08 and 0.12 for *B. plicatilis* and *B. rotundiformis*, respectively. The ACR of NH_3-N to *B. plicatilis* and *B. rotundiformis* was 15.66 and 58.53, respectively.

3.2. Life history parameters responsive to ammonia exposure and effective concentrations

The life history parameters for each NH_3-N treatment of the two rotifer species are shown in **Fig. 3-1**. In *B. plicatilis*, the survival rate (%) was not affected by NH_3-N treatments. For *B. rotundiformis*, there was no significant difference in survival rate between 0–17.0 mg/L NH_3-N, while 29.3 mg/L NH_3-N a significant difference ($p < 0.05$) from other treatments. Mortality was greatest (25%) on day 1. Higher ammonia concentrations significantly delayed the time for the first offspring (generation time) and modulated the reproductive period in two rotifer species, resulting in a reduction in fecundity and an inhibition of the intrinsic growth rate (*r*). For *B. plicatilis*, the generation

time, reproductive period, and fecundity of control rotifer were 1.7±0.04 days, 5.8±0.4 days, and 21.5±1.7 offspring/female, respectively. When the NH_3-N concentration reached 17.0 mg/L, the respective parameters were 2.0±0.02 days, 6.7±0.5 days, and 17.0±1.4 offspring/female. As well, the intrinsic growth rate (r) decreased significantly from 0.42±0.02 (control) to 0.32±0.02. For *B. rotundiformis*, the generation time, reproductive period, and fecundity of control rotifer were 1.2±0.01 days, 3.6±0.2 days, and 20.1±0.7 offspring/female, respectively. A significant delay in generation time and a reduction in reproductive period and fecundity were observed at NH_3-N > 8.5 mg/L. The intrinsic growth rate (*r*) was significantly inhibited at NH_3-N > 2.1 mg/L.

3.3. Reproductive parameters under ammonia exposure

The reproductive parameters under various concentrations of NH_3-N are shown in **Table 3-2**. The two rotifer species showed different NH_3-N tolerance in sexual reproductive parameters. For *B. plicatilis*, fertilization and resting egg production were significantly inhibited at 0.5 mg/L NH_3-N. For *B. rotundiformis*, significant inhibition of fertilization and resting egg numbers were observed at NH_3-N > 8.5 mg/L and > 1.1 mg/L, respectively. The population growth rate (asexual reproductive parameter) of the two rotifer species was significantly inhibited at NH_3-N ≥ 17.0 mg/L.

3.4. Swimming speed

NH_3-N induced a concentration-dependent increase in the swimming speed of *B. plicatilis* and *B. rotundiformis* at the concentrations tested (**Fig. 3-2**). In *B. plicatilis*, the swimming speed increased 1.2- and 1.5-fold ($p < 0.05$) at 1.1 and 8.5 mg/L NH_3-N, respectively, compared with that of control. In *B. rotundiformis*, 1.2- and 1.4-fold increase ($p < 0.05$) was observed at the respective levels of NH_3-N.

3.5. Neutral lipid droplets

NH_3-N induced different patterns of lipid accumulation in the reproductive organs of *B. plicatilis* and *B. rotundiformis* (**Fig. 3-3**). High NH_3-N (8.5 mg/L) induced 1.5-fold ($p < 0.05$) reduction in neutral lipid content in *B. plicatilis*. However, no significant difference in NH_3-N treatments (1.1 and 8.5 mg/L) was detected in *B. rotundiformis*

3.6. Intracellular ROS levels and enzymatic activities

NH_3-N exposure reduced ROS levels in both rotifer species (**Fig. 3-4 A**). In *B. plicatilis*, a 1.3-fold reduction ($p < 0.05$) was detected at 8.5 mg/L NH_3-N compared with the control. For *B. rotundiformis*, 1.3- and 4.7-fold reduction ($p < 0.05$) was induced by 1.1 and 8.5 mg/L NH_3-N, respectively, compared with control.

The patterns of oxidative and antioxidant enzymatic activities under different NH_3-N exposure are shown in **Fig. 3-4 B** and **C**. NH_3-N (8.5 mg/L) induced different patterns of MDA activity in two rotifer species. An insignificant increase ($p = 0.051$) was observed in *B. plicatilis*. A significant 1.4-fold decrease ($p < 0.05$) was observed in *B. rotundiformis*. AChE activity was significantly inhibited ($p < 0.05$) by 2.0-fold in *B. plicatilis* and by 1.1-fold in *B. rotundiformis* at 8.5 mg/L NH_3-N. For the antioxidant enzymes, the activity of SOD in *B. plicatilis* was stimulated 1.4 and 1.9-fold ($p < 0.05$) at 1.1 and 8.5 mg/L NH_3-N, respectively. In *B. rotundiformis*, a significant 1.2-fold reduction ($p < 0.05$) was observed at 1.1 mg/L NH_3-N. There were no significant changes in CAT activity in either species.

3.7. Activities of biomarkers

The transcript levels of the oxidative stress-sensitive biomarkers are shown in **Fig. 3-5 A**. High levels of NH_3-N (8.5 mg/L) induced significant upregulation ($p < 0.05$) of *Hsc70 like 1* in *B. plicatilis* (1.5-fold) and *B. rotundiformis* (1.8-fold). The expression of *NADPH oxidase 5* (*Nox5*) was significantly downregulated ($p < 0.05$) by 2.3-fold and 1.4-fold in *B. plicatilis*, and 2.5- and 2.5-fold in *B. rotundiformis* at 1.1 and 8.5 mg/L NH_3-N, respectively. *AChE* was downregulated 1.8-fold ($p < 0.05$) in *B. plicatilis* at 1.1 mg/L NH_3-N and was by 1.6-fold ($p < 0.05$) in *B. rotundiformis* at 8.5 mg/L NH_3-N.

The two rotifer species showed different reactivities in response to varying levels of NH_3-N in terms of antioxidant, lipid, and reproduction-related biomarker activities. Both NH_3-N levels (1.1 and 8.5 mg/L) caused significant responses in *B. plicatilis*, while the significant modifications in *B. rotundiformis* were observed at 8.5 mg/L.

The expression of antioxidant biomarkers following NH_3-N exposure is shown in **Fig. 3-5 B**. In *B. plicatilis*, significant downregulation (all $p < 0.05$) of the transcripts at 1.1 and 8.5 mg/L NH_3-N were evident for *CuZnSOD* (1.7- and 1.7-fold), *cat* (1.8- and 1.3-fold), and *calmodulin* (*CaM*) (1.6- and 1.1-fold). In *B. rotundiformis*, *CuZnSOD, cat, glutathione S-transferase* (*GSTs2*), and *MnSOD* were respectively upregulated by 1.3-, 2.1-, 1.6-, and 1.4-fold, respectively, (all $p < 0.05$) at 8.5 mg/L NH_3-N.

The activities of the lipid metabolic-related biomarkers are shown in **Fig. 3-5 C**. The expression of *citrate lyase* and *acetyl-CoA carboxylase* (*ACC*) was significantly downregulated in *B. plicatilis* at 1.1 mg/L NH_3-N (2.0- and 2.1-fold, respectively, $p < 0.05$). These biomarkers were upregulated 2.2- and 1.1-fold, respectively, ($p < 0.05$) in *B. rotundiformis* at 8.5 mg/L NH_3-N. Mitochondrial *CYP* was downregulated in two rotifer species at 1.1 and 8.5 mg/L NH_3-N: 1.9- and 1.7-fold, respectively, ($p < 0.05$) in *B. plicatilis* (*CYP3335A1*), and 1.7- and 2.2-fold ($p < 0.05$), respectively in *B. rotundiformis* (*CYP3047B1*).

The transcript levels of the rotifer reproduction-related biomarkers are shown in **Fig. 3-5 D**. In *B. plicatilis*, the expression of *cathepsin L*, *histone H_2A*, and *vasa* were downregulated 27.7-, 1.4-, and 2.1-fold, respectively, (all $p < 0.05$) at 1.1 mg/L NH_3-N. In *B. rotundiformis*, *cathepsin L* and *histone H_2A* were significantly upregulated by 1.5- and 2.1-fold, respectively, (both $p < 0.05$) at 8.5 mg/L NH_3-N.

3.8. IBR

IBR star plots and scores are shown in **Fig. 3-6**. A high IBR score indicates strong deterioration. In both rotifer species, the IBR score in the general stress functional group varied more than in the lipid and reproduction groups between the control and NH_3-N treatments. In *B. plicatilis*, the values changed from 8.3 (control) to 13.6 (1.1 mg/L NH_3-N), and 6.7 (8.5 mg/L NH_3-N). *AChE*, *CYP*, *MnSOD*, and *CaM* contributed to the high IBR

score with 1.1 mg/L NH_3-N. In *B. rotundiformis*, the IBR score in the stress-response functional group varied from 4.4 (control) to 5.4 (1.1 mg/L NH_3-N) and 8.3 (8.5 mg/L NH_3-N). *GSTs2*, *MnSOD, CuZnSOD,* and *cat* were the biomarkers that contributed to the high score in 8.5 mg/L NH_3-N.

4. Discussion

The results support our hypothesis that the tropical rotifer *B. rotundiformis* is more tolerant of higher ammonia levels than the temperate species *B. plicatilis*, reflecting different adaptive strategies to ammonia stress at the molecular and enzymatic levels. Rotifers with better nutritional quality, higher growth rate, and resistance to environmental stress are used to feed fish larvae, which could facilitate high survival rates and stable growth of the larvae (Hagiwara et al., 2007). Compared to temperate *B. plicatilis*, tropical *B. rotundiformis* is more tolerant to high temperatures (e.g., 35°C) (Hagiwara et al., 1995), high salinity (e.g., 30–35 ppt) (Fielder et al., 2000), and toxic substances like juglone (Tanaka et al., 2009) and iron (Han et al., 2021). In the previous study, 24 h LC_{50} of NH_3-N to *B. plicatilis* and *B. rotundiformis* was 17.0 mg/L (Yu and Hirayama, 1986) and 12.2 mg/L (Gao et al., 2021), respectively. These are lower than our data of 40.0 mg/L for *B. plicatilis* and 26.9 mg/L for *B. rotundiformis*. NH_3-N toxicity was sensitive to temperature and pH (Emerson et al., 1975; Miller et al., 1990), and thus different culture conditions might affect the lethal dosage responses with rotifer strains. Meanwhile, the tolerance of toxicants was significantly affected by animal size (Vesela and Vijverberg, 2007), therefore we transformed the LC_{50} and EC_{50} values by dividing the dry weights of rotifers employed (mg/L per ng). In this transformation, the NH_3-N tolerance in *B. rotundiformis* was greater than *B. plicatilis* in both acute and chronic exposure, with 1.5- and 1.7-fold higher LC_{50} and EC_{50} (for intrinsic growth rate), respectively (**Table 3-3**). The two rotifer species chronically exposed to 0–29.3 mg/L of NH_3-N induced inhibition of fecundity and delayed generation time, resulting in a reduced intrinsic growth rate (*r*). The *r* in *B. rotundiformis* was higher than that in *B. plicatilis* at all NH_3-N treatments, except 29.3 mg/L (**Fig. 3-1 D**), despite the experiment temperature of 25°C, which was lower than the optimum growth temperature of 30°C for *B. rotundiformis*. Moreover, *B. rotundiformis* showed stronger

ammonia tolerance for sexual reproduction parameters, including mixis, fertilization, and resting egg production, compared to *B. plicatilis*. Rotifer sexual reproduction is triggered by environmental factors, such as crowding, and both promote the development of haploid eggs into fertilized resting eggs and improve species adaptation to environmental changes, and/or facilitate rotifer evolution through clonal variation (Crow, 1994; Gilbert and Schröder, 2004). Pronounced sexual reproduction tolerance may improve rotifer stress resistance and maintain the population under harsh environments. As aforementioned, the tropical *B. rotundiformis* commonly exhibited higher reproduction and may encounter more NH_3-N than temperate *B. plicatilis* during mass culture. Therefore, we suspect that the higher ammonia tolerance in tropical *B. rotundiformis* may support their high population growth.

The difference in ammonia sensitivity between the two rotifer species may be attributed to evolved adaptations in their habitats. Compared with temperate species, tropical freshwater organisms can reportedly endure higher and more toxic levels of NH_3-N due to high-temperature exposure (Wang and Leung, 2015). The toxicity of NH_3-N to aquatic organisms increases with temperature because the water solubility of NH_3 and its uptake/circulation rate is generally high at elevated temperatures. Moreover, high water temperatures may promote the metabolic rate of animals, increasing ammonia accumulation and toxicity (Zhang et al., 2020). Zooplanktons, including rotifers, which have experienced high levels of habitat-resource abiotic stress, may preadapt to exogenous toxic substances and may evolve effective stress defense systems (Han et al., 2021; Tanaka et al., 2009). These events can enhance their stress tolerance. Thus, the stress conditions experienced by the two rotifer species in their original habitats may affect their adaptation to the environment.

The mechanisms of ammonia-induced sensitivity were determined by the synergistic responses in oxidative/antioxidant and lipid activities. Two NH_3-N concentrations (1.1 and 8.5 mg/L) were selected to explore the mechanisms based on the recorded data from rotifer cultivation (Hagiwara et al., 1993; Yoshimura et al., 1996) and the results of our reproductive experiments, which revealed the effects on rotifer sexual and asexual reproduction. NH_3-N induces oxidative stress in aquatic animals by increasing intracellular ROS levels. However, in the present study, exposure to 8.5 mg/L NH_3-N induced a

reduction of ROS in both rotifer species. Ammonia overload reportedly affects oxygen consumption, inducing low metabolic rate and decreased ROS in zebrafish *Danio rerio* (Conlin et al., 2018). Slowed metabolism causes growth and reproduction retardation in copepod *Paracyclopina nana* (Byeon et al., 2020). High levels of NH_3-N at ≥ 8.5 mg/L negatively affect rotifer maturation and fecundity with a gene expression disorder: the superoxide inducer biomarker *Nox5* was downregulated, while the oxidative sensitivity indicator *Hsc70* was upregulated, compared with control. Nevertheless, the antioxidant activities were different in the two rotifer species. The enzymes were inhibited in *B. plicatilis* with a low level of *CuZnSOD* and *cat*, but *B. rotundiformi*s showed an active antioxidant response. The associated SOD enzyme activity showed different patterns, with stimulation in *B. plicatilis* at both NH_3-N levels but inhibition in *B. rotundiformis* at 1.1 mg/L. We do not have a rational explanation for the lack of expression of the enzyme by its associated gene, which has been documented (Kim et al., 2015). The lipid metabolic patterns were also different between *B. plicatilis* and *B. rotundiformis* in the presence of NH_3-N. Higher NH_3-N levels reduced the accumulation of neutral lipids in the reproductive organs (vitellarium/ovary) of *B. plicatilis*, and stimulated lipid peroxidation with a high level of MDA, which was overproduced as free radicals increased (Gaweł et al., 2004). In contrast, *B. rotundiformis* showed a low level of MDA and an insignificant increase in lipid content in the reproductive organs. Neutral lipids are an important source of energy in the development and diapause of resting eggs because they can protect cells from various cellular stresses, such as lipotoxicity, and effectively maintain energy and redox homeostasis (Unger, 1995; Welte and Gould, 2017). The inhibition of lipid activities associated with the downregulation of lipid synthesis-related biomarkers (e.g., *citrate lyase*, *ACC*, and mitochondrial *CYP*) and rotifer reproduction-related genes (e.g., *cathepsin L*, *histone H_2A*, and *vasa*) may contribute to *B. plicatilis* reproductive sensitivity to NH_3-N stress (Hanson et al., 2013; Zhang et al., 2016b). However, in *B. rotundiformis*, positive expression of these biomarkers was detected. The IBR analysis of biomarkers in general stress, lipid, and rotifer reproduction functional groups revealed the sensitivity of *GSTs*, mitochondria *CYP*, *AChE*, CuZn*SOD*, and *MnSOD* in response to ammonia stress. These findings suggest that these genes might be valuable as ammonia-sensitive biomarkers. A

summary of the oxidative stress-response biomarkers found in the two tested rotifer species is shown in **Table 3-4**.

The increased swimming speed with the modulation of AChE activity implies the effects of NH_3-N on the modification of rotifer locomotion through the cholinergic-neuromuscular system in two rotifer species (Pérez-Legaspi et al., 2014). Oxidative stress is related to the regulation of AChE activity, which has a function in the locomotion regulation of invertebrates and vertebrates (Clement and Amsellem, 1989; Lovern et al., 2007) Imbalanced ROS inhibits AChE activity, alters neurotransmission, and lead to behavioral disorders, such as hyperactivity, paralysis, and a lack of coordination (Parolini et al., 2018; Roast et al., 2001). NH_3-N exposure triggered AChE inhibition, and rotifer fast swimming might be relevant to hyperactivity, which consumes more energy. This may affect the energy allocation for growth and reproduction, ultimately resulting in negative effects on rotifer reproduction, especially sexual reproduction. This is because rotifer sexual reproduction is more sensitive to external stimuli including NH_3-N (Snell and Boyer, 1988) and insecticide diazinon (Marcial and Hagiwara, 2007) than asexual reproduction. A similar pattern was observed in the present study. The sensitivity of NH_3-N to rotifer sexual reproductive characteristics, such as mixis rate and resting egg production, was higher than the asexual parameter (population growth rate), with a low LOEC (EC_{20}) value.

Taken together, the different NH_3-N tolerance and associated biochemical responses between the two marine rotifer sibling species indicate species-specific characteristics of ammonia toxicity. As an important water quality indicator, the acute and chronic criteria for NH_3-N in freshwater species are well established. The documented ACRs of NH_3-N for freshwater aquatic species, such as mussels (*Lampsilis fasciola*) and the crustacean *Daphnia magna*, ranged from 2.4 to 49.5 (US EPA, 2013). ACR is a fundamental hazard assessment parameter that uses acute toxicity data to gauge the chronic toxicity of chemicals to organisms (Kenaga, 1982). However, there is little information on marine species. Based on our data, the calculated NH_3-N ACR values for *B. plicatilis* and *B. rotundiformis* were 15.66 and 58.53, respectively. Furthermore, the stronger reproductivity of tropical species compared to temperate species may have an impact on the aquatic system. There is a developing tendency for poleward expansion of tropical species caused by climate changes, such as global warming. This may alter the species composition and

diversity of native communities due to competitors or predator-prey interactions among different species and may therefore affect aquaculture and even the entire ecosystem (Figueira and Booth, 2010; Osland et al., 2021).

5. Conclusions

The tropical rotifer *B. rotundiformis* shows a higher population density than temperate species *B. plicatilis*, with an expected reason of ammonia (NH_3-N) tolerance. The present molecular evidence confirmed the strong ammonia tolerance of tropical rotifers. Ammonia sensitivity has species-specific characteristics. Tropical rotifer species that experienced habitat-resource stressors were able to cope with ammonia stress more actively than temperate species, such as by enhanced antioxidant and lipid activities. The different modes of ammonia action in the reproduction of two rotifers suggest the importance of lipid/energy metabolism and environmental adaptations in rotifer stress resistance.

Table 3-1. The detected actual concentrations of ammonia (NH_4Cl) in the investigations.

	NH_4Cl concentrations (mg/L)							
Nominal	0	10	20	40	80	160	320	550
Actual	0.1±0.0	7.3±0.1	17.8±0.0	35.6±0.4	75.4±0.3	157.1±0.5	300.7±3.0	544.2±1.9

Values are mean ± standard error of three replicates.

Table 3-2. Sexual and asexual reproduction parameters of *Brachionus plicatilis* and *Brachionus rotundiformis* exposed to different concentrations of un-ionized ammonia (NH_3-N) during batch culture for 7 days.

	B. plicatilis				*B. rotundiformis*			
Treatment (mg/L)	Population growth rate (*r*)	Mixis (%)	Fertilization (%)	Resting eggs /mL	Population growth rate (*r*)	Mixis (%)	Fertilization (%)	Resting eggs /mL
0	0.72±0.00	4.8±1.6	35.5±8.5	4.7±0.8	0.92±0.01	10.5±0.7	68.8±3.3	25.8±2.0
0.5	0.72±0.01	3.5±0.3	14.4±4.0**	1.7±0.3***	0.89±0.00	8.9±0.5	49.8±4.0	19.7±2.4
1.1	0.72±0.01	1.9±0.1*	3.7±3.7***	0.3±0.3***	0.91±0.01	6.2±0.2***	55.4±6.6	10.3±0.8***
2.1	0.70±0.00	0.4±0.4***	6.7±6.7***	0.3±0.3***	0.91±0.00	5.4±0.6***	56.8±6.3	13.4±2.3**
4.2	0.69±0.01	0.4±0.2***	0.0±0.0***	0.0±0.0***	0.91±0.00	3.4±0.7***	55.3±5.8	7.7±0.2***
8.5	0.65±0.01	0.1±0.1***	0.0±0.0***	0.0±0.0***	0.84±0.03	0.6±0.2***	8.3±8.3***	0.5±0.4***
17.0	0.18±0.01***	0.0±0.0***	0.0±0.0***	0.0±0.0***	0.09±0.06***	0.0±0.0***	0.0±0.0***	0.0±0.0***
29.3	0.00±0.00***	0.0±0.0***	0.0±0.0***	0.0±0.0***	0.00±0.00***	0.0±0.0***	0.0±0.0***	0.0±0.0***

Data are mean ± standard error. ANOVA followed by Dunnett's test show significant differences from control treatment (*, **, *** indicate $p < 0.05$, 0.01, 0.001, respectively, n = 3).

Table 3-3. Twenty four hour lethal concentrations (24 h LC_{10}, LC_{20} and LC_{50}, mg/L) and effective concentrations (EC_{10}, EC_{20} and EC_{50}, mg/L) with 95% confidence intervals for life history and reproduction parameters of *Brachionus plicatilis* and *Brachionus rotundiformis* in response to un-ionized ammonia (NH_3-N) at 25°C and pH = 8.

	B. plicatilis	*B. rotundiformis*
24 h lethal concentrations (LCs)		
LC_{10}	26.9 (20.6–32.0)	19.6 (13.9–23.5)
LC_{20}	30.8 (24.7–36.2)	21.8 (16.4–25.8)
LC_{50}	40.0 (33.9–47.0)	26.9 (22.0–31.2)
LC_{50}/ng DW	0.08 (0.07–0.09)	0.12 (0.10–0.14)
Life-history parameters (Individual culture)		
Time (h) for the first egg		
NOEC	8.51	4.26
LOEC	17.02	8.51
Time (h) for the first offspring		
NOEC	8.51	4.26
LOEC	17.02	8.51
Fecundity		
EC_{10}	ND	2.40 (1.21–4.76)
EC_{20}	ND	3.13 (1.58–6.22)
EC_{50}	> 50.00	5.16 (2.60–10.26)
Lifespan		
EC_{10}	26.69 (25.54–27.83)	ND
EC_{20}	27.78 (26.64–28.92)	ND
EC_{50}	29.77 (28.63–30.92)	> 50.00
Intrinsic growth rate (*r*)		
EC_{10}	2.09 (2.01–2.17)	0.32 (-0.03–0.67)
EC_{20}	3.12 (3.04–3.20)	0.66 (0.31–1.01)
EC_{50}	6.88 (6.80–6.96)	4.85 (4.49–5.20)
EC_{50}/ng DW	0.013 (0.013–0.014)	0.022 (0.020–0.024)
Acute chronic ratio (ACR)		
MATC	2.55	0.46
ACR	15.66	58.53
Sexual and asexual reproduction parameters (Batch culture)		
Population growth rate (*r*)		
EC_{10}	9.09 (8.72–9.47)	8.84 (8.56–9.12)

EC_{20}	10.78 (10.35–11.24)	9.93 (9.62–10.25)
EC_{50}	14.65 (14.06–15.27)	12.18 (11.80–12.57)
Mixis		
EC_{10}	0.37 (0.30–0.47)	0.43 (0.14–1.32)
EC_{20}	0.49 (0.39–0.61)	0.77 (0.25–2.35)
EC_{50}	0.83 (0.66–1.04)	3.03 (1.00–9.22)
Fertilization		
EC_{10}	0.22 (0.21–0.23)	4.82 (2.99–7.77)
EC_{20}	0.29 (0.28–0.30)	5.39 (3.34–8.68)
EC_{50}	0.50 (0.48–0.53)	6.55 (4.07–10.56)
Resting egg production		
EC_{10}	0.24 (0.21–0.27)	0.27 (-0.04–0.59)
EC_{20}	0.29 (0.26–0.33)	0.56 (0.24–0.87)
EC_{50}	0.43 (0.39–0.49)	4.10 (3.78–4.42)

MATC indicates maximum acceptable toxicant concentrations. ND means no data.

Table 3-4. Biomarkers and enzymatic activity in response to stressful conditions in *Brachionus plicatilis* and *Brachionus rotundiformis*.

Species	Stressors	Biomarkers	Enzyme activities	References
B. plicatilis	Chlordecone (25, 50, 100 μg/L)	**Phase I detoxification system** Clan3 *CYPs* (*CYP3044B2*, *CYP3045A2*, *CYP3045C8*, *CYP3046A2*): upregulated		Lee et al., 2018
	Polybrominated diphenyl ethers (BDE-47, BDE-209) (200 μg/L)	**ROS pathway** *sod*: upregulated *cat*: no change **Calcium-signaling pathway** Ca^{2+} levels in the mastax, stomach and ovary: increased *calmodulin* (*CaM*): upregulated	Low concentration (8 μg/L) ethoxyresorufin-O-deethylase (EROD), GPx and GR: increased Moderate (80 μg/L) and high concentration (800 μg/L) EROD, GPx and GR: decreased SOD, GST: increased	Zhang et al., 2016a Jian et al., 2017
	Benzo[α]pyrene (B[α]P) (100 μg/L)	*CYP3047B1*, *CYP3045C3*: upregulated *CYP3042A1*, *CYP3043A1*: downregulated		Kim et al., 2017
	Chlorpyrifos (CHF) (1000 μg/L) 2-ethyl-PHE (700 μg/L)	Clan2 *CYPs* (*CYP3048A2*): upregulated Clan3 *CYPs* (*CYP3045B2*, *CYP3045A2*, *CYP3045C5*, *CYP3045C10*): upregulated Clan46 *CYPs* (*CYP3049B3*): upregulated		Han et al., 2019a
	Arsenic (As) 1000 μg/L	**Phase II detoxification system** *GSTo1*: upregulated Arsenite methyltransferase (*AS3MT1*, *AS3MT4*): upregulated **Phase III detoxification system** *ATP-binding cassette* (*ABC*) genes: upregulated	GSH contents: increased GST: increased ABC protein: increased	Byeon et al., 2020 Kang et al., 2021

Copper sulfate ($CuSO_4$) (25 μg/L)		Hsp58 protein: increased	Cochrane et al., 1991
Tributyltin (TBT) (30 μg/L)			
Nanoplastics (NPs) (2.5 μg/mL)		GSH contents: increased GST, CAT: increased ABC protein activity: inhibited	Kang et al., 2021
Harmful algae (*Phaeocystis globose*)		Low proportions (25% and 50%) of *P. globose* GSH contents: increased GPx: decreased High proportions (75% and 100%) of *P. globose* SOD, CAT: inhibited	Liang et al., 2020
Calorie restriction Hypoxia (< 0.1% oxygen)	*MnSOD*: upregulated *cat*: upregulated **Glycolytic metabolisms** *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*), *enolase* (*ENO*), *phosphoglycerate mutase* (*PGM*): upregulated	SOD, CAT: increased FOX family genes (e.g., DAF-16) Insulin/insulin-like growth factor signaling pathway	Denekamp et al., 2009 Kailasam et al., 2011 Ozaki et al., 2010
Temperature (15, 20, 25 [control], 30°C)	*CuZnSOD*, *cat*: downregulated *MnSOD*, *GSTs-7*, *GSTz-4*: upregulated	SOD, CAT: inhibited GST: increased	Han et al., 2020
Salinity (5, 15 [control], 25, 35 ppt)	*cat*: downregulated *MnSOD*, *GSTs-1*: upregulated	SOD, CAT: increased	Han and Lee, 2020

B. rotundiformis	Benzo[α]pyrene (B[α]P) (100 μg/L)	**Phase I detoxification system** Clan3 *CYPs* (*CYP3045A2*, *CYP3045B4*, *CYP3045C10*): upregulated Clan46 CYPs (*CYP3049A5*, *CYP3049E8*): upregulated Clan MT (*CYP3049A5*, *CYP3047A4*, *CYP3047B1*): upregulated **Phase II detoxification system** *GSTs* (1&4), *GSTo1*, *GSTz2*: upregulated **Phase III detoxification system** ATP-binding cassette (ABC) *ABCC-like* genes, *ABCG-like* genes: downregulated	MAPK pathway p-JNK, p-p38: increased	Han et al., 2019b
	Pharmaceuticals Acetaminophen (APAP, 100 μg/L) and Oxytetracycline (OTC, 100 μg/L)	APAP Clan3 *CYPs* (*CYP3045A2*, *CYP3045C10*): upregulated Clan46 *CYP* (*CYP3049B7*): upregulated *GSTs* (1&4), *GSTo1*, *GSTz1*: upregulated OTC *GSTs* (1, 2, 4, 7, 8&9), *GSTα1*, *GSTo* (1&2): upregulated		Park et al., 2018
Putative genes in both two species	Cadmium (Cd)	Autophagy-related (Atg) genes		Kang et al., 2018
	Benzo[α]pyrene (B[α]P)	DNA double-strand break repair (DSB) genes *ATM*, *RAD51B/C*, *SLX1*, *APTX*, *DNA-PKcs* *Ku70*, *Ku80*, *RECQL5*, *TOP3A*		Lee et al., 2020b

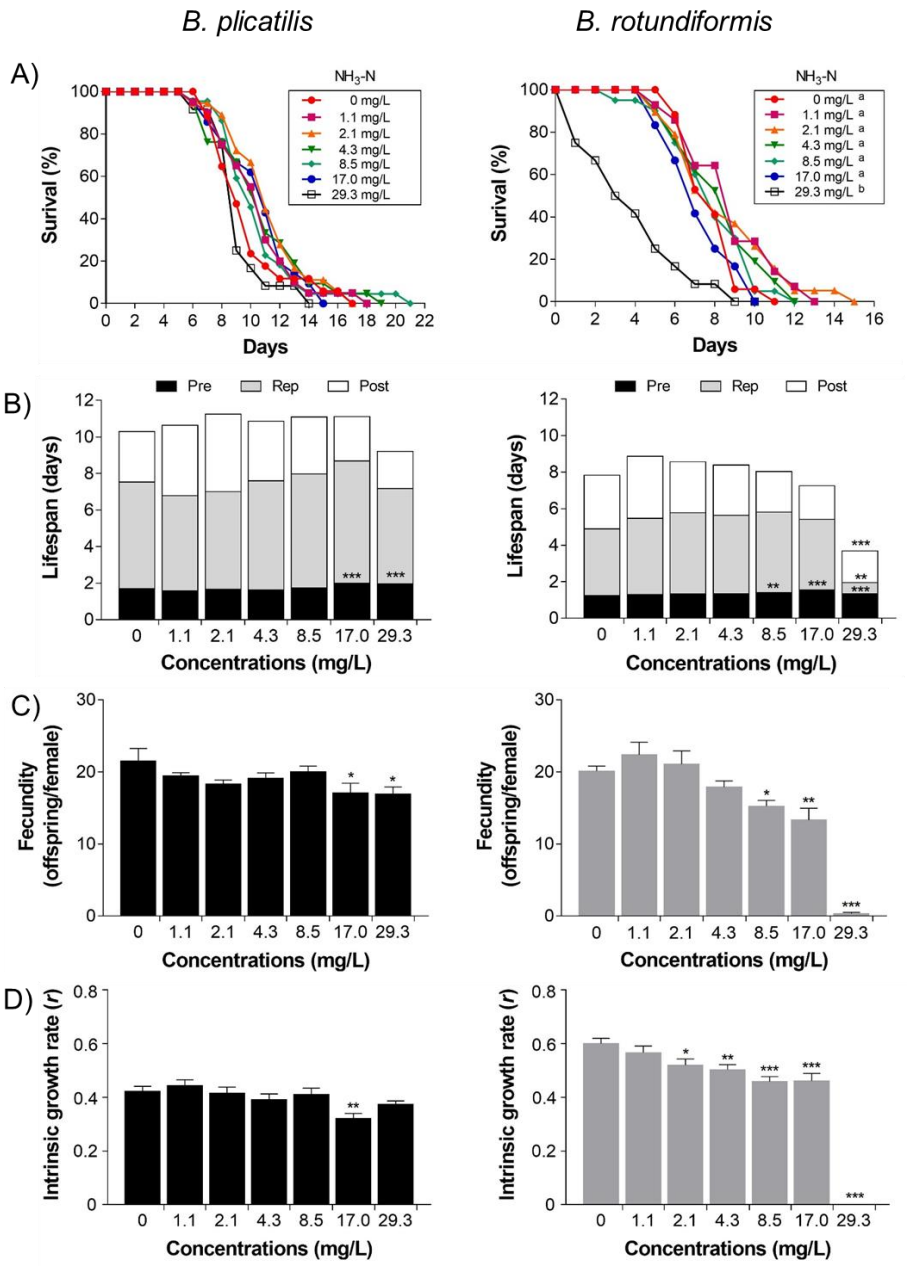

Fig. 3-1. (A) Age-specific survival rate (%), (B) lifespan (pre for pre-reproductive period; rep for reproductive period; post for post-reproductive period), (C) fecundity, and (D) intrinsic growth rate (*r*) of *Brachionus plicatilis* and *Brachionus rotundiformis* in response to different concentrations of NH_3-N (a>b, Log-rank test, $p < 0.01$; *, **, *** indicate $p <$ 0.05, 0.01, 0.001, respectively, Dunnett's test, n = 12–22).

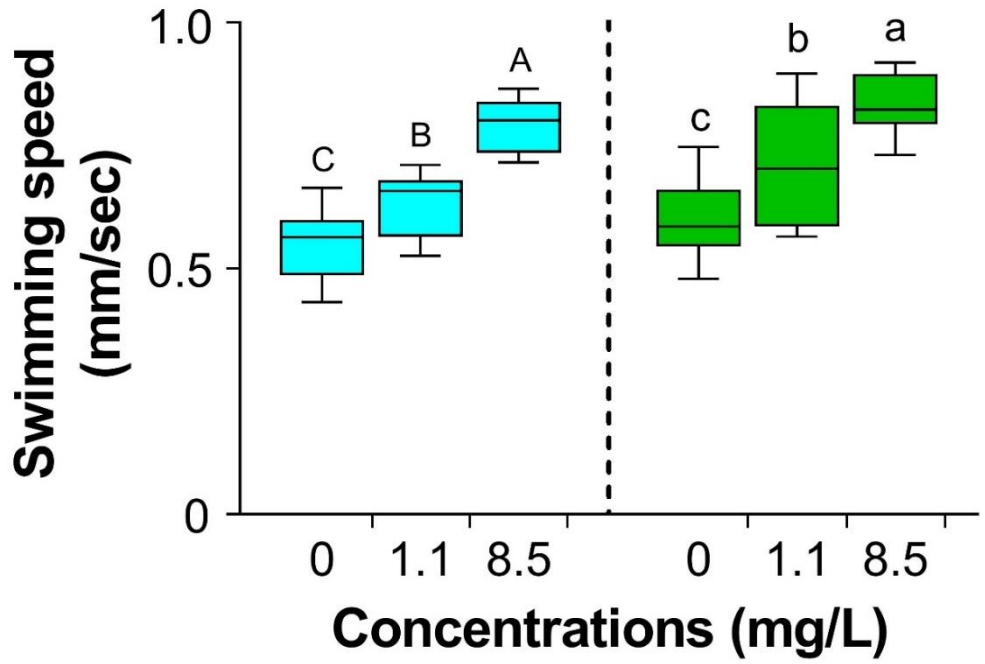

Fig. 3-2. Variation of swimming speed of *Brachionus plicatilis* (blue bar) and *Brachionus rotundiformis* (green bar) under different concentrations of NH_3-N (0 [control], 1.1, and 8.5 mg/L) exposure in 7 days batch cultures (A > B > C, a > b > c, Tukey HSD test, $p < 0.05$, n = 10–16).

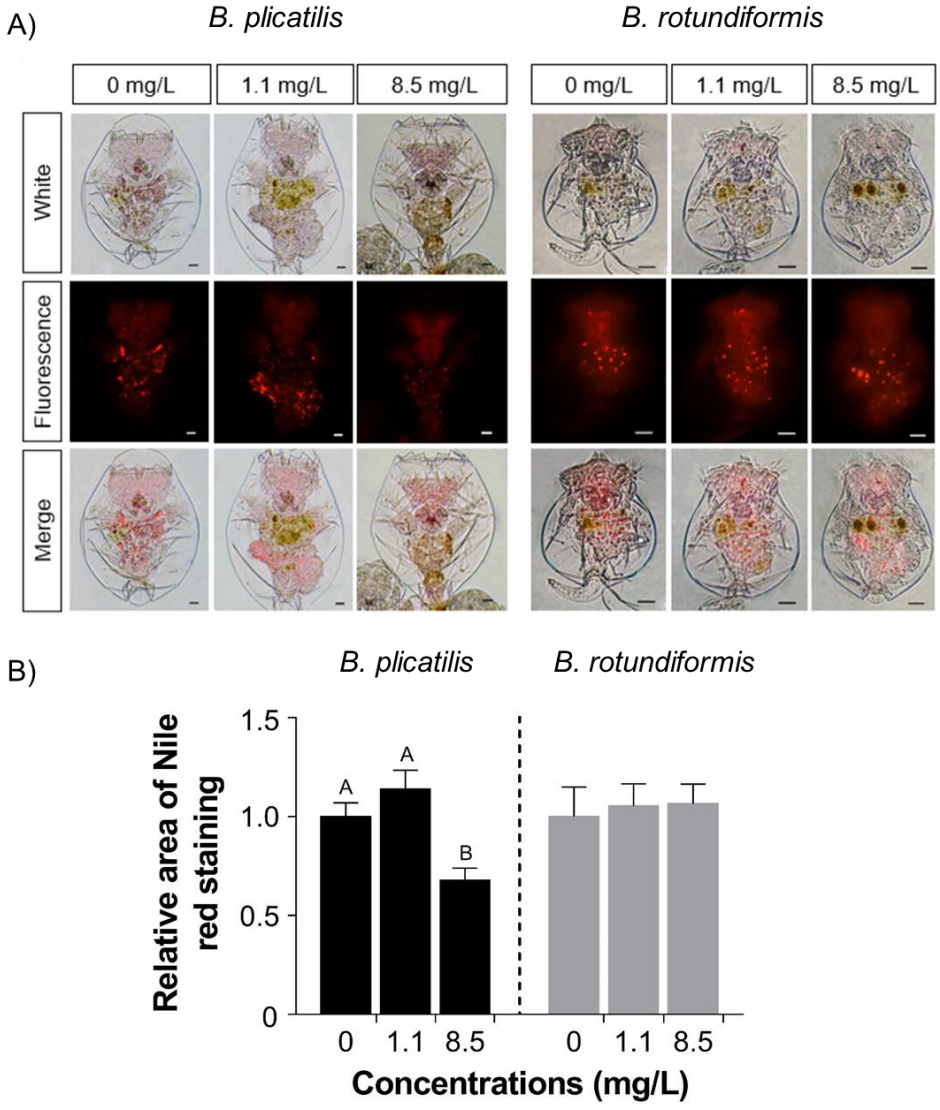

Fig. 3-3. (A) Lipid accumulations in *Brachionus plicatilis* and *Brachionus rotundiformis* under different concentrations of NH_3-N (0 [control], 1.1, and 8.5 mg/L) exposure during batch culture for 7 days. All scale bars represent 25 μm. (B) The relative area of lipid droplets in the two rotifer species (black bar for *B. plicatilis*, grey bar for *B. rotundiformis*) responding to different concentrations of NH_3-N (A > B, Tukey HSD test, $p < 0.05$, n = 15–30).

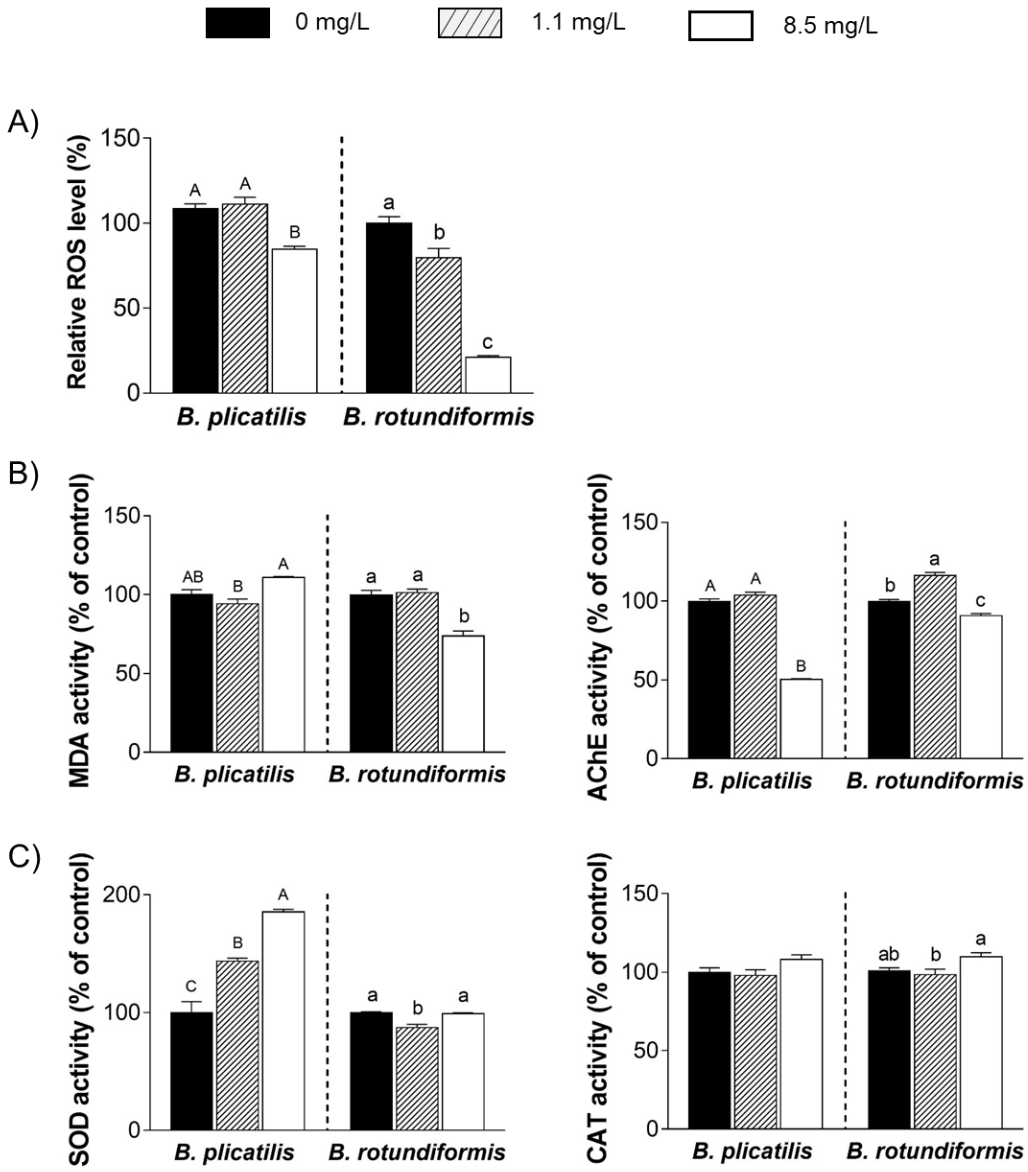

Fig. 3-4. (A) Intracellular reactive oxygen species (ROS) levels, enzymatic activity related to (B) oxidative stress, and (C) antioxidant responses in *Brachionus plicatilis* and *Brachionus rotundiformis* using different concentrations of NH_3-N (0 [control], 1.1, and 8.5 mg/L) in 7 days batch cultures (A > B > C, a > b > c, Tukey HSD test, $p < 0.05$, n = 3).

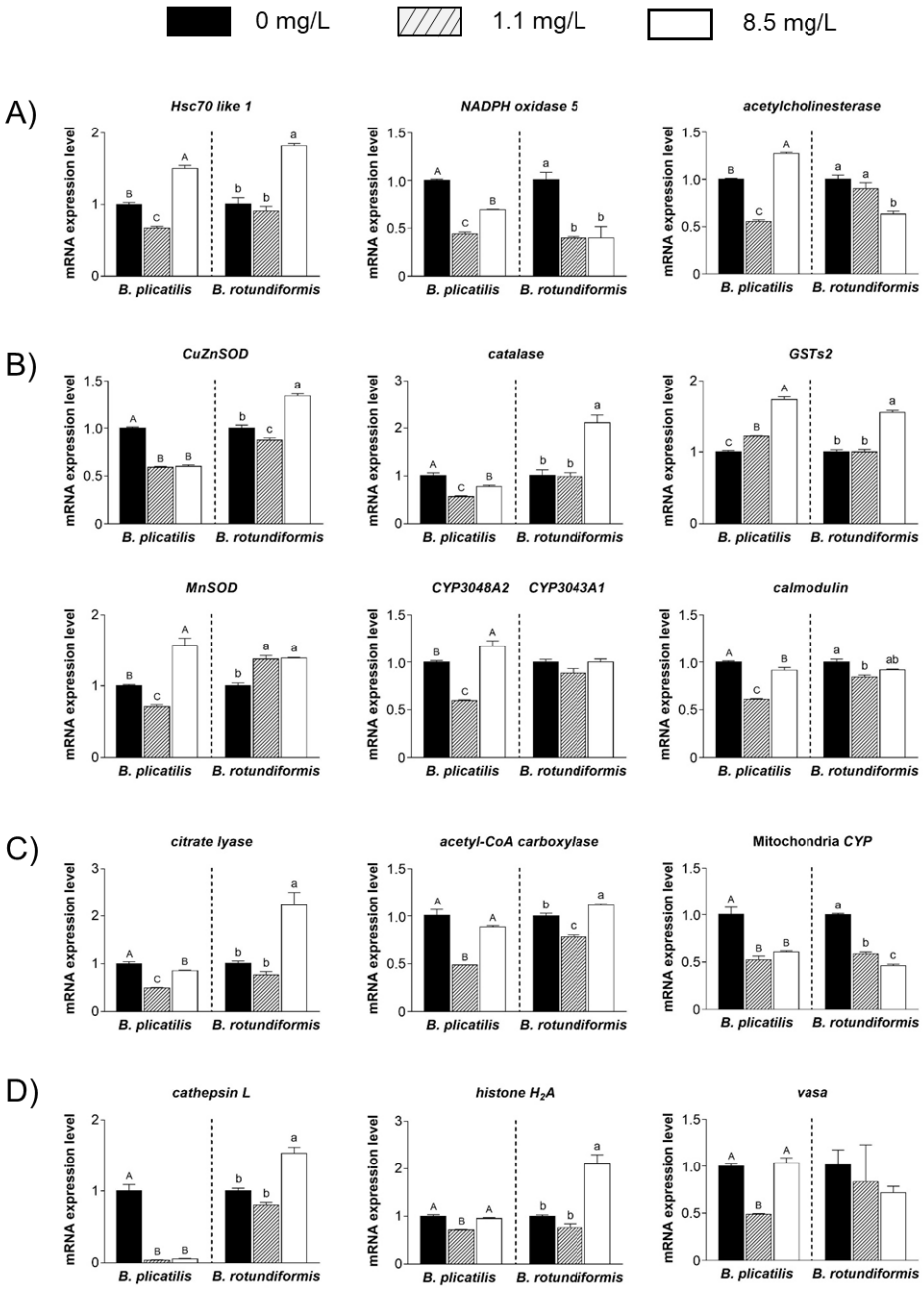

Fig. 3-5. Transcriptional levels of biomarkers involved in (A) oxidative stress, (B) antioxidant metabolisms, (C) lipid metabolic, and (D) rotifer reproduction in *Brachionus plicatilis* and *Brachionus rotundiformis* under different concentrations of NH_3-N (0 [control], 1.1, and 8.5 mg/L) in 7 days batch cultures (A > B > C, a > b > c, Tukey HSD test, $p < 0.05$, n = 3).

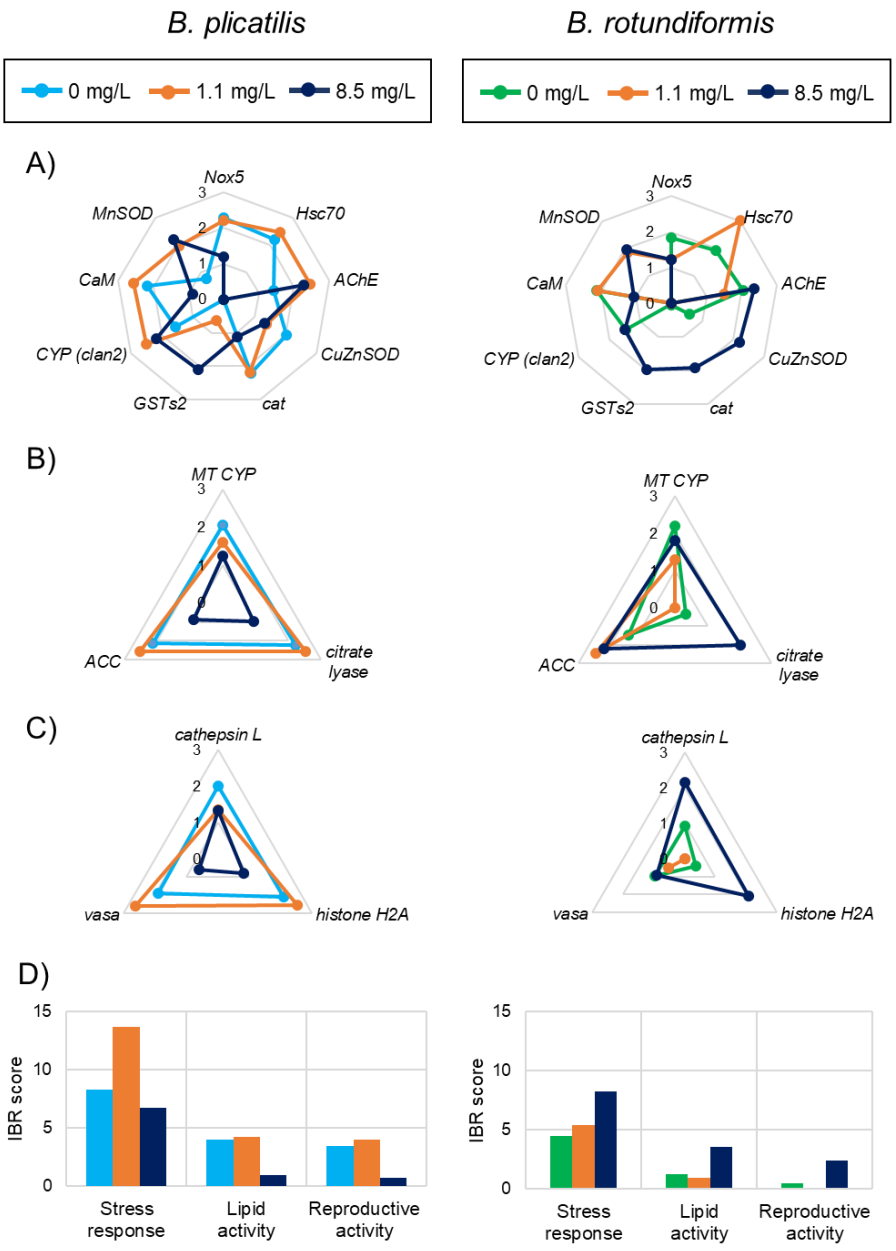

Fig. 3-6. Integrated biomarker response (IBR) star plot for (A) stress-responses related biomarkers, (B) lipid activity-related biomarkers, (C) reproduction-related biomarkers, and (D) IBR scores for three functional groups of biomarkers in *Brachionus plicatilis* and *Brachionus rotundiformis* using different concentrations of NH_3-N (0 [control], 1.1, and 8.5 mg/L).

Chapter V

General Discussion

The employed two rotifer sibling species, *B. plicatilis* and *B. rotundiformis*, exhibited differential modes of response in reproduction, swimming behavior, and stress defensive metabolisms following $FeSO_4 \cdot 7H_2O$, $ZnCl_2$, and NH_3-N exposure, as summarized in **Table 5-1**.

Reproductive responses of two rotifer species following exposure to stresses

First, the two rotifers exhibited specific tolerance in survival in response to acute $FeSO_4 \cdot 7H_2O$, $ZnCl_2$, or NH_3-N exposure, as demonstrated by variable values of 24 h LC_{50}. Because there was a correlation between animal size and toxicants tolerance, the larger the species, the greater toxicity tolerance, and vice versa (Sarma et al., 2007), and thus based on the different dry weights between the two species, *B. plicatilis* (495 ng/ind.) and *B. rotundiformis* (220 ng/ind.) (Yúfera et al., 1997), I transformed the 24 h LC_{50} values to LC_{50}/ng DW with a divide by rotifer dry weight. In this transformation, *B. rotundiformis* was shown to be more resistant to Fe and NH_3-N stress than *B. plicatilis*, with 1.9- and 1.5-fold higher values, respectively.

Second, regarding chronic exposure, this study identified the species-specific reproductive sensitivity, especially in sexual reproduction, associated with chemical concentrations in the two rotifer sibling species. It was extensively described that rotifer sexual reproduction was more sensitive to various environmental stimuli, including pesticides (Preston and Snell, 2001; Marcial and Hagiwara, 2007), organofluoride compounds (Zhang et al., 2014; Zhang et al., 2016), and metal elements (Preston and Snell, 2001; Xu et al., 2015), than that of asexual reproduction. Nonetheless, this study established different patterns of sexual reproductive sensitivity in the two rotifer sibling species. For example, the resting egg production of *B. plicatilis* was highly susceptible to either the Fe, Zn, or NH_3-N exposure. In contrast, *B. rotundiformis* showed greater resilience in response to the same concentrations of Fe and Zn exposure, with enhanced

mixis induction and resting egg generation. According to biological activity investigations, the different sexual reproductive responses between the two rotifers could be attributed to the expected influences on (1) sperm viability and (2) lipid droplet accumulation in female reproductive organs, both of which were important in regulating rotifer reproductive fitness and were easily affected by stressors.

The life cycle of *Brachionus* rotifer contains both asexual and sexual reproduction, the latter of which is established by the male-female mating process (Gómez and Serra, 1996). Male rotifers are short-lived, e.g., a lifespan of 2.6 days in *B. plicatilis* and 2.2 days in *B. rotundiformis* (**Chapter II.2**), and they have a limited number of sperm, e.g., an average of 30 sperm per *B. plicatilis* male (Snell and Hoff, 1987). Male fertility is largely limited by sperm numbers and motility (Snell and Childress 1987; Snell and Hoff 1987). As aforementioned in the General Introduction (**Chapter I**), Fe and Zn are essential for maintaining sperm viability, while high levels negatively affect the quality of spermatozoa (Clapper et al., 1985; Desouky, 2009; Yamaguchi et al., 2009). In this study, the sensitively inhibited sperm viability in *B. plicatilis* responded to Fe ($\geqslant$ 20 mg/L) and Zn ($\geqslant$ 1 mg/L) but enhanced/sustained viability in *B. rotundiformis* at the same treatments, which helped to explain the differences in sexual sensitivity between the two species.

Resting eggs, produced by the fertilization of male and mictic females, are essential to ensuring the persistence of the rotifer population under temporally harsh environmental conditions. While they require sufficient energy sources to maintain viability during diapause (García-Roger and Ortells, 2018). The storage of lipids as an energy resource, typically in the form of lipid droplets, has been suggested as playing an important role in the reproduction and diapause of zooplankton (Lee et al., 2006). Moreover, lipid droplets functioned as central managers in the lipid metabolisms, maintaining energy and redox homeostasis, and protecting cells against lipotoxicity by sequestering toxic lipids into their neutral lipid core (Petan, 2018; Welte and Gould, 2017). The reduced lipid accumulation in the reproductive organs of *B. plicatilis* rotifers was observed in response to the Fe, Zn, and NH_3-N exposure. It was reported that the fluctuations of lipids and fatty acids were closely related to sexual development of sea pansy *Renilla koellikeri*, and significant gonad fatty acids and lipids accumulation were observed prior to spawing (Pernet et al., 2002). Hence, the shortage of energy source might contribute to the sexual reproductive sensitivity

in *B. plicatilis* (García-Roger and Ortells, 2018). On the other hand, males did not feed, therefore their energy budget strongly depended on the maternal inputs (Epp and Lewis, 1979). It was observed that sperm viability was affected by maternal nutrition (Snell and Hoff 1987). Hence, to some extent, the reduced lipid storage in maternal *B. plicatilis* may contribute to the poor status of their offspring, such as suppressed sperm viability. In contrast, a stimulated or constant sustained lipid accumulation in the ovary/vitellarium of *B. rotundiformis* was observed, which supported the greater reproductive resilience of *B. rotundiformis* in response to the three chemicals.

The differential lipid response patterns between two rotifer species were evidenced by the following molecular modifications. Citrate lyase and acetyl-CoA carboxylase (ACC) are essential enzymes in lipid biosynthesis (Barber et al., 2005; Jones et al., 2017; Munday, 2002; Sun et al., 2010), and the mitochondrial CYP is involved in steroidogenesis, cholesterol, and steroid metabolism (Han et al., 2019a, 2019b; Midzak and Papadopoulos, 2016). The non-activated *citrate lyase*, and downregulated *ACC* and MT *CYP* in *B. plicatilis* suggested the inhibited lipid metabolism in this species in response to Fe, Zn, and NH_3-N exposure, respectively. Moreover, rotifer reproduction-associated biomarkers, such as asexual development-related *cathepsin L*, gametogenesis-involved *histone* H_2A, and *vasa* (Shi and Mello, 1998; Johnson, 1999; Hanson et al., 2013; Zhang et al., 2016b) were downregulated in *B. plicatilis* following exposure. However, they were upregulated in *B. rotundiformis*.

Swimming behavior modifications in two rotifer species following stress exposure

Swimming behavior is employed as a sensitive indicator in rotifer toxicological study. In this study, male and female rotifers in both species exhibited different behavioral responses to Fe (20–45 mg/L) exposure. That is male swimming speed decreased as Fe concentration increased, but female swimming speed were stimulated at relatively lower Fe concentrations. This may suggest that male performance is more vulnerable to Fe stress than female rotifers. Moreover, female rotifers with fast swimming speed are recorded after exposure to Zn (1–2 mg/L) and NH_3-N (1.1–8.5 mg/L). Stress inhibits rotifer swimming speed, which has been observed in both freshwater and marine rotifers, such as *B.*

calyciflorus (Janssen et al., 1994a; Guo et al., 2012), *B. plicatilis* (Sha et al., 2015), and *B. koreanus* (Won et al., 2022). Toxic stimulating swimming, on the other hand, is also noticed, which is considered as a hyperactivity or an escape strategy (Chen et al., 2014; Kim et al., 2020). Therefore, it is speculated that the stimulated female swimming speed in response to Fe/Zn/NH_3 stress may reflect a hyperactive behavior. This may be supported by an inhibition of AChE activity, an important enzyme in the nervous system, which is involved in the regulation of neuro-muscle movement (Perez-Legaspi et al., 2014). Acetylcholine is a neurotransmitter, which is released into synapses to excite the receiving neuron. The enzyme AChE is responsible for the breakdown of the acetylcholine for a normal movement response (Kwong, 2002). The inhibition of AChE increases both the level and duration of the neurotransmitter action, inducing behavior disorders, such as hyperactivity or paralysis. AChE regulatory effects on the behavior of aquatic species (Clement and Amsellem, 1989; Lovern et al., 2007; Jin et al., 2019), including rotifer (Rhee et al., 2013) were extensively documented. Moreover, AChE activity is easily to be affected by oxidative stress (Parolini et al., 2018; Roast et al., 2001). Therefore, Fe, Zn, and NH_3 stress trigger AChE inhibition, regulating rotifer swimming behavior.

The modifications in swimming behavior may have an indirect effect on rotifer reproduction. From one perspective, rotifer executes swimming motions depending on the anatomical configuration of the cilia-muscular locomotor system (Clement et al., 1983). This is an energy-consuming process. Hence, the stimulated rotifer swimming speed may affect the energy allocation for growth and reproduction, ultimately resulting in negative effects on rotifer reproduction, especially sexual reproduction. Because the occurrence of sexual reproduction largely depends on sufficient nutrition/energy resource (Gilbert, 2010). From another perspective, 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 (Rico-Martínez and Snell, 1997). 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 faster swimming speeds and greater sperm viability can search for females many times and choose appropriate individuals for insemination. Under the Fe situation, described as

slower males and faster females in swimming speed, the mate encounter rate is likely decreased, which could suppress rotifer sexual reproductivity.

Stress defensive metabolisms in two rotifer species: temperate and tropical habitat adaptations

Mitochondria play significant roles in maintaining normal physiological activities. It not only provides not only a source of ATP but also regulates lipid storage and utilization, as well as harbors an oxidative/antioxidant metabolic network that maintains a viable balance between ROS generation and detoxification (Adlam et al., 2005; Andreyev et al., 2005; Benador et al., 2019). For the biological metabolism of organisms, the importance of the balance between ROS generation and clearance has been extensively documented (Garg and Manchanda, 2009; Mittler et al., 2004). The recognized effective antioxidant systems include SOD, CAT, CYP, GST, and GPx (Covarrubias et al., 2008; Kim et al., 2017; Yang et al., 2013). Excessive ROS generation, along with poor scavenging defenses, results in the accumulation of ROS to levels exceeding the physiological threshold, thereby inducing oxidative stress. In contrast, overcompensated antioxidant activity will promote reductive stress (Rajasekaran et al., 2007; Zorov et al., 2014). Both excessively high (oxidative stress) and low (reductive stress) levels of ROS are deleterious.

The two rotifer species chronically exposed to Fe, Zn, and NH_3-N, showed both increased and reduced intracellular ROS production. *B. rotundiformis* exhibited greater tolerance to ROS variations, with effectively activated anti-stress components, such as SOD, CAT, CYP, and CaM. However, *B. plicatilis* has shown inhibited stress defensive activities in response to either Fe or NH_3-N exposure. Although the SOD and CAT activity were effectively stimulated under Zn treatments, rotifer reproduction was inhibited, which might be explained as a “reductive stress” induced by low levels of ROS.

As hypothesized in the General Introduction (**Chapter I**), the different stress-response patterns between the two rotifer species may be attributed to evolved adaptations in their habitats. *B. plicatilis* is found in temperate waters (15–30°C), with high performance at relatively lower temperatures of 20°C. Whereas the *B. rotundiformis* is mainly distributed in tropical or subtropical areas (20–35°C) and performs better at temperatures higher than

28°C (Hagiwara et al., 1995b, 2001; Jung et al., 1997). Furthermore, *B. rotundiformis* is more tolerant of high salinity (e.g., 30–35 ppt) than *B. plicatilis* (Lubzens et al., 1985; Fielder et al., 2000). Ecological factors, such as temperature, pH, salinity, and dissolved oxygen affect the bioavailability or toxicity of substances, including trace elements and ammonia. For example, the oxygen contents in aquatic waters decreased as the temperature increased (Cairns et al., 1975; Wetzel, 2001). Higher temperatures lead to significantly higher oxygen consumption rates and an increase in the metabolic rates of organisms, enhancing ROS production (Childress, 1976; Li et al., 2014). For Fe status, ferrous Fe (II) is considered more toxic to aquatic animals than ferric Fe (III), which is abundant in oxygenated conditions (Faust and Zepp, 1993). The overall ratio of Fe (II)/Fe (III) can be 1000-fold higher in mildly anoxic seawater than in oxygenated seawater (Shaked, 2008; Waite, 2001). Therefore, species inhabited in tropical waters may be subjected to higher levels of stress than that in temperate regions. Moreover, it was reported that tropical organisms can endure higher toxic levels of NH_3-N due to high-temperature exposure than temperate species (Wang and Leung, 2015). An adaptation response to high levels of local NH_3-N was reported in benthic crustacean *Scylla serrata* (Weihrauch et al., 1999; Romano and Zeng, 2007).

Moreover, the toxicants sensitivity has been shown to differ in species adapted to freshwater and seawater environments. A summary of toxicants' sensitivity in freshwater and marine rotifers revealed that freshwater species were more vulnerable to various toxicants, especially metals, than marine species, as shown in **Tables 5-2** and **5-3**. For example, cadmium (Cd) acute and chronic toxicity were reduced 87.4–306-fold in the marine rotifer *B. plicatilis* compared to freshwater *B. calyciflorus* (Snell et al., 1991a; Snell and Moffat, 1992; Hwang et al., 2016). Fe tolerance to population growth was 24-fold higher in the two marine rotifers studied than in the freshwater rotifer *E. dilatata* (Hernández-Flores et al., 2020). This might be attributed to the differences in salinity, metal complexation capacity, alkalinity, and pH between freshwater and marine ecosystems. Salinity has a substantial impact on metal toxicity in aquatic invertebrates, with metal toxicity increasing as salinity decreases, due to the abundant availability of free metal ions at lower salinities (Hall and Anderson, 1995; McLusky et al., 1986). Whereas, in seawater systems, metal inorganic (Cl^-, CO_3^{2-}, and PO_4^{3-}), organic, proteins, and humic acid

complexations reduced free available metals, resulting in the inhibition of metal toxicity or bioavailability (Neubecker and Allen, 1983). Furthermore, pH variations would affect the oxidation status of metals. For instance, Fe (II) oxidation was faster in high pH conditions (González et al., 2010). The pH of most natural freshwater ranges between 6 and 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 (Marion et al., 2011). Thus, lower salt ions, poor organic complexation capacity, and variable pH in freshwater ecosystems may contribute to the vulnerability of metal toxicity in freshwater species. These observations suggest the significance of ecological experience in regulating stress tolerances and adaptations in aquatic species. Moreover, previous studies have shown that rotifers confer adaptability through epigenetic (histone) modifications (Lee et al., 2020) and phenotypic plasticity, such as improved mixis induction (Serra and Carmona, 1993) in coping with environmental variations.

Finally, on the basis of the current results and the previous publications (Serra and Carmona, 1993; Kim et al., 2014; Vargas et al., 2017; Franch-Gras et al., 2018; Lee et al., 2020), further studies on habitat-specific adaptations in regulating reproductive strategies of aquatic species are important for a better understanding of ecosystem evolution in the face of climate changes, such as ocean acidification and global warming (Figueira and Booth, 2010; Osland et al., 2021).

Conclusions

This study established (1) the differences in stress tolerance and reproductive response patterns, especially in sexual reproduction between two marine rotifer sibling species. (2) *B. plicatilis* sexual reproduction was more vulnerable to Fe, Zn, or NH_3-N stress, compared to *B. rotundiformis*, which exhibited higher stress resilience, with effectively activated ROS scavenging systems (e.g., SOD, CAT, GST, and CYP). (3) different sexual reproductive sensitivity associated with distinct (inhibited/stimulated) lipid metabolic patterns in the two species, implying the significance of lipids in maintaining rotifer sexual reproduction. (4) stress stimulating effects on rotifer swimming behavior were observed in both species, and a sensitive biomarker of AChE was identified. (5) the different modes of

stress responses in the two rotifers might be attributed to temperate and tropical habitat adaptations, which deserved to investigate further.

Table 5-1. Summary of the stress responses in reproduction, behavior, and biological metabolisms of *Brachionus plicatilis* and *Brachionus rotundiformis* in response to $FeSO_4 \cdot 7H_2O$, $ZnCl_2$, and NH_3-N exposure, respectively.

	B. plicatilis		***B. rotundiformis***	
Short-term exposure				
24 h LC_{50} (mg/L)		**LC_{50}/ng DW**		**LC_{50}/ng DW**
$FeSO_4 \cdot 7H_2O$	469.6 (438.3–501.0)	0.9 (0.8–1.0)	363.6 (323.4–406.0)	1.7 (1.5–1.8)
$ZnCl_2$	29.9 (25.4–34.8)	0.06 (0.05–0.07)	13.3 (10.5–16.4)	0.06 (0.05–0.07)
NH_3-N	40.0 (33.9–47.0)	0.08 (0.07–0.09)	26.9 (22.0–31.2)	0.12 (0.10–0.14)
Long-term exposure				
Reproduction				
$FeSO_4 \cdot 7H_2O$ (Exposure concentrations: 0–96 mg/L)	–High levels of 96 mg/L Fe inhibited rotifer maturation and fecundity but extended the lifespan. –Fe concentrations within 12–48 mg/L did not affect asexual reproduction but significantly inhibited resting egg production.		–Fe levels within 1.5–12 mg/L positively affect rotifer reproduction. –6 mg/L Fe stimulated both the population growth and resting egg production.	
$ZnCl_2$ (Exposure concentrations: 0–2 mg/L)	–Individual-level: Zn concentrations higher than 1 mg/L accelerated rotifer maturation and shortened life expectancy. –Population-level: Zn exposure (≥ 1 mg/L) inhibited mixis induction and resting egg production.		–Individual-level: 2 mg/L Zn inhibit rotifer survival and fecundity, and shorten lifespan. –Population-level: Zn (≥ 1 mg/L) stimulated mixis induction and resting egg formation.	

NH_3-N (Exposure concentrations: 0–29.3 mg/L)	–Individual-level: higher levels of NH_3-N (≥ 17 mg/L) delayed rotifer maturation and shorten lifespan, resulting in reduced fecundity. –Population-level: rotifer sexual reproduction (fertilization and resting egg production) was sensitively inhibited by ≥ 0.5 mg/L NH_3-N.	–Individual-level: NH_3-N ≥ 8.5 mg/L resulted in delayed maturation, shorter lifespan, and decreased fecundity. –Population-level: significant inhibition of fertilization and resting egg numbers were observed at NH_3-N > 8.5 mg/L and > 1.1 mg/L, respectively.
Swimming behavior		
$FeSO_4 \cdot 7H_2O$	–Fe (20–45 mg/L) induced different swimming responses in male and female rotifers. Male: showed a decreasing pattern as Fe concentrations increased. Female: the swimming speed was stimulated by 20 mg/L Fe.	–A similar pattern with *B. plicatilis* was observed. Male: swimming speed significantly decreased when the concentrations are ≥ 20 mg/L. Female: an insignificant increase was observed at 30 mg/L Fe.
$ZnCl_2$	–Zn exposure (1–2 mg/L) stimulated the swimming behavior of both the female and male rotifers, with 1.4–1.5-fold increases in the swimming speed.	–1.3–1.8-fold higher swimming speed for females and males were detected in response to 1 and 2 mg/L of Zn exposure.
NH_3-N	–The swimming speed of female rotifer increased 1.2- and 1.5-fold in response to 1.1 and 8.5 mg/L NH_3-N, respectively.	–1.2- and 1.4-fold higher swimming speed than that of control was observed at 1.1 and 8.5 mg/L NH_3-N, respectively.
Biological metabolisms		
Neutral lipid accumulation		

$FeSO_4 \cdot 7H_2O$	–Fe ≥ 6 mg/L negatively affected lipid droplet synthesis in the reproductive organs (vitellarium/ovary), supported by downregulated lipid metabolism-associated genes (*acetyl-CoA carboxylase* and mitochondrial *cytochrome*).	–Fe exposure stimulated lipid droplets synthesis, with 1.3–2.1-fold higher levels than control at 6 and 12 mg/L treatments. The mRNA levels of *citrate lyase* and mitochondrial *CYP* were upregulated.
$ZnCl_2$	–2 mg/L Zn exposure reduced lipid contents in the vitellarium/ovary of rotifers. Whereas upregulated *acetyl-CoA carboxylase* and mitochondrial *CYP* expression were detected.	–No significant changes in the neutral lipid contents were detected among treatments.
NH_3-N	–8.5 mg/L NH_3-N induced a 1.5-fold reduction in neutral lipid contents, supported by downregulated *acetyl-CoA carboxylase* and mitochondrial *CYP*.	–No significant changes in the neutral lipid contents were detected among treatments. However, upregulated *citrate lyase* and *ACC* expression were detected.
Sperm viability		
$FeSO_4 \cdot 7H_2O$	–Fe exposure (≥ 20 mg/L) suppressed sperm viability.	–The inhibitory effect on the sperm viability was observed at ≥ 45 mg/L Fe.
$ZnCl_2$	–Zn exposure (1–2 mg/L) significantly inhibited (1.4–2.4-fold) sperm viability.	–A 2.7–2.9-fold enhanced sperm viability was observed in response to 1 and 2 mg/L Zn.
ROS and antioxidant activities		

$FeSO_4 \cdot 7H_2O$	–Fe acute and chronic exposure induced 1.3–2.6-fold higher ROS production than that of controls. –The key antioxidant components, such as SOD and CYP were inhibited.	–1.3–2.6-fold increased ROS production was detected following acute and chronic Fe exposure. –The antioxidant biomarkers and associated enzymes, including SOD, CAT, CYP, and GST were effectively activated.
$ZnCl_2$	–2 mg/L Zn exposure activated mitochondrial membrane potential (ΔΨm), with enhanced mitochondrial complex IV (cytochrome c oxidase) and V (ATPase) activity. –1.3–2.3-fold decrease in ROS production was detected in response to 1 and 2 mg/L Zn exposure. –The antioxidant biomarkers and associated enzymes were activated, such as SOD, CAT, CYP, and GSTs2.	–Zn exposure activated ΔΨm, with upregulated *cytochrome c oxidase*. Moreover, increased ATP levels were measured. –A 2.1-fold higher ROS level than that of control was determined at 2 mg/L Zn. –For the antioxidant components, the activity of SOD was activated, while there were no changes in CAT and CYP.
NH_3-N	–8.5 mg/L NH_3-N exposure caused a 1.3-fold decrease in ROS production. –The transcription of antioxidant SOD and CAT were inhibited.	–Decreased ROS levels (1.3–4.7-fold) were detected following 1.1 and 8.5 mg/L NH_3-N exposure. –Enhanced antioxidant activity was determined.

Table 5-2. Comparion of the acute toxicity investigations in freshwater rotifer *Brachionus calyciflorus* and marine rotifer *Brachionus plicatilis*.

		24 h LC_{50} (mg/L)			
		Marine ***B. plicatilis***	**Freshwater** ***B. calyciflorus***	**Sensitivity factor** ***B. p*** **(LC_{50})/*B. c*** **(LC_{50})**	**References**
Metals	Mercury	0.061	0.06	1.0	Persoone et al., 1989
	Silver	0.12	0.0075	16.0	Snell et al., 1991a, b
	Copper	0.13	0.19	0.7	Snell and Persoone,
	Lead	> 4.0	> 4.0	ND	1989b
	Zinc	> 4.8	1.3	> 3.7	
	Selenium	17	16	1.1	
	Nickel	> 20	4	> 5	
	Potassium	22.2	ND	ND	
	Cadmium	56.8	0.65	87.4	
	Iron	469.6	0.23	2041.7	Present study
		363.6 (*B. rotundiformis*)		1580.9	Santos-Medrano and Rico-Martínez, 2013
Water quality parameter	Free NH_3	17.7–38	3.21	5.5–11.8	Yu and Hirayama, 1986; Snell and Persoone, 1989b
Pesticides	Pentachlorophenol (NaPCP)	1.36–1.9	0.62–1.2	1.6–2.2	Ferrando and Andreu-
	Fenitrohion	8.9	6.7	1.3	Moliner, 1991

	Chlorpyrifos	10.7	11.9	0.9	Marcial et al., 2005
	Diazinon	26.9	29.2	0.9	Snell et al., 1991a, b
	Methylparathion	ND	29.2	ND	Snell and Persoone,
	Lindane	35.9	22.5	1.6	1989a, b
	Malathion	45.5	35.3	1.3	
	Trichlorfon	274.9	51.9	5.3	
	Dichlorophenoxy-	598	117	5.1	
Chemical	Tributyltin	0.3	0.19	1.6	Calleja and
compounds	NaOCl	1.2	0.37	3.2	Persoone, 1992
	Chlotoform	2.4	2	1.2	Snell and Persoone,
	Sodium dodecyl sulfate (SDS)	4.4–5.6	1.4	3.3–4.1	1989a
	Acetone	75	51	1.5	
	Hexane	156	68	2.3	
	Phenol	> 400	> 150	> 2.7	
	Xylene	496	33	15.0	
	Ethanol	36,840	ND	ND	
	Methanol	49,680	ND	ND	

Table 5-3. Comparsion of the chronic toxicity assessments in freshwater rotifer *Brachionus calyciflorus* and marine rotifer *Brachionus plicatilis*.

EC_{50} (mg/L) of growth rate (*r*)					
		Marine ***B. plicatilis***	**Freshwater** ***B. calyciflorus***	**Sensitivity factor** ***B. p* (EC_{50})/*B. c* (EC_{50})**	**References**
Metals	Copper	0.12	0.0025	48	De Schamphelaere and Janssen, 2010
		ND	0.034 (ingestion rate)	ND	
	Zinc	6.15	0.19–1.1	5.6–32.4	Hwang et al., 2016
	Arsenic	12.98	ND	ND	Janssen et al., 1994b
	Cadmium	21.41	0.07	305.9	Lee et al., 2016
	Chromium	82.34	2.9	28.4	Snell and Moffat, 1992
	Plumbum	110.14	ND	ND	
	Iron	59.6	2.49	23.9	
		55.6 (*B. rotundiformis*)	(*Euchlanis dilatata*)	22.3	Present study Hernández-Flores et al., 2020
Water quality parameter	Free NH_3	13.2	3–5 (*B. rubens*, LOEC)	ND	De Araujo et al., 2000, 2001
		2.3 (swimming)			
		2.4 (LOEC, fecundity)			Yu and Hirayama, 1986
		4.9 (LOEC, lifespan)			
Pesticides	Pentachlorophenol (PCP)	ND	0.27–0.4	ND	

Chlorpyrifos	0.3 (NOEC)	0.36	0.8	Fernandez-Casalderrey et al., 1992
Diazinon	1.53	5.2	0.29	Janssen et al., 1994b
	1.28 (fecundity)	ND	ND	Marcial and Hagiwara, 2007
	1.62 (lifespan)			Snell and Moffat, 1992

$EC_{50:}$ Effective Concentration for 50% of inhibition. LOEC: Lowest Observed Effective Concentration. NOEC: No Observed Effective Concentration. ND: No Data.

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Appendix

Table A1. GenBank accession numbers and the primer sets used in this study.

Species	GenBank Accession No.	Gene	Primer sequence (5′ to 3′)
B. plicatilis	Bp_014740T	*iron oxidase*	F: CCTGAAGAAGTGCCCGAGTTG
			R: AACCAAGACTAAGAGCCAAGCC
	Bp_145010T	*iron-sulfur Protein*	F: TGGCTGCTCAATTTGTTCCTC
			R: TCCTGAAGTACCACCAGTATCA
	Bp_027720T	*iron-sulfur cluster*	F: CTGGTCGTTGAGAAGCAAGGAT
			R: TTATGCTGGCTTGTTCCGAGTT
	Bp_056850T	*NADPH oxidase 5*	F: CATCGCCTCTTCGCCTACTTG
			R: CACCATCGCTGTCCACATCAA
	Bp_CYP3335A1	*Mitochondrial cytochrome*	F: GATCCTGAAATTGCAGAACAAG
			R: GTTCTTAAAATTCGCCAACG
	Bp_167200T	*copper/zinc superoxide dismutase*	F: AAGCAGTGGCAGTCCTAATTGG
			R: CGCCGAACTGGTGAATGTGA
	Bp_025300T	*calmodulin*	F: CCAAGATCACTCGTTCCAAGGT
			R: CTCTGAAGCGACACTCTAAGGT
	Bp_107550T	*citrate lyase*	F: GCCTTGAGTCTATCTGCTGAA
			R: GAGTCCTTGGTTCTTGATGCT
	Bp_011640T	*acetyl-CoA carboxylase*	F: GCATTCAGCACACGCAAGA
			R: GAGGAGGAAGAGGAGCAGTC
	Bp_148890T	*p300/CREB-binding protein*	F: TGCTCATATTCGGCTCACACTT
			R: TGTCATTGGAACCAGTGGCTAA
	Bp_184930T	*cdc42p*	F: TGTAGTAGGAGACGGAGCAGTG
			R: AGACGGTCGTAGTCCTCTTGG
	Bp_042560T	*histone H2A*	F: ATCGGCTGTCCTGGAATACTTG
			R: ACACCACCTTGAGCAATAGTGA
	Bp_102670T	*septin-2*	F: TGCGGTAGTTGGTTCTAGTCAG
			R: GGAGCCTAGCCGTTCAGATC
	Bp_082650T	*cathepsin L*	F: CAAGTTCAATGGCACGCTCAG
			R: CAGCATCCACCGCAGTCAA
	Bp_195460T	*sirtuin-2*	F: GCGAATGCTTGCCAACTGAATT

			R: CAGCCAGCGGTAGCCTAGAA
	Bp_CYP3048A2	*CYP* (*Clan2*)	F: GACAGAAGCGCCTCATATAA
			R: AAGATGTGAAGCGAGACTTT
	Bp_CYP3045A2	*CYP* (*Clan3*)	F: GCTGACTATGGACTCGCTTTG
			R: CAGATCGGGTCCAACTCG
	Bp_191940T	*cytochrome c oxidase*	F: TGGAGTTCGATTCAGCTCTACC
			R: TTCGGCACCATACAACAAGTC
	Bp_110990T	*caspase-2*	F: CCTAGACGACATATTCACCAT
			R: ATCCGCCTCATCTGACTC
	Bp_084940T	*caspase-7*	F: CGATGGAACCGATGTGGATG
			R: CGACTTGGCGTATCTAAGCATT
	Bp_105060T	*acetylcholinesterase*	F: TCCATCTGAGGCAGGTAGGT
			R: TTCGCACGCATTCAATTCCA
	AB513493.1	*elongation factor 1-alpha 1*	F: GACGCCATTGTTCCACCATCA
			R: GGCTGGAGCAAAAGTGACAAC
B. rotundiformis	BRGM016580T0	*iron oxidase*	F: GCCTGGCTCTAAGTCTTGGTT
			R: ACCGTAATCTGTATGCTCTCCA
	BRGM018560T0	*iron-sulfur protein*	F: TGCTTCAGCCTTCAACGATTCA
			R: TCACTACGGCGGCATTCCT
	BRGM028040T0	*iron-sulfur cluster*	F: ACCAAAGTCCTCCGTCACAAAT
			R: AGCAGCAGTTCTCATCCATACA
	BRGM016560T0	*NADPH oxidase 5*	F: CTGGCTCGTTCTGGCAATCAT
			R: AAGAATGGCAGGACCGATGAAC
	Br_CYP3047B1	*Mitochondrial cytochrome*	F: CTACAGCCACAGCCATTCATTA
			R: TGTTATCTCGCCGCTATTGC
	BRGM100200T0	*copper/zinc superoxide dismutase*	F: TCGCTTGGTTCGTCTCAATGG
			R: TTGCTCAACTCAGTTCCACCTT
	BRGM068540T0	*citrate lyase*	F: GCCTTGAGTCTATCTGCTGAA
			R: GAGTCCTTGGTTCTTGATGCT
	BRGM001480T0	*acetyl-CoA carboxylase*	F: CGGCTCCAACAACAACAACTA
			R: CGGTCCTATGAACGCAATGTC
	BRGM072140T0	*calmodulin*	F: AGTGACCTGCCATCGTCCAA

			R: CCAGTCCCGTCCAAATCAAACA
	BRGM014040T0	*p300/CREB-binding protein*	F: TTCTATGTCAGTGGTGCTCGTA
			R: CCGTTGTCTAGTGCTCATAACC
	BRGM082810T0	*cdc42p*	F: GCTGGTTGTTGTAGGTGATGG
			R: CTAAGTCGGTCGTATTCCTCCT
	BRGM014830T0	*histone H2A*	F: GCTGGCAATGCTGCTAGAGATA
			R: CACCACCTTGAGCGATTGTTAC
	BRGM023250T0	*septin-2*	F: CCAGGAAAGGAGCAAGCAACT
			R: GCTATTGTTACCAGGCCGTCTT
	BRGM019140T0	*cathepsin L*	F: GCTACTTGGCTGGGATTGTGAA
			R: CCGATGCTGGTTCCTGTATTCC
	BRGM084160T0	*sirtuin-2*	F: CTAGCGGAATTGTTAAGCCTGA
			R: AGCCTAGAGAATGGAGCAACTT
	BRGM034860T0	*elongation factor 1-alpha 1*	F: CATTCTCGGCAGTCTTCACAAT
			R: CGTGGTCAGTCGCTAGAAGT
	Br_CYP3043A1	*CYP* (Clan2)	F: AGGTAGAGCCGCCCAATTAC
			R: AGCTTCGGTCAGTGCATAGG
	Br_CYP3045C10	*CYP* (Clan3)	F: GGTTTTGGAGCTGGACCTCG
			R: TTCCTTCCCAGGCGTCAATG
	BRGM048110T0	*cytochrome c oxidase*	F: AACTCAATGGCTGGTCTAC
			R: GCGTTCATCTCACACTTAG
	BRGM065410T0	*caspase-2*	F: TCGTCCTTAGCCAACTCCAA
			R: GCGTTCATATACCAACTGCCTA
	BRGM008330T0	*caspase-7*	F: GAGACTTGATGCTTAGATACG
			R: GCCTCTACACGCTTGAAT
	BRGM030420T0	*acetylcholinesterase*	F: CCGAACTTCTGTCATCTGGTAG
			R: ATCCTGTGGAAGAACATTGAGT
Two species	BAH28837	*catalase*	F: GGAATCGAGCCATCACCAGA
			R: GCATTGTGGACCATCACGTT
	RNA21457	*manganese superoxide dismutase*	F: AGCACCAACTGGAACTTTGA
			R: CTGATGGCCAGAGATCCGTC
		GSTs2	F: TGCAAGAGGTCGAGCTGAG

		R: TGACCCAGAGGAGCTTTCTT
BRGM023370T0	*GSTo1*	F: TTGCCGAAGACTTGCTCAAA
		R: CCAATCTCTCGATCCACGGC
GU969245.1	*vasa*	F: GAGTCAGTTGAGCGGCATGC
		R: AGCCGAAGAAAACACCACCG
AB076052.1	*Hsc 70 like 1*	F: CGACAACCGACTGGTCAACC
		R: GCTCTGGTGATGCTCGTGTAG