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Physiological responses in crustacean zooplankton to abiotic and biotic stresses, especially lowering pH and crowding

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Acknowledgments

The studies were conducted in the Laboratory of Aquatic Ecosystem in Department of Ecosystem Studies, **The University of Shiga Prefecture**, Japan. All experiments complied with the current laws regarding the treatment of animals of the country in which they were performed.

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Abstract

Zooplankton play a key role in aquatic food webs as both primary consumer and secondary producer. Physiological activities, such as metabolism, ingestion, growth and production, contribute to total energetic flux in an ecosystem. Zooplankton metabolism through biological processes were largely influenced by environmental factors including temperature, pH, food conditions, and population density, in turn may influence population dynamics. Therefore, clarifying the process that control the zooplankton metabolism is a major objective for understanding aquatic ecosystem productivity.

Cladocerans and copepods are two important components of zooplankton in lake ecosystems, and can be good candidates to clarify responses of metabolism to the different environmental parameters in aquatic organisms. In this study, I examined effects of both abiotic and biotic environment factors, i.e., starvation, food shortage, crowding, acidification, low temperature, on respiration rate and life history traits in three different zooplankton taxa, to clarify how zooplankton respond to these environmental stresses.

In cladoceran *Daphnia magna*, metabolic rate as respiration rate decreased under starvation conditions, especially for juveniles. The starvation resistance ability largely depends on the body size. In acclimatizing food limited conditions, metabolic rates were significantly depressed with somatic growth and reproduction, and consequently net growth efficiency decreased. *D. magna* also showed lower metabolic rates in the crowded condition at both high and low temperatures with interaction effects. The

density-mediated depression of metabolic rate may be related to lowering food uptake, and the crowding effect may be decelerated at cold water. Acidic stresses to survival and metabolic rates varied among the three zooplankton taxa, *Daphnia pulicaria*, *Eodiaptomus japonicus* and Cyclopoida spp.; *D. pulicaria* did not respond acidic stress while two copepod taxa reduced respiration rates in low pHs, <7. This depression in the copepods might be related to lowering swimming activity under lowering pH. These results will provide some metabolic parameters of zooplankton under these abiotic and biotic environmental stresses which are essential for learning and predicting the quantic ecological processes.

Keywords

Zooplankton, Cladocera, copepod, metabolism, respiration, growth efficiency, starvation, food shortage, crowding, population density, acidification, acute acidic stress, proton concentration, swimming behavior, freshwater lake

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CONTENTS

General Introduction

1. Zooplankton in aquatic ecosystem

Zooplankton play an important component in aquatic ecosystems as primary consumer or secondary producer, collect organic matters such as phytoplankton, bacterioplankton, organic detritus, and even smaller zooplankton, and then deliver the organic matters and energy to higher trophic level organisms such as fish. Therefore, zooplankton determine the distribution, abundance, and productivity of higher trophic levels. In global scale, zooplankton production has been estimated at more than 4.2 billion tons carbon in a year, suggesting that the zooplankton biomass plays a large part of temporary stocked carbon as their bodies in carbon fluxes through food chain, and long-term storage carbon via delivering organic matters into the deep floor after sinking their dead bodies (Del and Williams 2005; Steinberg and Landry 2017; Bar-On et al. 2018). Zooplankton can detect the subtle alterations in some chemicals particularly at lower levels, and therefore can be used as sensitive biomarkers for toxicity assessment and monitoring of water quality (Duquesne and Küster 2010; Asghari et al. 2012; Bownik 2017).

2. Environmental factors affecting zooplankton physiology

Physiological activities, such as metabolic, growth and reproduction rates were influenced by several environmental factors such as temperature, pH, food

concentration, and population density. These interactions consequently affect abundance, distribution, and community structure of zooplankton, and then impact aquatic ecosystems through food web.

Zooplankton generally encounter to food shortage and/or starvation, especially in both oligotrophic and mesotrophic lakes due to low nutritional loading (Goldman 1988; Winder and Sommer 2012). Urabe (1988) found decreases in growth and reproduction of *Daphnia galeata* under lower food concentration. Gliwicz and Guisande (1992) showed that starvation significantly decreased the survival rate of *Daphnia pulicaria* and *Daphnia hyaline*. Cressler et al. (2014) reported starvation stress reduced fitness of *Daphnia magna*.

Zooplankton population density varies spatio-temporarily in lakes and ponds, and extremely high densities often found in several cladoceran species from spring to summer. In summer, more than 1000 ind. L^{-1} has been recorded in *Daphnia hyalina lacustris* in the gravel pits adjacent to the river Great Ouse, UK (Davies 1985), *Daphnia pulex* in lake Cisó, Spain (Jürgens et al. 1994) and *Daphnia longispina* in Lake Myravatn, Norway (Kvam and Kleiven 1995). It has been shown that the growth and reproduction of some zooplankton decreased with increasing population density (Lee and Ban 1998; Burns 2000; Ban et al. 2009).

Acidification has been shown to reduce the biomass and species richness of zooplankton (Beamish et al. 1975; Hendry and Brezonik 1984; Jeffries et al. 2003). In an individual level, high proton concentrations can disrupt its physiological functions, such as the acid–base balance and ion–regulation (Henry and Wheatly 1992; Pequeux

1995), and damage important sense organs (gills and heart muscle), negatively affecting zooplankton productivity (Brett 1989). For the community level, Holt et al. (2003) investigated the crustacean zooplankton communities of various 47 acidic lakes in south-central Ontario, Canada, and reported a pH 6.0 to be a threshold level for species richness and abundance.

Temperature also regulates filtering rate, development time, reproduction, survival rate in zooplankton (Heinle 1969). Growth and reproduction rates in *D. magna* reached a maximum value at 20 ˚C in the experimental range between 15 and 30 ˚C (Giebelhausen and Lampert 2001). In temperature range from 10 to 25 ˚C, the life history parameters, i.e., average lifespan, age at first reproduction, body size, number of neonates per female, in *Daphnia parvula* showed significantly different between the temperatures at an unlimited food condition (Orcutt and Porter 1984). At temperatures between 3 to 15 ˚C, the post-embryonic development times of marine copepod *Pseudocalanus newmani* exponentially increased with decreasing temperature, while the weight-specific growth rates linearly increased with temperature until 15˚C (Lee et al. 2003).

3. Metabolism of zooplankton

Metabolism includes several biological processes, e.g., energy uptake and transformation, allocation of chemicals and energies in an individual organism (Brown et al. 2004). All forms of the activities, such as feeding, growth and reproduction, contribute to total energetic flux in an organism, and therefore, nearly all interactions

between an organism and its surrounding environment are reflected in its metabolic rate (Ikeda and Motoda 1978; DeLong et al. 2014).

Previous studies showed that the metabolic rate responses to starvation varied in zooplankton species. Ikeda (1977) shown that 2 - 49 days starvation progressively decreased the respiration rates of marine zooplankton. However, Klumpen et al. (2021) reported that respiration rates of un-fed *D. pulex* were comparatively stable during 64 hours from start of the experiment. Lampert (1986) found that respiration rate of previously well-fed *D. magna* quickly reduced its respiration rate after cutting off the food supply. These differences for metabolic responses to starvation may depend on fasting time and its energy reserves in zooplankton (Ikeda 1977). For the energy reserves in zooplankton, Bychek et al. (2005) reported that *D. magna* tolerated short-term starvation without major changes in lipid metabolism. Tessier et al. (1983) showed that the quantity of visible lipid contents in its body positively increased with body mass in *D. magna* and *D. galeata mendotae*.

It has been also shown that food quantity affects metabolic rate of zooplankton (Kees and Corrie 1976; Urabe and Watanabe 1990; LaRow et al. 2017). Kees and Corrie (1976) showed that the respiration rates of *D. ma*gna increased to a maximum value with increasing food concentration when the food concentration below a certain critical level, then decreased if the food concentrations continuously increased over the certain critical level. Porter et al. (1982) reported that high food concentration (above the incipient limiting concentration) may cause an over-collection of food in *D. magna* and resulted in a high respiration loss. Urabe and Watanabe (1990) found that respiration

rates of *D. galeata* and *Bosmina longirostris* increased with increasing food concentration and reached to a plateau at a certain food density and suggested that it may be caused by specific dynamic action (SDA), energetic cost required to process food. Kiørboe et al. (1985) also showed that respiration rates of copepod *Acartia tonsa* in well-fed condition were 4 times higher than those in starved one and suggested that it is mostly attributed to increasing the costs of assimilation and biosynthesis.

High population density has been shown to negatively affect zooplankton physiology and life-history traits (Lee and Ban 1998; Ban et al. 2009). However, it remains unclear how the population density influences its metabolic rate. DeLong et al. (2014) showed that metabolic rates of organisms including primary producers to consumers decreased with increasing population density even though temperature and body mass were main factors on influencing variation of metabolic rate. Whereas Yashchenko et al. (2016) showed no effect of population density on metabolic rate in *D. magna*.

It has been shown that acidification reduces the biomass and species richness of zooplankton (Hendry and Brezonik 1984; Jeffries et al. 2003). However, information about the effect of acute acidic stress on zooplankton metabolism are not so much yet, and vary with species. The threshold pHs to survive after 48 h exposure are different among species in cladocerans (Bulkowski et al. 1985; Price and Swift 1985; Bruns and Wiersma 1988). The metabolic rates of *D. magna* decreased with increasing pH from 7.3 to 4.0 (Alibone and Fair 1981), whereas those of *D. pulex* are similar between pH 5.5 and 7.8 (Weber and Pirow 2009).

4. The technique for respirometry of zooplankton

Metabolism is sum of all biochemical reactions and that reactions requiring adenosine triphosphate (ATP) to drive (Brown 2004). ATP is generated from the tricarboxylic-acid (TCA) cycle and oxygen is demanded to join the cycle. Therefore, oxygen consumption or respiration rate is generally used as a good proxy of metabolic rate (Roger et al. 2000). Whereas the respiration of micro-size animals such as zooplankton due to their small body size and low oxygen demand (Liu et al. 2017). The method mostly used for measuring respiration rate of zooplankton, such as Winkler titration method (Lee et al. 2001), oxygen electrode method, and method of indirectly measuring the total enzymes related to respiration (Devol, 1979), are sensitive and complicated on procedures and required a large number of animals (Nakamura and Turner 1997). These methods easily introduce some biases and hardly to exclude such as crowding effect during measurement. Recently, optical oxygen sensor was developed to measure the dissolved oxygen concentration in water. The new method with light quenching technique using near infrared light is contactless, non-destructive, convenient for measuring oxygen consumption rate of zooplankton in a small volume of water from outside a measurement chamber. Therefore, I used this technique throughout my study.

5. The scientific approach and Thesis contents

Cladocerans and copepods are two mostly important components of crustacean zooplankton in freshwater lakes (; Makarewicz and Likens 1979; Liu et al. 2020). For

example, *Daphnia* spp. as cladoceran, *Eodiaptomus japonicus* and Cyclopoida spp. as copepod have been shown to be dominant, > 90% of the total crustacean biomass in Lake Biwa, the largest lake in Japan (Liu et al. 2020). Cladoceran, especially *Daphnia* spp., is one of the most widely distributed zooplankton taxa in freshwater lakes and plays an important role in the trophic dynamics of aquatic ecosystems.

In this thesis, *Daphnia magna* was used as an experimental animal because of its breeding pattern of parthenogenetic allows it as a clone to maintain the same genome. I measured the metabolic rates of *D. magna* under starvation, after acclimatization of food shortage and at different population densities at two different temperatures, to clarify how zooplankton physiologically respond to those abiotic and biotic environmental stresses, being helpful to learn the energy allocation on individual level and the competitive strategy on population level in zooplankton. Previous studies shown that effect of acidic stresses on zooplankton varied with its genotype and chemical composition of surrounding water (Hendry et al. 1984; Price et al. 1985). Therefore, using zooplankton species and water from same lake is more meaningful. I determined the acute effects of acidic stress on survival and metabolic rate in three dominant zooplankton colonizing from Lake Biwa (*D. pulicaria*, *E. japonicus* and Cyclopoida spp.) at two temperatures and swimming behavior of *E. japonicus* to clarify effect of acidic stress on individual level and to evaluate the potential effect on zooplankton community. These basic knowledge about the effects of environmental stresses on zooplankton metabolism may allow us to learn and predict the ecological processes in aquatic ecosystems.

This thesis is structured as follow:

Chapter 1: Effect of starvation on metabolic rate of juvenile and adult *Daphnia magna*

Chapter 2: The effect of food shortage on growth, reproduction, and metabolic rates in *Daphnia magna*

Chapter 3: Density-mediated metabolic rate in *Daphnia magna* interacted with temperature

Chapter 4: Effect of acute acidic stress on survival and metabolic activity of zooplankton from Lake Biwa, Japan

Chapter 1:

Effect of starvation on metabolic rate of juvenile and adult

Daphnia magna

1. Introduction

Metabolic rate is a rate of biological processes of energy and materials, and nearly all interactions between an organism and its environment are reflected in its metabolic rate (Brown et al. 2004; DeLong et al. 2014). Therefore, the metabolic rates of zooplankton were used to predict the life history attributes, population dynamics and ecological processes (Price et al. 2010; Price et al. 2012; O'Connor and Bernhardt 2018). Starvation is common for pelagic zooplankton in both oligo- and mesotrophic lakes because of low nutritional supply (Goldman 1988; Winder and Sommer 2012; Liu et al. 2021). Besides, to evaluate the production changes in an aquatic ecosystem, many researchers estimated the metabolic rate of zooplankton with traditional method, "water bottle method" (Omori and Ikeda 1984). Since no foods are generally supplied during the measurement in the traditional method, the metabolic rates may vary during the experiment, and consequently make some biases for evaluating the production from the metabolic rates.

In long-term experiment for metabolic rates under starvation, those of unfed zooplankton are variable during the experiment. For example, respiration rates of marine copepod *Calanus plumchrus* under starvation decreased to nearly zero during the first 12 days, then increased from day 13 to day 16, and decreased thereafter. Those in unfed *Euphausia pacifica* increased within first two days, but decreased thereafter until day 7 (Ikeda 1977). Respiration rates in unfed *Parathemisto pacifica* also increased within a day from the start of the experiment, and then decreased thereafter, while those in unfed *Pleurobrachia pileus* constantly decreased until day 8 (Ikeda 1977).

In short-term experiment, Lampert (1986) showed that respiration rates of the well-fed *D. magna* decreased within first one hour after replacement to no-food water, while those of starved one increased within first 2 hours after food was resupplied. Folgado (2010) also found that respiration rates of *D. magna* decreased in the first 2 hours after fasting, then kept constant for the following 3 to 12 hours. Whereas, Klumpen et al. (2021) found that respiration rates of un-fed *Daphnia pulex* were comparatively stable during 64 hours from start of the experiment.

Besides, the energy allocation under starvation was different between the juvenile and adult zooplankton. It has been shown that the respiration loss of energy was different between juvenile and adult in daphnids (Tessier et al 1983; Bradley et al. 1991). Amount of the storage in well-fed daphnids are also different between juvenile and adult; triacylglycerol and lipid in *Daphnia galeata mendotae* and *D. magna* were stored more in adult than those in juveniles (Tessier et al. 1983; Yang et al. 2021). Zooplankton in different ages may therefore make a different tolerance for starvation.

In this chapter, I determined respiration rates of juveniles and adults in well-fed *D. magna* after 2 to 24 h and 1 to 7 days, to clarify how such medium-term starvation period affect zooplankton metabolism.

2. Material and Methods

2.1 Stock cultures

Stock cultures of *D. magna* were maintained in a 500-mL glass jars filled with tap

water filtered with a glass fiber filter (pore size: 0.7 μm, Whatman, GF/F) autoclaved (120˚C for 15 min) and well oxygenated for ~24 h (FTW). The stock cultures were placed in an incubator (Sanyo, MLR-350) with a photoperiod of 12L:12D and light intensity of ca. 130 μmol m^{-2} s^{-1} at 20 ˚C. All animals were fed with fresh green alga *Chlamydomonas reinhardtii* (IAM C-9) at cell concentration of ~ 5 × 10^5 cells mL^{-1}, equivaled to 20.5 μg C mL^{-1} (Lampert 1987; Mitchell et al. 1992). The fresh food suspensions were supplied every 2 days. Algal cultures of *C. reinhardtii* were maintained in 1-L conical flasks with the autoclaved C medium (Ichimura 1971) under the same temperature and light conditions as the zooplankton culture.

2.2 Experimental animals

In order to exclude density effects during the stock culture, a single female sorted from the stock culture was individually reared in a 50-mL glass bottle under the same temperature and food conditions as the stock culture. Algal food suspensions were exchanged every day (Laabir et al. 1995). The animals grew up adult, produced eggs, and then the new-born neonates from the third clutch were reared at the same manner of the 1st generation. After the animals in 2nd generation matured, new-born neonates from the third clutch were also reared at the same manner until the respiration measurement. In the following experiments, respiration rates in juveniles (3-day old animals) and adults (6-day old animals) were determined after 2 – 24 h and 2 – 7 days starvation periods.

2.3 Measurement of respiration

Respiration rate as a good proxy for metabolic rate in zooplankton was generally expressed as the oxygen consumption rate over time (Del and Williams 2005; Liu and Ban 2017). Oxygen consumption rates of juvenile and adult animals were determined at different starvation periods at 20 ˚C to clarify how such medium-term starvation periods affect zooplankton metabolism. All measurements were finished within 4 hours before the dissolved oxygen (DO) concentration declined to < 80% of the initial value to exclude the effect of low DO concentration on respiration of *D. magna*. There were no food supplies during the measurements.

In the measurements, five gas-tight glass bottles (20-mL) were used for measuring the oxygen consumption of the experimental animals. The DO concentration in each bottle was measured using a fiber-optic oxygen meter (PyroScience, FirestingO_2), fitted with a spot-fiber oxygen sensor (PyroScience, SPFIB). This allowed semi-continuous (every minute) measurements using four oxygen sensors (three for experimental bottles with animals and one for control bottles without animals) with a submersible temperature sensor (PyroScience, TSUB21) in the remaining bottle. An oxygen sensor spot (PyroScience, OXSP5) was glued to the inner wall of each experimental bottle to non-invasively and non-destructively measure the DO concentrations with the oxygen sensors from outside the bottles.

Prior to the measurement, more than 25 well-fed juveniles (3-day old animals) or adults (6-day old animals) were sorted from experimental culture and gently washed at

six times using FTW to remove algae and bacteria to avoid any contamination. After washing, the well-fed animals transferred to new glass bottle filled with FTW without food and settled this moment as the fasting 0 h. Each 5 individuals that experienced different fasting times were transferred into an experimental bottle filled with FTW, and monitoring the DO concentration was started. During the 4 hours of measurement, DO concentrations in the experimental bottles usually fluctuated within the first 2 hours probably due to increasing activity of the animals (Liu and Ban 2017; Gao et al. 2022), then the DO concentration decreased linearly for the following two hours. The data for linearly decreasing DO concentration were used for calculating oxygen consumption rate.

After the respiration measurements, the all five animals in each experimental vessel for the 3-day old *D. magna* females were transferred to a pre-combusted and pre-weighed aluminum pan, and dried at 60 ºC for 24 hours with a muffle furnace (DK600, YAMATO). Then, the total weight was measured with an electro-balance (MT5, Mettler Toledo, accuracy: 1 μg), and a body dry-weight of an experimental animals (W_t, μg ind.$^{-1}$) was calculated for excluding the weight of the aluminum from the total weight and dividing by the number of the animals tested (five). The 6-day old *D. manga* female started to carry eggs/embryos in their brood pouch, and it may induce overestimation of respiration rates because of including those in the eggs and/or embryos. After the respiration measurement, therefore, if the eggs were found, number of the eggs in the brood pouch for each female was directly counted under a microscope (Olympus, SZX12, Japan). After that, the dry weight of the animals including eggs were measured

as the same manner as those in 3-day old animals.

Respiration rate (R, $\mu LO_2\ ind^{-1}\ h^{-1}$) was calculated from oxygen consumption over time, using the coefficients of oxygen consumption (slope of the linear regression line of DO concentration in both experimental (ΔO_{exp}) and control bottle (ΔO_c) against incubation time) and the following equation (1-1);

$$R = \frac{(\Delta O_{\mathrm{exp}} - \Delta O_{\mathrm{c}}) \times V}{N} \times 1000 \qquad (1\text{-}1),$$

where V is a volume (L) of the experimental bottle, N is number of the animals in an experimental bottle. Weight-specific respiration rate (R_w, mgC mgC-weight^{-1} h^{-1}) of 3 or 6-day old *D. magna* was calculated using following equation (1-2);

$$R_w = \frac{(R - R_{\mathrm{b}}) \times 0.536}{(W_{\mathrm{t}} - W_{\mathrm{b}}) \times 0.447} \qquad (1\text{-}2).$$

The factors of 0.447 and 0.536 were used for converting μg body dry weight and μL oxygen consumption to carbon body mass (μgC) and carbon loss rate for respiration (μgC h^{-1}) (Dumont et al. 1975; Liu and Ban 2017). W_b is the dry-weight of eggs/embryos (μg brood^{-1}) in each female and calculated using the following equation (1-3);

$$W_b = N_{\mathrm{e}} \times W_{\mathrm{e}} \qquad (1\text{-}3),$$

where N_e is number of eggs, W_e is dry-weight of an egg or embryo. The weights of an egg and embryo carried by a 6-day old *D. magna* was 8.00 and 7.62 μg, respectively (Dumont et al. 1975; Sobral et al. 2001). R_b is respiration rate of the eggs/embryos in a brood pouch ($\mu LO_2\ brood^{-1}\ h^{-1}$) which was calculated using the following equation (1-4);

$$R_b = W_\mathrm{b} \times R_\mathrm{e} \quad (1\text{-}4),$$

where the R_e is weight-specific respiration rate of egg or embryo from the literatures, 1.94 and 4.92 μLO_2 mg-dry-weight^{-1} h^{-1} in an egg and embryo, respectively (Glazier 1991; Sobral et al. 2001).

2.4 Statistical analysis

Differences in the body dry-weight excluding eggs/embryos and weight-specific respiration (R_w) of both juveniles and adults among different starvation periods were tested with one-way analysis of variance (ANOVA). The post-hoc Tukey-Kramer tests were also conducted when the ANOVA revealed significantly different. All statistical analyses were performed with SPSS software (IBM 2015) and R computing environment, version 4.1.0 (R Core Team 2021) at a significance level of $P < 0.05$.

3. Results

In the starvation periods within 24 h, R_w were 0.007–0.015 mgC mgC-weight^{-1} h^{-1} in juveniles, and 0.005–0.011 mgC mgC-weight^{-1} h^{-1} in adults (Fig. 1-1a, b). Average R_w of the juveniles was 0.014 mgC mgC-weight^{-1} h^{-1} after < 12 h starvation while 0.009 mgC mgC-weight^{-1} h^{-1} after > 13 h starvation, being significantly higher in former one than those in later one (One-way ANOVA, $P < 0.05$); There were neither significantly different among the starvation periods of <12 h nor those of >13 h (The post-hoc Tukey-Kramer test, $P > 0.05$). Whereas, there were no significant differences in R_w of the adults among any starvation periods (One-way ANOVA, $P > 0.05$). For the

starvation periods of 2-7 days, R_w of adults decreased from 0.01 to 0.002 mgC mgC-weight^{-1} h^{-1} with increasing the starvation periods (Fig. 1-2c). Average R_w of adults was 0.009 mgC mgC-weight^{-1} h^{-1} in the starvation period within 24 h. R_w was significantly different between the starvation periods of 1-3 days and 4-7 days (One-way ANOVA, $P < 0.05$).

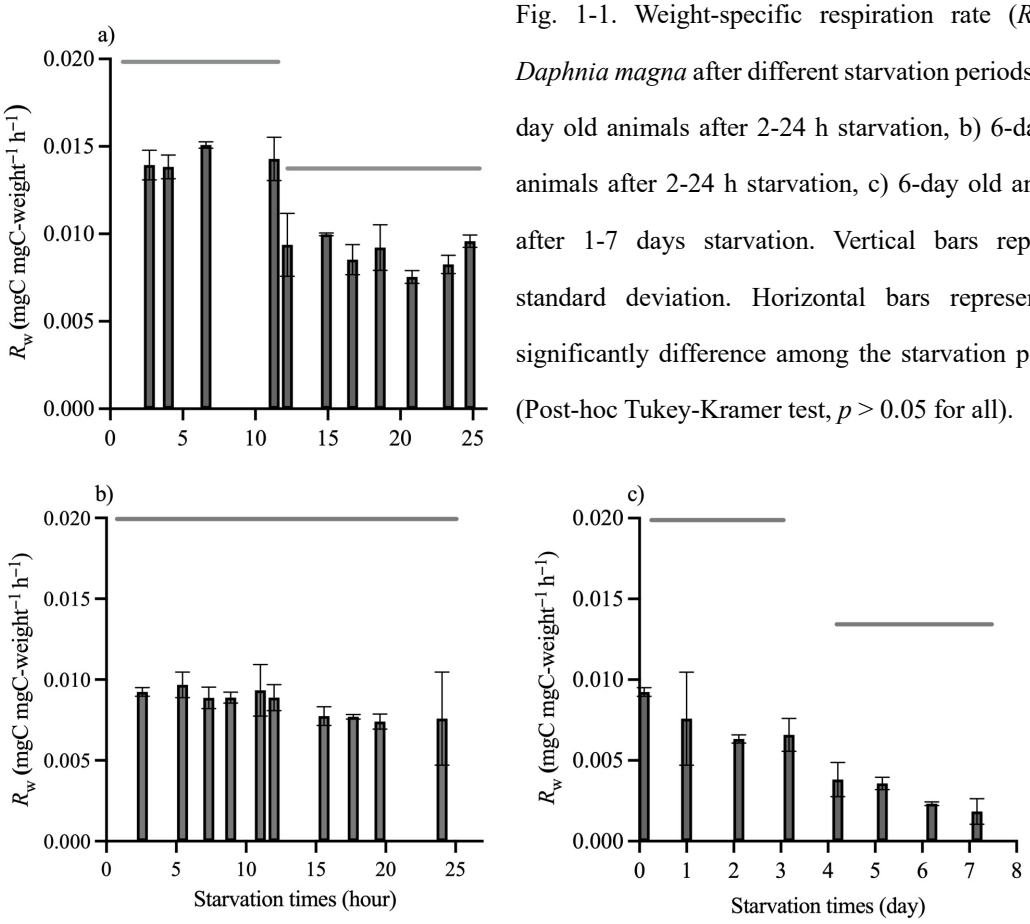

Fig. 1-1. Weight-specific respiration rate (R_w) of *Daphnia magna* after different starvation periods. a) 3-day old animals after 2-24 h starvation, b) 6-day old animals after 2-24 h starvation, c) 6-day old animals after 1-7 days starvation. Vertical bars represent standard deviation. Horizontal bars represent no significantly difference among the starvation periods (Post-hoc Tukey-Kramer test, $p > 0.05$ for all).

The post-hoc Tukey-Kramer test showed that R_w were neither significantly different among 1–3 days and 4–7 days starvation periods ($P > 0.05$).

A body dry weight of *D. magna* varied 22.3–32.3 μg ind.$^{-1}$ and 45.1–110.2 μg ind.$^{-1}$ for juvenile (3 days old) and adult (6 days old), respectively (Fig. 1-2). Those in

both juveniles and adults were not significantly different among the starvation periods within 24 h, while those after 3-days starvation periods were significantly lower than those after less than 2 days starvation.

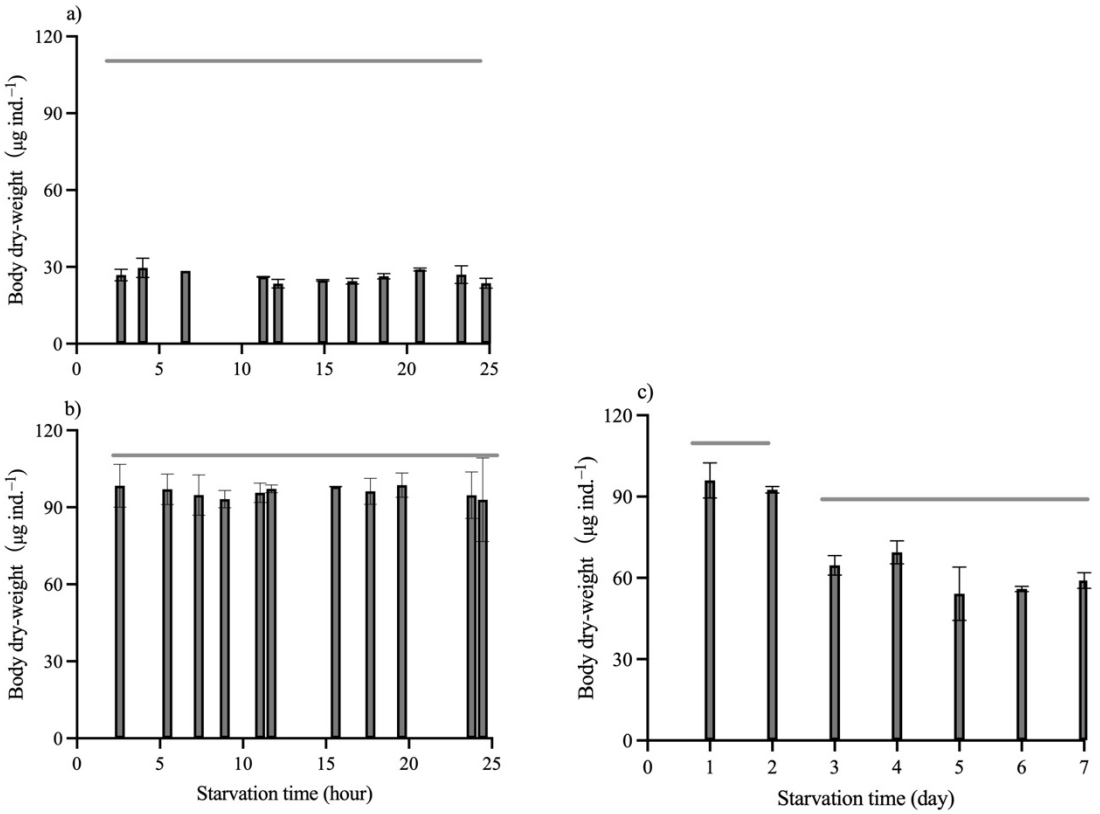

Fig. 1-2. Body dry-weights of *Daphnia magna* after different starvation periods. a) 3-day old animals after 2-24 h starvation, b) 6-day old animals after 2-24 h starvation, c) 6-day old animals after 1-7 days starvation. Error bars represent standard deviation. The result at one day starvation in this panel is the average of the results within 24 h data in b). Horizontal bars represent no significant difference among the starvation periods (Post-hoc Tukey-Kramer test, $p > 0.05$ for all).

To more easily understand the effect of starvation, the R_w of *D. magna* females in starvation also compared with those in well-fed ones (the data from well-fed individuals in chapter 2 and 3). The values of R_w in juveniles were depressed from those in well fed ones after > 13 h starvation, while the body weights were almost the same under

the starvation (Fig. 1-3). For the adults, the values of R_w were significantly depressed under the starvation periods of 2 days, while the body weights also did after 3 days starvation.

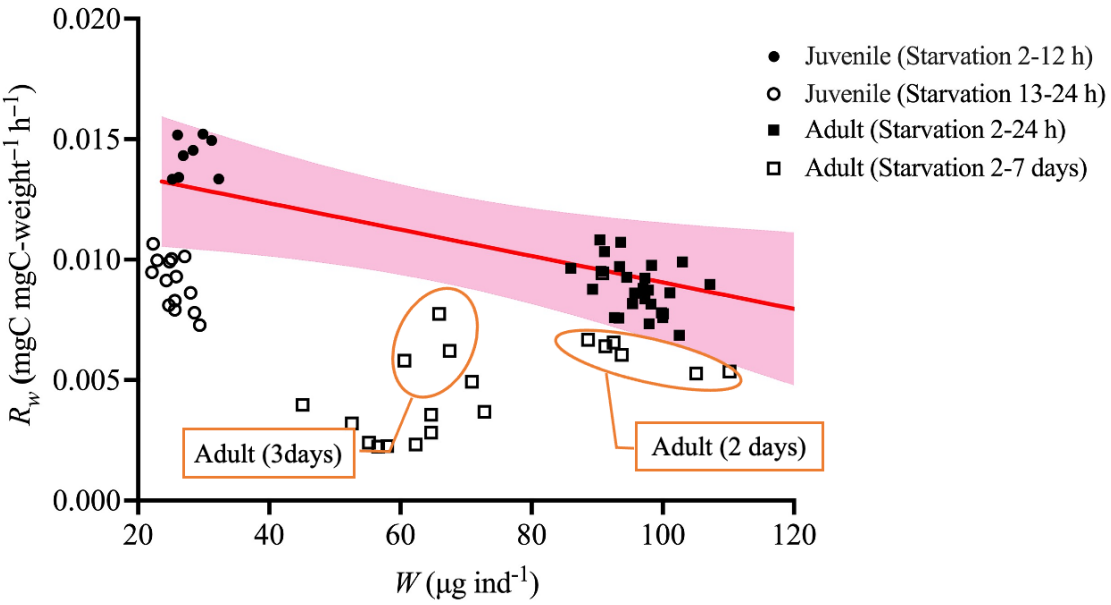

Fig. 1-3 Relationship between body dry weight and respiration rate in staved and well-fen *Daphnia magna*. Closed and open circles represent starvation period of 2–12 h and 13–24h for juvenile, respectively. Closed and open squares represent starvation period of 2–24 h and 2–7 days for adult, respectively. The red line indicates a regression line from well-fed *D. magna* and red area represents the 95% confidence interval of the regression line (the data from Chapter 2 and 3).

4. Discussion

Starvation-mediated respiration rates of zooplankton has been reported in many zooplankton species, such as copepod *C. plumchrus*, *P. parvus*, *Parathemisto pacifica* and *Pleurobrachia pileus* (Ikeda 1977). Respiration rates of well-fed *Acartia tonsa* decreased to 58% of initial value after 36 h starvation (Thor 2003). In this study, we also confirmed that respiration rates of *D. magna* decreased after starvation, but the effective period was different between juvenile and adult. Respiration rates of juveniles

declined after 13-h starvation, while those of adults declined after 4 days starvation. This result suggests that starvation resistance ability largely depends on body size, i.e. small animals can keep its respiration rate stable only in several hours, but large animals can keep its respiration rate stable for several days.

Maintaining the bioenergetic supply-demand function in aquatic animals depends on a balance of inputs (e.g., ingestion, food assimilation) and outputs of energy (e.g., growth, reproduction, metabolic loss) (Carlotti et al. 2000; Ban et al. 2008). Metabolic rates in zooplankton decline when the starvation time reach to a critical point. Energy allocation model under starvation showed that metabolic maintenance in zooplankton are supported by the energy source stores and maintain priority for growth (Bradley et al. 1991).

Difference of starvation resistance ability is related to quantity of the stored energy and release rate of sources from their internal stores (Maino et al. 2014). It has been known that degradation of glycogen stores provides glucose, which fuels glycolysis as a first response to a lack of food (Saudek and Felig 1976). Subsequently, lipid species such as triacylglycerols was provided from fat cells are activated by enzymes and degraded to both free fatty acids and glycerol to maintain homoeostatic physiological processes (Nielsen 1997; Finn and Dice 2006; McCue 2012). In *D. magna*, lipid content increased with age until 8-day old (Mckee and Knowles 1987). Triacylglycerols content was 2.832 μg mg-wet-weight^{-1} at 3-day old animals, but increased to 60%, being 4.521 μg mg-wet-weight^{-1} in adult (Bychek and Gushchina 1999). Quantities of visible lipid increased with body mass in many cladoceran zooplankton such as *D. magna* and *D.*

galeata mendotae (Tessier et al. 1983). Weight-specific respiration rates generally decreased with body size (Ikeda 1970), and we also observed the same response in this study. I observed effects of low food supply on somatic growth and egg production in the next chapter, though the starvation effects were not investigated, since mature animals may tend to maintain their basal metabolism to keep survival.

Chapter 2:

The effect of long-term food shortage on growth, reproduction, and metabolic rate in *Daphnia magna*

1. Introduction

In aquatic ecosystem, zooplankton as a secondary producer play a critical role in connecting primary producers to higher trophic levels and delivering organic matters into the deep floor (Steinberg and Landry 2017; Liu et al. 2020). On the other hand, pelagic zooplankton are often exposed to food shortage and/or even starvation in oligo- and mesotrophic lakes depending on nutritional supply (Goldman 1988; Winder and Sommer 2012; Liu et al. 2021). Food-shortage affects zooplankton in different ways through survival, growth and reproduction (Gliwicz and Guisande 1992; Burns 1995; Cressler et al. 2014), and obviously regulate zooplankton population dynamics (Threlkeld 1976; Kirk 1997). Measuring the changing of life history parameters and metabolism in zooplankton under food shortage may be useful for understanding the consequences of food shortage in zooplankton community level.

Both growth and reproduction of zooplankton generally reduce at low food concentration (food shortage). For example, Burns (1995) showed that body length and growth rate in *Daphnia galeata*, *Daphnia hyaline* and *Daphnia magna* reduced by lowering food concentration. Urabe (1988) also showed that body weight, brood size, and net production rate of *D. galeata* decreased with decreasing food concentration. The egg production and adult body size of calanoid copepod *Eurytemora affinis* were depressed under food shortage (Ban 1994). Liu et al. (2015) found that the adult body size and clutch size of *Eodiaptomus japonicus* declined under food shortage, though the first naupliar stage was not affected by food shortage due to using storage of the egg yolk. The energy allocation is different between the juvenile and adult in

cladocerans (Mccauley et al. 1990). Therefore, the effect of food shortage on body size and weight may also different ontogenetically.

In the previous studies, the respiration rate of zooplankton generally increases with increasing food concentration. Kees and Corrie (1976) and Porter et al. (1982) showed that respiration rate of *D. magna* increased with increasing food concentration to a critical level, and suggested that food concentration might affect respiration rate through feeding behavior. In *D. galeata* and *Bosmina longirostris* (Urabe and Watanabe 1990) and *D. magna* (Bohrer and Lampert 1988), respiration rates increased with increasing food concentration and reached a plateau at a critical food density, suggesting that such food-concentration-mediated increase of respiration rate may be attributed to increasing the specific dynamic action (SDA). Generally, respiration rate of zooplankton increases with increasing body size, while the weight specific respiration rate decreases (Gillooly et al. 2001).

In this chapter, I measured life history traits of *D. magna*, i.e., growth and reproduction, with its metabolic rate as respiration rate, under food satiated and food limited conditions, to clarify how the animals respond to food shortage through energetic allocation or energetic balance among growth, reproduction and respiration loss.

2. Material and Methods

2.1 Stock cultures

*Daphni*a *magna* were maintained in a 500-mL glass jars filled with tap water

filtered with a glass fiber filter (Whatman, GF/F) autoclaved (120˚C for 15min) and well oxygenated at least for 24 h (FTW) as stock cultures. The stock cultures were placed in an incubator (Sanyo, MLR-350) with a photoperiod of 14L:10D and light intensity of ca. 130 μmol m^{-2} s^{-1} at 20˚C (Gao et al. 2022). All animals were fed with fresh green alga *Chlamydomonas reinhardtii* (IAM C-9) at cell concentration of 5×10^5 cells mL^{-1}, equivalent to 20.5 μgC mL^{-1}, which was far above the incipient limiting food concentration and allow them to reach at the maximum growth (Lampert 1987; Mitchell et al. 1992). Experimental container and fresh food suspensions were exchanged every two days. Algal cultures of *C. reinhardtii* were grown in 1-L conical flasks with the autoclaved C medium (Ichimura 1971) under the same photoperiod and light intensity as the stock culture.

2.2 Flow-through cultivation system and preparation for experimental animals

The flow-through cultivation system which can provide a stable food supply for experimental animals and avoid any potential effect of accumulated metabolites released from the animals themselves has been used for acclimatized and treated animals in this study (Ban et al. 2009). Animals were individually reared in 50-mL transparent polystyrene chambers (Corning, NY, USA) in the flow-through cultivation system. In each culture chamber, same food suspension was provided through a 20-μm mesh from a 12-L reservoir tank, which was aerated during the experiment to avoid lowering dissolved oxygen at night. The fresh food suspension in the tank was continuously provided to the culture chamber through a silicone tube (ARAM, OSAKA,

Japan) with a peristaltic pump (EYELA, MP2000) at a flow rate of 70 mL h^{-1}, to continuously supply the algal suspension at sufficient food (5 x 10^5 cells mL^{-1} or 20.5 µgC mL^{-1}) and food limited (2.5 x 10^3 cells mL^{-1} or 0.1025µgC mL^{-1}) conditions for growth of *D. magna* (Ban et al. 2009).

Neonates of *D. magna* from a single female sorted from stock culture were acclimatized at 1 ind. 50-mL^{-1} with the same food conditions as the stock culture in the flow-through cultivation system at least two generation acclimatization to exclude any per-cultured histories. After two generation acclimatization, neonates from the 3rd clutch of the animals were used for the experiments.

2.3 Measurement of respiration rate

The experimental animals started to carry eggs in their brood pouch at 6 days from hatching. After carrying eggs, all eggs were carefully removed with flushing out using a micro-pipette and counted under a dissecting microscope (Olympus, SZX12, Japan) at 24 h before start of the respiration measurements. It has been confirmed that this removing procedure does not affect respiration measurement in *D. magna* (Glazier 1991). All experiments were conducted at 20 ˚C.

Respiration rate as a good proxy for metabolic rate was expressed as the oxygen consumption rate over time (Del and Williams 2005; Liu and Ban 2017). The procedure of the respiration rate measurement was described in chapter 1.

In each of the two food conditions, healthy and active individuals of *D. magna* that reached 9 ages, from 2 to 18 days old at 2 days interval, equivalent to 1.1 to 4.4

mm in carapace length, were sorted from the flow-through cultivation system and used for measuring the respiration rates at different body sizes. Prior to the measurements, the animals sorted were gently washed six times to remove algae and bacteria with FTW. Then, an experimental animal was transferred to a gas-tight glass bottle (20-mL) filled with FTW, and DO concentration monitoring was started. All measurements were finished within 6 hours before the DO concentration declined to < 80% of the initial value. In the 6 hours monitoring, DO concentrations in the experimental bottles usually fluctuated within the first two hours probably due to varying activity of the animals influenced by handlings (Liu and Ban 2017; Gao et al. 2022), then DO concentration decreased linearly for following hours in the experimental bottles.

After the respirometry, the carapace length (L, mm) of the animal, from tip of head to base of tail spine, was immediately measured under a dissecting microscope (Olympus, SZX-ILLK100) with a digital micrometer (Wraycam, NF500) at a magnification of ×20. Then, the animal was wrapped in pre-combusted and pre-weighed aluminum foil and dried at 60 ºC for 24 hours using a muffle furnace (DK600, YAMATO). The body dry weight (*W*, μg) was measured using an electronic balance (MT5, Mettler Toledo, accuracy, 1 μg). After the animals matured (6 days old), the eggs in the brood pouch were carefully removed through flushing out using a micro-pipette and counted number of eggs under a dissecting microscope (Olympus, SZX12, Japan).

2.4 Data transformation

Respiration rate (*R,* μLO_2 ind^{-1} h^{-1}) of *D. magna* was calculated from oxygen

depletion over the period for measurement using following equation (2-1):

$$R = \frac{(\Delta O_{\mathrm{exp}} - \Delta O_{\mathrm{c}}) \times V}{N} \quad (2\text{-}1),$$

where ΔO_{exp} and ΔO_{cont} are the coefficients of oxygen consumption ($\mu LO_2\ L^{-1}\ h^{-1}$) in experiment and control, i.e. slopes of the regression lines in DO concentrations to incubation duration, which was obtained with a least square method. V is volume (L) of the experimental bottle, and N is number of animals in each experimental bottle, i.e. one.

Then, weight-specific respiration rate (R_{w}, mgC mg-C-weight^{-1} h^{-1}) was calculated using following equation (2-2):

$$R_{\mathrm{w}} = \frac{R \times 0.536}{W \times 0.447} \quad (2\text{-}2),$$

where W is animal body-dry weight (μg) measured using an electronic balance.

To evaluate physiological efficiency of *D. magna* under different food conditions, the net growth efficiency (K_2, %) in the two food conditions was calculated using a following equation (2-3):

$$K_2 = \frac{G + M_e}{G + M_e + R_{\mathrm{m}}} \times 100 \quad (2\text{-}3),$$

where G (μgC) is the mass for somatic growth of *D. magna*, calculated from an equation (2-4):

$$G = (W_2 - W_1) \times 0.447 \quad (2\text{-}4),$$

where W_1 and W_2 are the body dry weights (μg) at 4- and 16-days old, respectively. M_e (μgC) is the mass for egg production, and calculated from an equation (2-5):

$$M_e = n \times 8.0 \times 0.447 \quad (2\text{-}5),$$

where n is the cumulative number of eggs in an individual from 4- to 16-days old, and a factor of 8.0 is an average dry weight (μg) of an egg in *D. magna* from the literature (Dumont et al. 1975).

R_m (μgC) is the total respiration loss during development of *D. magna* and calculated from the equation (2-6):

$$R_m = \sum_{n=i}^{\omega} R \times 24 \times D_{ci} \times RQ \times 0.536 \quad (2\text{-}6),$$

where D_{ci} is a period between the two measurements (days). RQ is a respiratory quotient of *D. magna*, 1.1, and 0.75 for well-fed and food shortage conditions, respectively (Lampert 1984). The factors of 0.447 and 0.536 were used for converting μg body dry weight and μL volume of oxygen gas to carbon weights (μgC), respectively (Dumont et al. 1975; Liu and Ban 2017).

2.4 Statistical analysis

The difference in carapace length, body dry-weight, and the cumulative number of neonates for *D. magna* between food satiated and limited conditions were tested with Student's *t*-test. Linear regression analysis of Ln-transformed R (LnR) against LnW was conducted with a least-square method. Statistical difference of LnR between two food conditions was tested with analysis of covariance (ANCOVA). Linear regression analysis between R_w and W was also conducted with a least-squares method. The difference of net growth efficiencies between juvenile and adult at two food conditions were tested with one-way ANOVA. All statistical analyses were performed with SPSS software (IBM 2015).

3. Results

The carapace lengths of *D. magna* increased from 1.120 to 4.373 mm in food satiated condition, while those in food limited condition were almost the same until 8 days from hatching but significantly lower than those in food satiated one from 2.490 to 3.473 mm after 10 days from hatching (Fig. 2-1). The body dry weights increased from 0.029 to 0.611 mg in food satiated condition, while those in food limited condition were almost the same until 4 days from hatching, but significantly lower than those in food satiated one from 0.069 to 0.311 mg after 6 days from hatching.

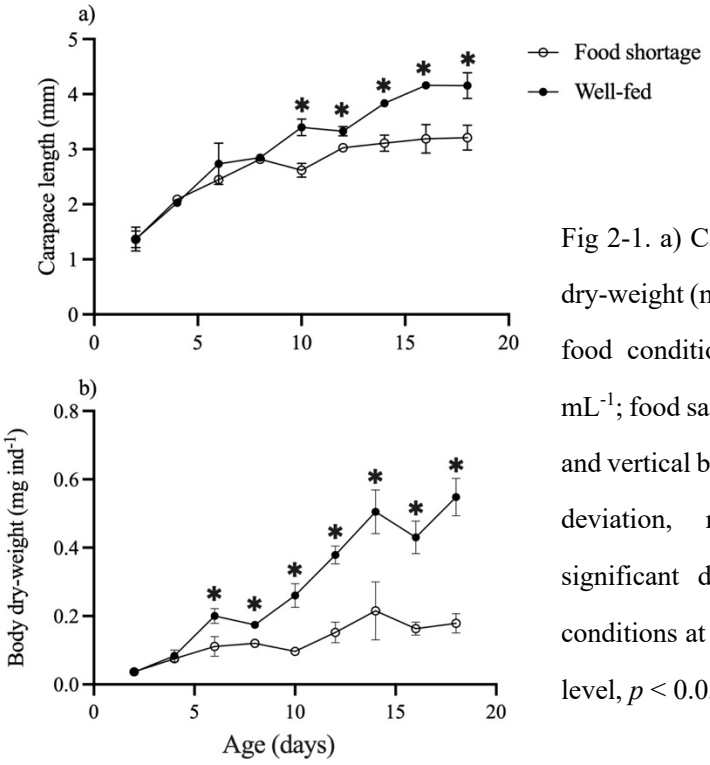

Fig 2-1. a) Carapace length (mm) and b) body dry-weight (mg ind^{-1}) of *Daphnia magna* at two food conditions (food limited, 2.5×10^3 cells mL^{-1}; food satiated, 5×10^5 cells mL^{-1}). Symbols and vertical bars represent average and standard deviation, respectively. Asterisks denote significant different between the two food conditions at each age with *t*–test at significant level, $p < 0.05$.

Number of neonates in first clutch in food limited and satiated conditions were 10.8 and 12 eggs, respectively, and not statistically significant (Fig. 2-2). Cumulative number of eggs increased with development up to 96 eggs until 4th clutch in food satiated condition, while being significantly lower in food limited one, from 12 to 41

eggs (22 on average) (Fig. 2-2). The clutch sizes of well-fed animals increased with increasing carapace length, whereas those of food-limited animals decreased with increasing the carapace length (Fig. 2-3).

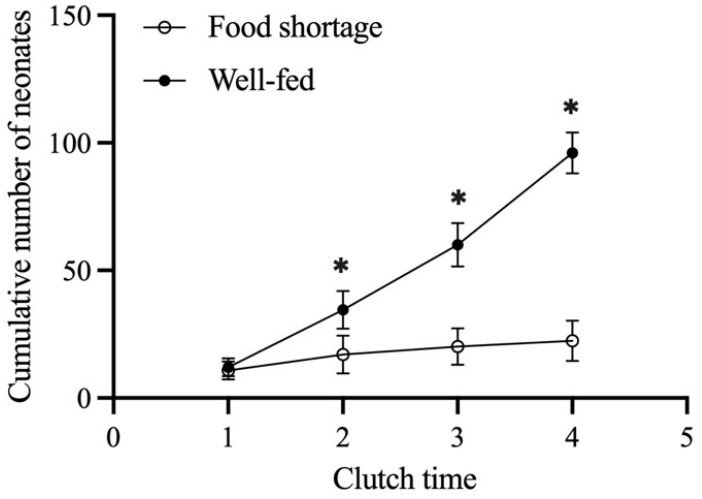

Fig 2-2. Cumulative number of neonates in a female of *Daphnia magna* in food shortage (open circle) and food satiated (closed circle) condition. Symbols and vertical bars represent average and standard deviation, respectively. Asterisks denote significant different between the two food treatments at each clutch with *t*–test at significant level, $p < 0.05$.

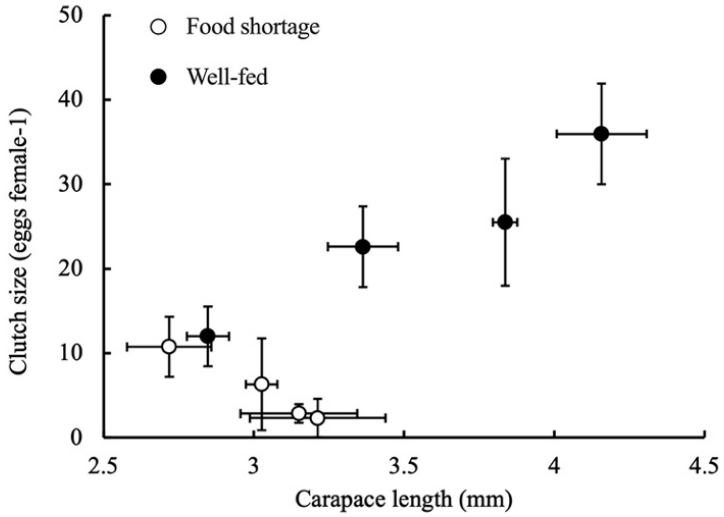

Fig 2-3. Relationship between clutch size and carapace length (mm) of female *Daphnia magna* at two food concentrations. Vertical and horizontal bars represent standard deviations.

Respiration rates (*R*s) of *D. magna* in food satiated condition increased with increasing body weight from 0.452 to 2.468 μLO_2 ind.$^{-1}$ h^{-1}, while those in food limited one were always lower than those in food satiated one, varying from 0.281 to 0.627 μLO_2 ind.$^{-1}$ h^{-1} (Fig 2-4a). Regression analysis showed that Ln*R* of well-fed animals linearly correlated with Ln-transformed body dry-weight (Ln*W*), while those in food limited animals were variable and not correlated to the Ln*W* (Fig 2-4a, and Table 2-1). ANOVA showed significant difference of Ln*R* between the two food conditions (df = 1, 46, F = 61.972, p < 0.01).

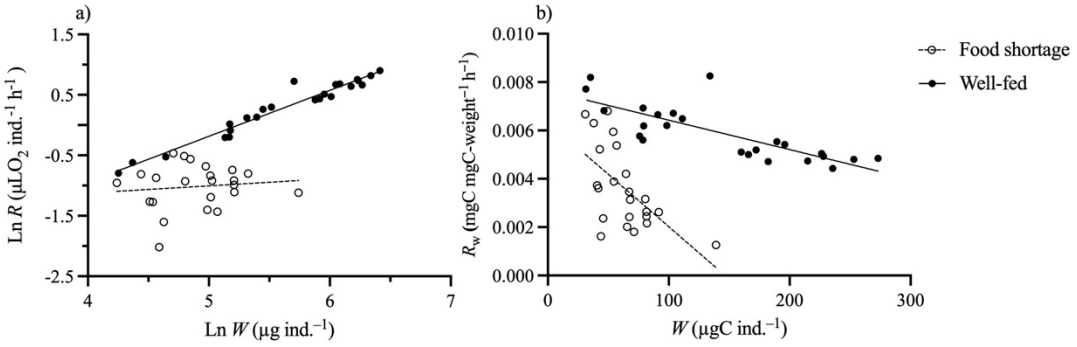

Fig. 2-4 a) Relationship between Ln-transformed respiration rate (Ln*R*, μLO_2 ind.$^{-1}$ h^{-1}) and body dry-weight (Ln*W*, µg ind.$^{-1}$) and b) relationship between weight-specific respiration rates (R_w, mgC mgC-weight $^{-1}$ h^{-1}) and body dry-weight (*W*, µgC) in *Daphnia magna* in food satiated and food limited conditions. Regression line in each food condition is shown in a) and b), and fitted parameters are showed in Table 2-1.

R_w of well-fed animals decreased with body weight from 0.004 to 0.008 mgC mg-weight^{-1} h^{-1}, while those of food limited ones were always lower than those of well-fed ones, ranging from 0.001 to 0.007 mgC mgC-weight^{-1} h^{-1} (Fig. 2-4b). Regression slope in food limited animals showed steeper than that in well-fed one (Fig 4b and Table 1).

Table 2-1. Parameters of linear regression analysis of respiration rate (R) and weight-specific R (R_w) against body dry-weight (W) in *Daphnia magna* under two food conditions.

Metabolic rate	Food condition	Slope	Intercept	n	r^2	t	P
R	Well-fed	0.760	1.264	24	0.951	20.761	< 0.001
	Food limited	0.119	0.777	23	0.013	0.517	0.611
R_w	Well-fed	-4.534	6.369	24	0.626	-6.063	< 0.001
	Food limited	-16.134	5.292	23	0.365	-3.476	0.002

The average net growth efficiencies (K_2) calculated from carbon accumulation and metabolic loss in *D. magna* were 77.6% and 67.3% for "Juvenile", and 59.2% and 71.7% for "Adult" in food satiated and food limited conditions, respectively. One-way ANOVA showed that there was not significantly different between K_2 values in two food condition for "Juveniles" (df = 1,4, F = 1.417, p = 0.319), whereas significantly different between K_2 values for "Adult stages" (df = 1,5, F = 84.802, p < 0.01).

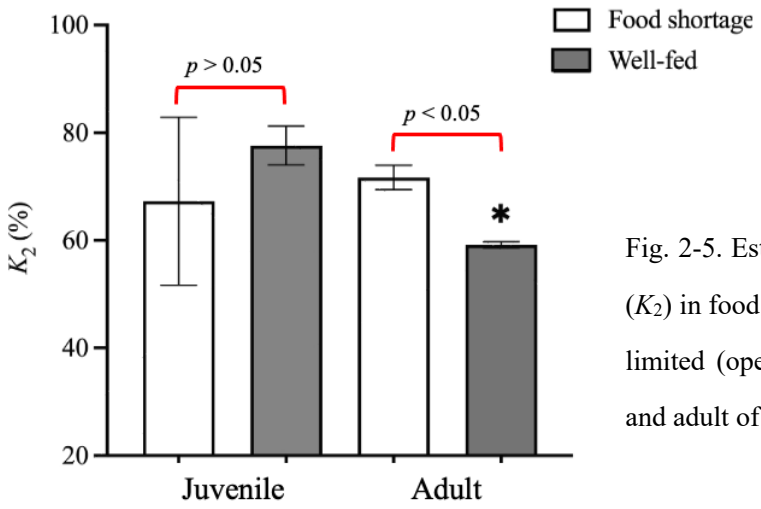

Fig. 2-5. Estimated net growth efficiencies (K_2) in food satiated (closed bars) and food limited (open bars) conditions in juvenile and adult of *Daphnia magna*.

4. Discussion

In the present study, both of the growth and reproduction of *D. magna* were significantly reduced in food shortage (0.1025 mgC L^{-1}) compared with those in food

satiated condition (20.5 mgC L^{-1}). Gorbi et al. (2011) investigated the effect of food shortage in two different clones of *D. magna*, and found that the body dry-weight at 36 days from hatching was 0.103 mg in low food concentration of 0.44 mgC L^{-1} while 0.514 mg in high food concentration of 2.20 mgC L^{-1}, being ca. 80% lower in low food concentration. Depression of the growth and reproduction in the food shortage was also found in other zooplankton species, i.e., Cladocera *D. hyaline*, *D. pulicaria* (Burns 1995) and *D. galeata* (Urabe 1988), and copepod *E. affinis* (Ban 1994) and *E. japonicus* (Liu et al. 2015).

However, the different developmental stages of zooplankton may differently respond to food shortage. In this study, the carapace length, body dry-weight and clutch size of *D. magna* were significantly lower in food shortage after 2nd clutch (9 days old) in adult stages, while did not show significant difference among the juvenile stages. Hanazato (1996) found that the body length of *D. magna* in low food concentration (*Chlorella* sp., 0.2 μg dry wt mL^{-1} or 0.3 mgC L^{-1}) did not differ from those in high food concentration (2 μg dry wt mL^{-1} or 3 mgC L^{-1}) during the juveniles of < 6^{th} instar at around 7 days old, but significantly different after 7^{th} instar. Burns (1995) showed that body lengths of *D. hyaline* and *D. magna* within 6 days old were not significantly different between low food condition of 0.2 mgC L^{-1} and high food one of 1 mgC L^{-1} using *Scenedesmus acutus*. Glazier (1992) showed that body mass of *D. magna* at < 4 days old was not significantly different between high food concentration of 1.5 mgC L^{-1} and low food concentration of 0.3 mgC L^{-1} using *Chlorella vulgaris*, but significantly different in the older ages. Urabe (1988) showed that brood size of *D. galeata* in fist

clutch was not significantly different between two different food concentrations of 2.5 mgC L^{-1} and 0.25 mgC L^{-1}.

Food shortage generally depresses the respiration rate of zooplankton (Porter et al. 1982; Bohrer and Lampert 1988; Urabe and Watanabe 1990), and I also observed same response in the present study. Muck and Lampert (1980) found that a depression in filtering rate of *Daphnia longispina* at low food concentration. Bohrer and Lampert (1988) reported that the assimilation rate of *D. magna* also decreased when food concentrations were below the incipient limiting level. The respiration rates in zooplankton were generally associated with its filtering and assimilation rates (Helgen 1987; Ban et al. 2008). In the present study, net growth efficiencies in adult increased under food limited condition, while those in juvenile stages were not significantly different between the food conditions. This suggests that the adult daphnids might adapt food shortage and reduce metabolic loss to increase its net production.

Chapter 3:

Density-mediated metabolic rate in *Daphnia magna* interacted with temperature

1. Introduction

Metabolic rate is one of the most fundamental biological processes (Brown et al. 2004; Del Giorgio and Williams 2005). Variation in metabolic rate is strongly tied to a range of individual characteristics (Ikeda 1985; Vézina et al. 2006; Einum et al. 2019) as well as environmental factors (Heinle 1969; LaRow et al. 2017; Gao et al. 2022), and therefore can provide some basic information at all levels of organization from individuals such as survival, growth and reproduction to biosphere such as life history attributes, population interactions and ecosystem processes (Brown et al. 2004).

Brown et al. (2004) developed metabolic theory of ecology (MTE), which is widely accepted and used to predict the life history attributes, population interactions and ecological processes by a lot of researchers (Price CA et al. 2010; Price CA et al. 2012; O'Connor and Bernhardt 2018). MTE explicitly deems that body size, temperature and stoichiometry substantially contribute variation in metabolic rate, and that food supply as phenotypic variation is not a primary factor. Whereas, DeLong et al. (2014) claimed that although body size and temperature are major drivers of metabolism, resource supply as a source of variation in metabolic rate was possibly overlooked and it caused some mismatch between the predictions of MTE and empirical observations. Chalcraft and Resetarits (2004) showed a mismatch on rates of fish predation to tadpoles between the prediction of MTE and observation of experiment, and suggested that the mismatch might be caused by unconsidered predator density in MTE. DeLong et al. (2014) used data for a wide range of organisms including zooplankton *Daphnia ambigua* from the literatures and found that the metabolic rates

were negatively correlated with population density. Similar responses were found in *Simocephalus vetulus* (Hoshi 1957). Yashchenko et al. (2016), however, showed that population density did not necessarily influence metabolic rates of zooplankton; weight-specific metabolic rates increased with population density in *Daphnia magna*, but not in *Daphnia pulex*. Goss and Bunting (1980) also did not show any relationships between metabolic rates and population density in both of the two species.

D. magna as an amenable model organism used in many scientific investigations, showing the different metabolic responses to population density in the previous studies as mentioned above. We therefore used it as experimental animal in this study and determined its respiration rates in three different density conditions (1, 10 and 20 ind. 50-mL^{-1}) at two temperatures (10 and 20 ˚C) using a high accurate optical oxygen meter, to clarify effects of population density with its temperature-mediated response on metabolic rate of the cladoceran.

2. Material and Methods

2.1 Stock cultures

Daphnia magna were maintained as stock cultures using a 500-mL glass jars filled with aged tap water filtered with a glass fiber filter (Whatman, GF/F), autoclaved at 120˚C for 15 min and well oxygenated for at least 24 h (FTW). The stock cultures were placed in an incubator (Sanyo, MLR-350) under photoperiod of 14L:12D and light intensity of ca. 130 μmol m^{-2} s^{-1} at 20˚C. All animals were fed with fresh green alga *Chlamydomonas reinhardtii* (IAM C-9) at 5×10^5 cells mL^{-1}, equivalent to 20.5 μgC

mL^{-1}. Food suspensions were exchanged twice a week. *C. reinhardtii* was cultured in a 1-L conical flasks filled with autoclaved C medium (Ichimura 1971) under the same photoperiod and light intensity as the daphnid stock culture.

2.2 Flow-through cultivation and preparation for experimental animals

Experimental animals were reared in 50-mL transparent polystyrene chambers (Corning, NY, USA) with a flow-through cultivation system. This flow-through cultivation system can provide a stable food supply and avoid any potential effect of accumulating metabolites or info-chemicals released from the animals themselves (Ban et al. 2009). In each culture chamber, same food suspension as used in the stock culture was provided through a 20-μm mesh from a 12-L reservoir tank, which was aerated during the experiment to avoid lowering dissolved oxygen at night. The food suspension in the tank was continuously provided to the culture chamber through a silicone tube (ARAM, OSAKA, Japan) with a peristaltic pump (EYELA, MP2000) at 70 mL h^{-1} of flow rate, to supply sufficient food for growth and reproduction of *D. magna* (Ban et al. 2009).

Three different population densities (1, 10 and 20 ind. 50-mL^{-1}) were used in this study. In each density treatment, newborn neonates from the stock culture were placed in each new chamber filled with the food suspension and reared at 20 ˚C with the flow-through system. After two generations rearing under each treatment for acclimatization to exclude any stock-culture histories, the animals were used in experiments. The animals sorted from the stock culture grew up adulthood, the neonates from the third

clutches of the animals were sorted, and reared again as 2nd generation. The neonates from the 3rd clutch in the 2nd generation were used for respiration measurement experiment at 10 and 20 ˚C. In the 10 ˚C experiments, the animals were acclimated for 4 hours with sufficient food supply at two temperature steps prior to the experiment, i.e. at 15 ˚C for 2 hours and then transferred to 10 ˚C for 2 hours to avoid suddenly temperature shock. At 6-day old, the animals started to carry eggs in their brood pouch, which induced overestimation of respiration rate because of including egg respiration. In these cases, all eggs were carefully removed through flushing out using a micro-pipette under a dissecting microscope (Olympus, SZX12, Japan) at 24 h before start of the experiments. It has been confirmed that this removing procedure does not affect respiration measurement in *D. magna* (Glazier 1991).

2.3 Measurement of respiration rate

Respiration rate as a good proxy for metabolic rate in zooplankton was generally expressed as the oxygen consumption rate over time (Del and Williams 2005; Liu and Ban 2017). Detailed method for measuring respiration rate is described elsewhere (Liu and Ban 2017; Gao et al. 2022). In each measurement, five 50-mL gas-tight glass bottles were used for measuring the oxygen consumption of the animals. Dissolved oxygen (DO) concentration in each bottle was measured using a fiber-optic oxygen meter (PyroScience, Firesting O_2), fitted with a spot-fiber oxygen sensor (PyroScience, SPFIB). This allowed semi-continuous (every minute) measurements using four oxygen sensors (three for experimental bottles with animals and one for control bottles

without animals) with a submersible temperature sensor (PyroScience, TSUB21) in the remaining bottle. An oxygen sensor spot (PyroScience, OXSP5) was glued to the inner wall of each experimental bottle to non-invasively and non-destructively measure the DO concentrations with the oxygen sensors from outside the bottles. In each measurement, no food supply during the measurement because of short measurement period.

In each density treatment, healthy and active individuals of *D. magna* that reached several ages, from 1.2 to 4.6 mm in carapace length, were sorted from the flow-through cultivation system, then acclimatized them to experimental temperature (acclimation method we mentioned before) in a 50-mL glass bottle (the population density same as them in flow-through cultivation system) with sufficient food supply. After 4 hours acclimation with a sufficient food supply, experimental animals were sorted and gently washed six times to remove micro-organisms such as algae and bacteria with FTW prepared in each temperature. After washing, the animals were transferred to a gas-tight glass bottle (50-mL) filled with FTW (no food algae), and then DO concentration monitoring were started. DO concentrations in the experimental bottles fluctuated during the first a couple of hours during the incubation, probably due to increasing activity of the animals (Liu and Ban 2017; Gao et al. 2022). Then, DO concentration decreased linearly for next 2 hours, and the data monitored were used for calculating oxygen consumption rate. All measurements were finished within 8 hours before the DO concentration declined to at least 80% of the initial value.

Just after the measurement, the carapace length (L, mm) of the animal, from tip of

head to base of tail spine, was immediately measured under a dissecting microscope (Olympus, SZX-ILLK100) with a digital micrometer (Wraycam, NF500) at a magnification of ×20. Then, animals were carefully returned to the flow-through cultivation system and reared continuously until next measurements.

2.4 Data analysis

Respiration rate (R, μLO_2 ind^{-1} h^{-1}) of *D. magna* was calculated from oxygen depletion over time using following equation (3-1):

$$R = (\Delta O_{exp} - \Delta O_{cont}) \times V / N \qquad (3\text{-}1),$$

where ΔO_{exp} and ΔO_{cont} are the coefficients of oxygen consumption (μLO_2 L^{-1} h^{-1}) in experiment and control, i.e. slopes of the regression lines in DO concentrations to incubation duration, which was obtained with a least square method. V is volume (L) of the experimental bottle, and N is number of animals in each experimental bottle.

Then, weight-specific respiration rate (R_w, mgC mgC-$weight^{-1}h^{-1}$) was calculated using following equation (3-2):

$$R_w = (R \times 0.536) / (W \times 0.447) \qquad (3\text{-}2),$$

where W is animal body-dry weight (μg) calculated from its carapace length (L, mm) using the length–weight equation, $\ln W = 2.48 + 2.4 \ln L$ (authors' unpublished data). The factors of 0.447 and 0.536 were used for converting μg body dry weight and μL oxygen consumption to carbon body mass (mgC) and carbon loss rate (mgC h^{-1}), respectively (Liu and Ban 2017).

Q_{10} coefficient, which represents the degree of temperature dependent metabolic

rate (Del Giorgio and Williams 2005), was calculated using equation (3-3):

$$Q_{10} = \left(\frac{k_1}{k_2}\right)^{\frac{10}{(t_1 - t_2)}} \quad (3\text{-}3),$$

where k_1 and k_2 are average R_w at temperature t_1 (20 ˚C) and t_2 (10 ˚C), respectively.

2.5 Statistical analysis

Linear regression analysis of ln-transformed R (ln$R$) against ln$W$ was conducted with a least-square method. After homogeneity of the regression slopes was confirmed, statistical difference of ln$R$ among the density treatments with ln$W$ as covariant was tested with analysis of covariance (ANCOVA) in each temperature. The post-hoc Turkey-Kramer tests were also conducted when the ANCOVA showed significant difference. The differences of R_w among the two factors of treatments and temperatures were tested with two-way ANOVA. Linear regression analysis between R_w and W were also performed with a least-squares method. All statistical analyses were performed with R computing environment, version 4.1.0 (R Core Team 2021).

3. Results

Daphnia magna always showed higher respiration rates (*R*s) at 20 ˚C compared to those at 10 ˚C (Fig. 3-1). *R* increased with increasing body dry weight in all treatments tested. *R*s at 10 ˚C ranged 0.19-1.18, 0.04-0.78 and 0.05-0.66 μLO_2 ind^{-1} h^{-1} in 1, 10 and 20 ind. 50-mL^{-1} treatments, respectively, while 0.19-3.24, 0.17-1.38 and 0.15-1.16 μLO_2 ind^{-1} h^{-1} at 20 ˚C (Fig. 3-1). The regression slopes were not significantly different among the density treatments at both temperature (df = 2, 64, F=0.034, P = 0.967 for

10 ˚C, and df = 2, 97, F = 0.513, P = 0.6 for 20 ˚C). Then, ANCOVA showed significant differences of ln$R$ among the density treatments at both temperatures (df = 2, 64, F = 16.64, P < 0.001 at 10 ˚C; df = 2, 97, F = 45.93, P < 0.001 at 20 ˚C). Post-hoc tests showed that ln$R$s in 1 ind. 50-mL^{-1} were significantly higher than those in two crowding treatments at both 10 and 20 ˚C, while no significantly different in ln$R$s between the 10 and 20 ind. 50-mL^{-1} at both temperatures (Table 3-1).

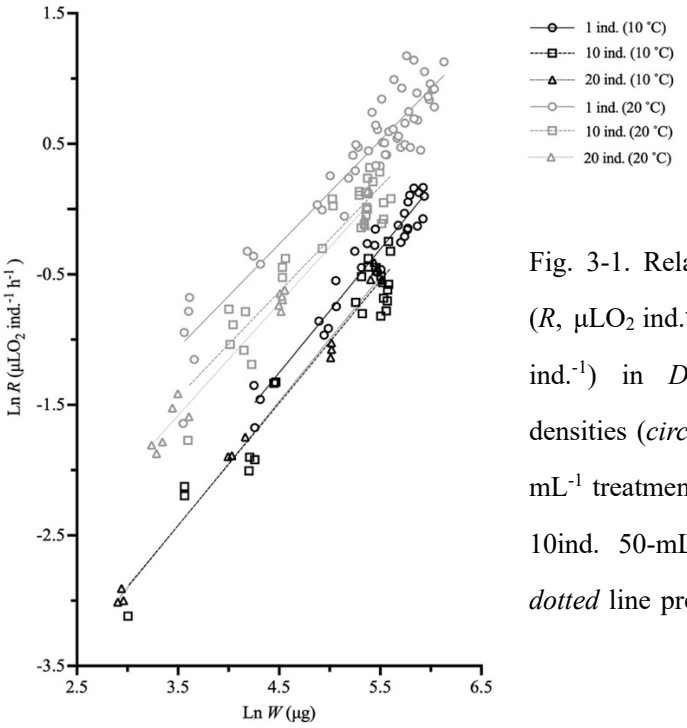

Fig. 3-1. Relationship between respiration rates (R, μLO$_2$ ind.$^{-1}$ h^{-1}) and body dry-weight (W, μg ind.$^{-1}$) in *Daphnia magna* among different densities (*circles* and *solid* line present 1ind. 50-mL^{-1} treatments; *squares* and *dashed* line present 10ind. 50-mL^{-1} treatments; and *triangles* and *dotted* line present 20ind. 50-mL^{-1} treatments) at

Regression analysis showed that R_W at 20 ˚C slightly decreased with increasing body weight in all density treatments, while those at 10 ˚C were not significantly different among the body weights (Fig 3-2 and Table 3-1). Average values of R_W were 0.00366, 0.00299, 0.00306 h^{-1} at 10 ˚C, and 0.00884, 0.00665, 0.00661 h^{-1} at 20 ˚C, in 1, 10 and 20 ind. 50-mL^{-1} treatment, respectively. R_W values in the crowded treatments were depressed to 81.7-83.6 % of those in single treatment at 10 ˚C, and 75.2 % and

74.8 % at 20 ˚C.

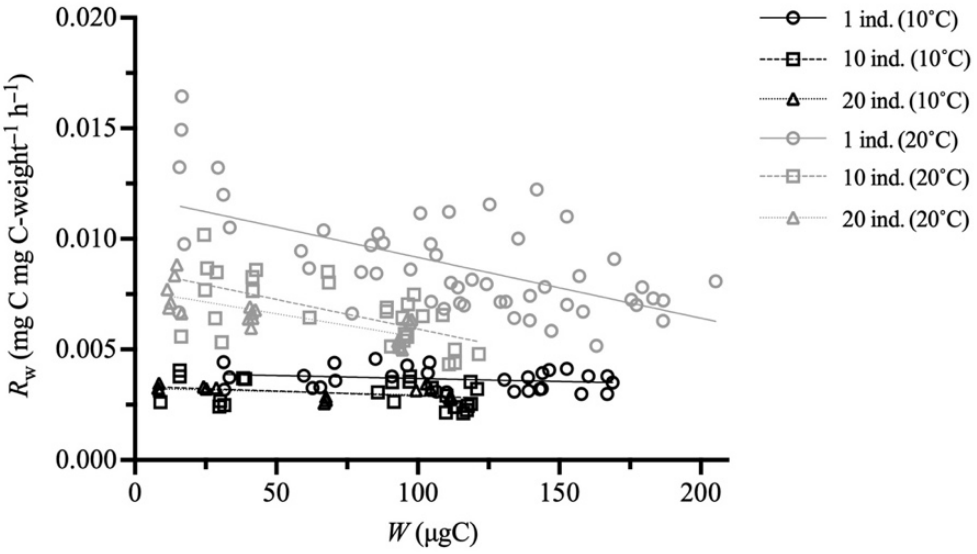

Fig. 3-2. Relationship between weight-specific respiration rates (R_w, mgC mgC-weight $^{-1}$ h^{-1}) and body dry-weight (W, μgC) in *Daphnia magna* among different densities (*circles* and *solid* line present 1ind. 50-mL^{-1}; *squares* and *dashed* line presented 10ind. 50-mL^{-1}; and *triangles* and *dotted* line present 20ind. 50-mL^{-1}) at 10 (*black symbols*) and 20 ºC (*gray symbols*). Fitted linear regression line in each treatment was also provided (10 ˚C for *black line* and 20 ˚C for *gray line*), all fitted parameters showed in Table 1.

Table 3-1. Linear regression parameter of weight-specific respiration rate (R_w) against body dry-weight (W) in *Daphnia magna* under different treatment conditions.

Temp	Density (50-mL^{-1})	Slope	Intercept	n	r^2	t	P
10 ˚C	1	-0.001	3.295	28	0.058	-1.283	0.210
	10	-0.002	2.780	22	0.087	-1.418	0.171
	20	-0.001	2.709	14	0.207	-1.843	0.088
20 ˚C	1	-0.010	9.923	52	0.358	-5.332	< 0.001
	10	-0.010	7.164	28	0.371	-3.988	< 0.001
	20	-0.008	6.394	17	0.556	-4.478	< 0.001

Two-way ANOVA showed a significant interaction between crowding and temperature on R_w (df = 2, 163, F = 5.124, P < 0.01), indicating the metabolic response to crowding varied with temperature. Therefore, Q_{10} values were also different among the temperature, i.e. 2.29, 1.98 and 1.63 in 1, 10 and 20 ind. 50-mL^{-1} treatment, respectively, when temperature increased from 10 to 20 ˚C (Fig. 3-3).

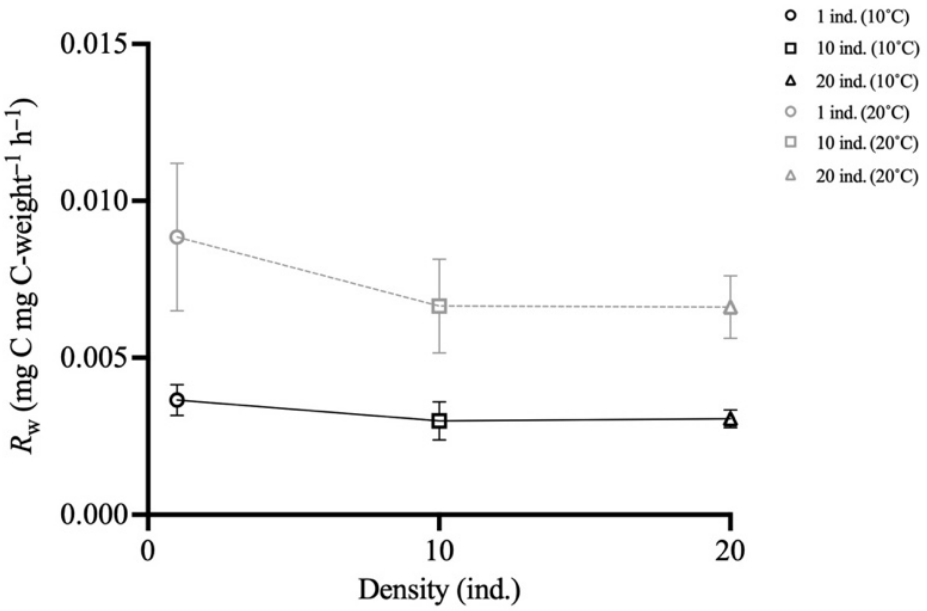

Fig. 3-3. Relationship between average weight-specific respiration rates (R_w, mgC mgC-weight^{-1} h^{-1}) and population densities (1, 10 and 20 ind. 50-mL^{-1}) in *Daphnia magna* at two temperatures (10 and 20 ˚C) (*circles* present 1ind. 50-mL^{-1}; *squares* presented 10ind. 50-mL^{-1}; and *triangles* present 20ind. 50-mL^{-1}) at 10 (*black symbols*) and 20 ºC (*gray symbols*). Vertical bars represent standard deviation.

4. Discussion

In the present study, the respiration rates (*R*s) of *Daphnia magna* in two crowded treatments were lower than those in a single treatment at both 10 and 20 ˚C, but not significantly different each other. This suggests that metabolic rates of *D. magna* decline at crowding conditions. In terms of weight-specific respiration rate (R_w), the

values of R_w were significantly depressed under crowding situation to 75 – 84 % of those in a single treatment. In marine copepod *Acartia steueri*, respiration rates also decreased to 80% with increasing population density from 100 to 2000 ind. L^{-1} (Takayama et al. 2020). DeLong et al. (2014) found that respiration rates in *Daphnia ambigua* decreased with increasing population density from 1700 to 6700 ind. L^{-1}. Respiration rates in *Simocephalus vetulus* at 8000 ind. L^{-1} were also lower than those in 1000 ind. L^{-1} (Hoshi 1957).

Such density-mediated responses on respiration rate might be associated with swimming behavior (Ambler 2002; Takayama et al. 2020) and/or competitive depression of feeding rates through foraging behavior under crowded condition (DeLong et al. 2014). It has been shown that, however, swimming activities of *D. magna* are not affected by the animal densities (Sereni and Einum 2015). Whereas, depression of ingestion rate with population density in cladocerans has been well known in a lot of previous studies. Helgen (1987) found that feeding rates of *D. pulex* at 30 ind. L^{-1} were significantly higher than those at 270 ind. L^{-1}. Hayward and Gallup (1976) showed that the feeding rate of *Daphnia schoedleri* at 5×10^3 ind L^{-1} was lower than that at 2×10^4 ind. L^{-1}. Ban et al. (2008) showed that the ingestion rates of *D. pulex* decreased to 40-80 % with increasing number of animals in an experimental container irrespective of the volume of the container under excess food supply, suggesting such depression of ingestion rate under crowding may be physically mediated response but not associated with food depression with competitive interaction.

The density-mediated reduction of respiration rate found in this study may

therefore be related to depression of ingestion rate due to physical interference even under excess food supply, and could be explained by lowering specific dynamic action (SDA), i.e., additional energy expenditure for processing food ingested, i.e. digestion, absorption, nutrient distribution, and synthesis of new tissues, under low food uptake (Ikeda 1977; Kiørboe et al. 1985; Urabe and Watanabe 1990).

Previous studies showed different responses of metabolic rates on population density as described in Introduction. Such discrepancy even in the same species may be attributed to methodological issues which makes some biases of the observations. Experimental procedures in the previous studies can be divided into two categories; one is that zooplankton are exposed to different densities just during respirometry, and another one is that zooplankton are exposed to different densities prior to respirometry but perform with a single density during the measurement. In the present study, we selected 3rd category in which zooplankton were exposed to different densities throughout the period of both acclimatization and measurement. If high population densities can depress metabolic rate through reducing SDA due to decreasing feeding rate, pre-respirometry conditions might be important. In the first category, it seems to be similar SDA conditions for the experimental animals irrespective of population density during the respirometry, because the animals were reared in the excess food condition and same population density prior to the measurement. It might be the reason why most cases of no crowding effect on metabolic rate were found in the studies that selected first category. In Yashchenko et al. (2016), the experimental animals were starved for 18 hours prior to the experiment to minimize the SDA. This may cause

lowering SDA, and therefore no crowding effect in *D. magna* even used category two. Yashchenko et al. (2016) also found that metabolic rate slightly increased with increasing population density with category one and lasted the respirometry within 1 hour. DO concentrations in the experimental bottles usually fluctuated over the first few hours of incubation, and that may lead to a bias of the result due to such kind of reasons.

The value of Q_{10} is frequently used for learning the relationship between metabolic rates and temperature in poikilotherms. Vollenweider and Ravera (1958) investigated effect of temperature on respiration rates in some limnetic zooplankters, *Daphnia obtuse*, *D. longispina* var. *hyalina*, *Sidacristallina* var. *limnetica*, *Cyclops strenuus*, *Mixodiaptomus laciniatus*, and found that the values of Q_{10} mostly ranges from 2 to 3. Ikeda and Fay (1981) reported that the average Q_{10} value for the weight-specific respiration rates in 13 species of family Calanidae and 6 species of genus *Euphausia* was 2.18, ranging from 1.78 to 2.67, with the temperature ranges from 5.0 to 27.6 ˚C. However, the values of Q_{10} for weight specific respiration even in same species vary among the literatures. Paul et al. (1997) reported a Q_{10} value is 2.4 when the temperature increased from 5 to 15 ˚C, and 1.3 when temperature increased from 15 to 25 ˚C for weight specific respiration rate of *D. magna*. The value of Q_{10} varied with the tested temperature because the metabolism of zooplankton exponentially increases with temperature to a maximum value then suddenly dropped (Goss and Bunting 1980; Brown et al. 2004). The Q_{10} values of zooplankton were not only affected by temperature range, but also acclimation period and body mass. Lamkemeyer et al. (2003) showed that Q_{10} of *D. magna* for the oxygen consumption with a short-term

acclimation at 10˚C prior to transferring to 20 ˚C temperatures was approximately 2.0, while it was 1.3 with long-term acclimation at 10˚C. Simčič and Brancelj (1997) determined oxygen consumptions of juvenile and adult *D. magna* at 5 and 20 ˚C and found that the Q_{10} values were 1.5 for juveniles and 1.7 for adults.

In the present study, the values of Q_{10} were 2.29, 1.98 and 1.63 in 1, 10 and 20 ind. 50-mL^{-1} treatment, respectively, when temperature increased from 10 to 20 ˚C. This implies that Q_{10} value is also influenced by animals' densities that used in the experiment. This suggests that temperature mediated metabolic rate is influenced by crowding and decelerated under crowding. In other words, crowding effect on metabolic rate of *D. magna* might decreased with lowering temperature.

Chapter 4:

Effect of acute acidic stress on survival and metabolic activity of zooplankton from Lake Biwa, Japan

This section is mainly based on the manuscript:

"The effect of long-term food shortage on growth, reproduction, and metabolic rate in *Daphnia magna*" by Huanan Gao, Xin Liu, and Syuhei Ban published in ***Inland Waters*** (2022), https://doi.org/10.1080/20442041.2022.2058861.

1. Introduction

The release of nitrogen and sulfur oxides into the atmosphere through anthropogenic activities causes acid rain, which is particularly damaging to aquatic ecosystems in lakes, streams, and rivers (Mohajan 2018; Zhou et al. 2019; Garaga et al. 2020; Roșca et al. 2020) because it threatens biodiversity and productivity (Brett 1989; Charles et al. 1989; Brönmark and Hansson 2017). Elevated acidity (pH < 4) in pristine forest lakes in Scandinavia may be because of sulfur dioxide from industries in northwestern Europe causing acid rain (Almer and Dickson 2021). Acid rain caused by atmospheric pollution from nearby industrial cities directly decreased pH of the lake water to < 5.0 in Lake George, USA (Beamish et al. 1975), and < 6.0 in Lake Ontario, Canada (Roff et al. 1977), and consequently the biomass and species richness of aquatic organisms such as rotifers, crustaceans and fishes have been reduced by the acidification (Beamish et al. 1975; Hendry and Brezonik 1984; Keller et al. 2002). Most aquatic animals are adapted to neutral or slightly alkaline conditions, but at pH < 4.0 they cannot survive (Sandine 1992; Weber and Pirow 2009) because high acidic stress can induce biochemical and/or physiological failures (Rosseland 1994).

Zooplankton do not only play a crucial role for linking primary producers to higher trophic levels in aquatic ecosystems but also an important biological pump in carbon recycle (Steinberg and Landry 2017; Liu et al. 2020, 2021). Because they are extremely sensitive to environmental stresses, they can be considered a good bio-indicator of environmental changes (Dodson et al. 1995; Bownik 2017). Acid rain inflicts acute acid shock on zooplankton from individual to community levels. For the individual level,

high proton concentrations can disrupt its physiological functions, such as the acid–base balance and ion–regulation (Henry and Wheatly 1992; Pequeux 1995; Whiteley et al. 1999), and damage important sense organs (gills and heart muscle), negatively affecting zooplankton productivity (Brett 1989). For the community level, Holt et al. (2003) investigated the crustacean zooplankton communities of 47 various acidic lakes in south-central Ontario, Canada, and reported a pH 6.0 to be a threshold level for species richness and abundance.

The responses of zooplankton to acidic stress vary by species (Locke 1991), possibly because of different physiological adaptations (Havas 1985; Price and Swift 1985). Thresholds to survive 48 h for the cladoceran species, *Daphnia galeata mendotae*, *Daphnia pulex* and *Simocephalus serrulatus* are pH 4.8, 4.4 and 4.2, respectively, while those for cyclopoid copepods, *Cyclops vernalis*, *Mesocyclops edax* and *Cyclops fuscus* are pH 4.0, 4.4 and 5.0, respectively (Bulkowski et al. 1985; Price and Swift 1985; Bruns and Wiersma 1988). Metabolic rates of *Daphnia magna* decreased with increasing acidity from pH 7.3 to 4.0 (Alibone and Fair 1981), whereas the metabolic rates of *D. pulex* is stable between pH 5.5 and 7.8 (Weber and Pirow 2009). Life history traits such as survival, somatic growth and neonate production in *D. magna* decrease with increasing acidity from pH 4.7 to 4.4 (Ghazy et al. 2011).

Temperature also regulates zooplankton metabolism (Brown et al. 2004). Because the energy budget of zooplankton depends on temperature, metabolic rate generally correlates positively with increasing temperature until a lethal level is reached, after which it dramatically decreases (Yurista 1999; Liu and Ban 2017). Lowering the

metabolic activities of zooplankton in cold water could reduce their acidic tolerance by depressing physiological responses. Previous studies have been shown effects of several environmental factors on life history traits of zooplankton, such as temperature, food availability (Ban 1994; Liu et al. 2015), salinity (Devreker et al. 2007; Garreta-Lara et al. 2018), heavy metal toxicity (ObuidAllah et al. 2005) and effects of CO_2-induced acidification (in relatively narrow pH ranges within lethal levels) on the growth and reproduction of marine copepods (Cao et al. 2015; Lee et al. 2020). No study has focused on the synergistic effects of temperature and acidic stress to the survival and metabolic rates of freshwater zooplankton yet.

The ability of zooplankton to move correlates with acidity. For example, the swimming speed of *D. magna* decreased at pH < 5.5 after 8 h exposure (Chen et al. 2012), and the swimming behavior of *Eurytemora affinis* and *Temora longicornis* are also affected by decreased pH (Seuront 2010). Because metabolic rate correlates positively with swimming behavior (Vlymen 1970; Porter et al. 1982), the impact of pH on zooplankton metabolic rate could be assessed by examining changes in swimming behavior.

Lake Biwa is the largest and oldest lake in Japan. The lake as a main water resource for fisheries, agricultural industry, and as a drinking water to support approximately 14.5 million people living in Kansai area (Kawanabe et al. 2012). On average, the water in Lake Biwa has always been kept at neutral or slightly alkaline, but the pH of the lake water varied from 7.3 to 9.4 in different areas and seasons of the lake (https://www.pref.shiga.lg.jp/file/attachment/1044204.pdf, March 7, 2022). Acid rain

of pH 4.5−5.0 has been reported in the cities surrounding the lake during the last three decades (https://www.lberi.jp/learn/atmosphere/asid_rain, March 7, 2022), and the snowmelt water of pH 3.8 has been also shown from the catchment area of the lake (Fushimi 1994). Acid rain and/or snowmelt water could drastically reduce the lake water pH in certain areas at a short period, especially in the surface of the lake which is the main habitat of planktonic plants and animals. It is important, therefore, to clarify effects of short-term acidic stress on zooplankton living there for assessment and forecasting the lake ecosystem.

We determined survival and metabolic rates of three dominant zooplankton taxa (*D. pulicaria*, *E. japonicus* and Cyclopoida spp.) from Lake Biwa, Japan, exposed to a range of acidities and two temperatures to clarify how the zooplankton respond to acidic stress under cold and warm waters, and measured the swimming behavior of *E. japonicus* exposed to a range of acidities to confirm effect of acidity on metabolic rate by swimming activity.

2. Methods

2.1. Field collection and stock culture

Zooplankton were collected with a Norpac plankton net (mouth diameter, 45 cm; mesh size, 200 μm) hauled vertically from 20 m to the surface at a pelagic site (35°18′32.6″N, 136°8′38.9″E, depth of 70 m) in the north basin of Lake Biwa between 6 August 2020 and 15 April 2021. Zooplankton samples were kept cool in an insulated box and delivered to our laboratory within 1 h.

More than 100 individuals of each of *Daphnia pulicaria*, *Eodiaptomus japonicus* and Cyclopoida spp. were sorted from the plankton samples collected under a dissecting microscope (Olympus, SZX12, Japan) at ca. ×7 magnification. Animals were transferred to 1-L glass jars filled with lake water pre-filtered through a glass fiber filter (Whatman, GF/F), autoclaved (120 ºC for 15 min), and well oxygenated for at least 24 h (FLW) at individual density of ca. 70 ind. L^{-1}. Animals were reared in a stock culture at 20 ºC in an incubator (Sanyo, MLR-350) with a 12L:12D photoperiod, light intensity of 130 μmol m^{-2} s^{-1}, and fed sufficiently to avoid any potential physiological responses to starvation. *Daphnia pulicaria* were fed fresh green alga *Chlamydomonas reinhardtii* (IAM C-9) at 5×10^5 cells mL^{-1} (Lampert 1987), and the two copepod taxa were fed a 1:1 (cell:cell) fresh algal mixture of *C. reinhardtii* and *Cryptomonas tetrapyrenoidosa* (NIES 282) at 5×10^4 cells mL^{-1} (Liu et al. 2015). Experimental container and food suspensions were exchanged every two days. Animals were cultured for at least two or three generations to avoid any bias due to wild population variability (Laabir et al. 1995, Liu et al. 2014).

Both algal cultures were maintained in the laboratory according to the procedures in Liu et al. (2014); cultures of *C. reinhardtii* were grown in C medium (Ichimura 1971) and *C. tetrapyrenoidosa* in VT medium (Provasoli and Pintner 1959) in 1-L conical flasks under the same temperature and light conditions as the zooplankton stock culture.

2.2 Preparation of pH and experimental animals

pH in experimental media was adjusted to desired levels using appropriate

amounts of 1 mol L^{-1} of hydrogen nitrate (FUJIFILM Wako Pure Chemical, Reagent, 149-02886) to FLW and measured with a pH meter (HORIBA, Lab pH meter F-72) connected to a micro pH electrode sensor (HORIBA, 9618S-10D) immediately prior to and following experiments.

After two generations acclimatization, healthy and active individuals of juvenile *D. pulicaria*, adult female *E. japonicus* and juvenile Cyclopoida spp. whose body sizes were 1.2 ± 0.2 mm of carapace length, 1.0 ± 0.1 and 0.5 ± 0.1 mm of prosome length, respectively, were sorted from the stock cultures and used for experiments. Because stock cultures were maintained at 20 ºC, to avoid temperature shock the temperature in 10 ºC treatments were progressively decreased in a two-step process over 4 h (with the same food supply as in stock cultures); 15 ºC for 2 h, then 10 ºC for 2 h. To prevent food and bacteria from influencing the pH of the experimental media, animals were not fed during experiments.

2.3 Survival

To evaluate the acute acidic tolerance for each taxon, survival rates were determined at various pH values between 4.0 and 8.0, at two temperatures (10 and 20 ºC) (Table 1). Prior to each experiment, more than 20 animals were sorted from the stock cultures and gently washed with each pH medium to remove food algae and bacteria from their body. Then, 3−5 individuals were transferred to each of four 20-mL glass vials filled with each pH medium; the total number of animals tested in each pH treatment ranged 17–20. Animals were incubated in an incubator (Mitsubishi, CN-25C)

in the dark at 10 and 20 ºC. All experiments lasted 24 h; dead animals were counted hourly and removed from the experimental vials. Pooled data in each pH treatment was used to calculate survival rate (S_t, %) using equation (4-1):

$$S_t = \frac{n_t}{N} \times 100 \qquad (4\text{-}1),$$

where n_t is the number of surviving individuals at time t, and N is the total number of individuals tested in each pH treatment. The pH of experimental medium was checked every 2 hours and exchanged with new medium if the pH was out of target value; variance from a target pH was kept on ± 0.1 during each experiment.

2.4 Respiration rate

Respiration rate is expressed as the oxygen consumption rate over time (Del Giorgio and Williams 2005). Methodology of measuring respiration rate is mainly referred to Liu and Ban (2017). Because different taxa have different acidic tolerances (see Results), pH ranges used in experiments differed between taxa: *D. pulicaria* and Cyclopoida spp. (pH 4.6−8.0), and *E. japonicus* (pH 5.4−8.0) (Table 4-1). Oxygen consumption rates were measured at 20 ºC for each taxon and additionally at 10 ºC for *D. pulicaria*.

Prior to the experiment, more than 30 individuals of each taxon were sorted from stock cultures and gently washed six times with each pH medium. Then, 10 individuals of each species were separately transferred into one of three 10-mL gas-tight glass bottles filled with experimental medium at each pH. The three gas-tight glass bottles with animals and two more without animals were placed in an incubator (Mitsubishi,

CN-25C) and maintained at constant temperature (either 10 or 20 ºC, depending on the experiment and taxon). Dissolved oxygen (DO) concentration in each experimental bottle was measured using a fiber-optic oxygen meter (PyroScience, Firesting O_2), fitted with a spot-fiber oxygen sensor (PyroScience, SPFIB). This allowed for semi-continuous (every 1 minute) measurements using four oxygen sensors (three for experimental bottles with animals and one for control bottles without animals), with a submersible temperature sensor (PyroScience, TSUB21) in the remaining bottle. An oxygen sensor spot (PyroScience, OXSP5) was glued to the inner wall of experimental and control bottles to non-invasively and non-destructively measure the DO concentration with oxygen sensors from outside the bottles (Liu and Ban 2017).

DO concentrations in experimental bottles fluctuated over the first few hours of incubation, probably because of increased animal activity (Teuber et al. 2013, Liu and Ban 2017). Therefore, we limited data for analyses to those collected after 2 h from the start of incubation, after which DO concentrations decreased linearly with incubation time. All measurements were completed within 6 h before the DO concentration declined to < 80% of the initial concentrations. After measurement, DO data from 2−6 h were used to calculate oxygen consumption rates using regression analysis. pH of the experimental medium was measured prior to and following experiment, any differences from target pH were within ± 0.1.

After the experiment, animals were preserved in 5% neutral sugar formalin, after which measurement of carapace length in *D. pulicaria* (from tip of head to the base of tail spine), and prosome length of *E. japonicus* and Cyclopoida spp. were measured

under a dissecting microscope (Olympus, IX70) using a digital micrometer (Wraycam, NF500) at ×20 magnification.

Weight-specific respiration rates (R*, μLO_2 mg-dry-weight^{-1} h^{-1}) of species were calculated from total oxygen consumption rates over time, determined from the slope of the linear regression line of DO concentration in both experimental and control bottles against incubation time using the following equation (4-2) (Liu and Ban 2017):

$$R = \frac{(\Delta O_{\mathrm{exp}} - \Delta O_{\mathrm{c}}) \times V}{N \times \mathrm{W}} \times 1000 \qquad (4\text{-}2),$$

where ΔO_{exp} and ΔO_{c} are coefficients of oxygen consumption (μLO_2 L^{-1} h^{-1}) estimated from the slope of the linear regression in experimental and control bottles, respectively, using the least-square method; and V is the volume (L) of the experimental bottle, N is the number of animals in an experimental bottle, and W is the average body dry weight (μg). The body dry weight of the experimental animal was calculated from body length (mm) using the length-weight equation (Liu et al. 2020).

Generally, respiration rates increase in a regular manner with increasing temperature within the range that aquatic animals can tolerate (Brown et al. 2004; Liu et al. 2017). The magnitude of the acceleration of the respiration rate can be characterized by the ratio of rates resulting from a temperature increase of 10 ºC (Q_{10}) (Del Giorgio and Williams 2005). Compared the Q_{10} coefficient in each pH treatment may be useful to understand the synergistic effect of pH and temperature on metabolism of zooplankton. Because the R* of *D. pulicaria* did not differ significantly among the pH treatments at 10 and 20 ºC (see Results), Q_{10} was calculated using equation (4-3)

(Del Giorgio and Williams 2005):

$$Q_{10} = \left(\frac{k_1}{k_2}\right)^{\frac{10}{(t_1 - t_2)}} \qquad (4\text{-}3),$$

where k_1 and k_2 are average R^* values among the pH treatments at temperature t_1 (20 ºC) and t_2 (10 ºC), respectively.

2.5 Swimming behavior in *E. japonicus*

To identify relationship between swimming behavior and acidity, the swimming velocity of female *E. japonicus* was monitored in experimental media at pH 5.4, 6.0, 7.0 and 8.0 at 20 ºC using a two-dimension (2D) video-recording technique. Prior to the experiment, healthy and active animals were sorted from stock culture, and carefully washed with experimental media at the appropriate pH for a given treatment. One animal was placed into a well (16-mL) of a polystyrene tissue-culture plate (IWAKI, 1810-006-MYP) filled with 10 mL of experimental medium for each pH treatment. Swimming behavior was recorded from the upper side of the culture plate using a digital camera (Huawei LEICA, frame speed: 30 frames s^{-1}). Experimental water pH was measured prior to and following experiment; any differences from a target pH was within ± 0.1. Nine replicates were conducted for each pH treatment.

After 4 h acclimation to each pH condition, the swimming behavior of *E. japonicus* female was recorded for ~20 min, and 90 s of well-focused scenes from the recordings were used to estimate swimming velocity. Copepod position coordinates x and y were manually tracked using ImageJ software included a manual tracking

package (Version 1.53j, https://imagej.nih.gov/ij/index.html, March 7, 2022). The instantaneous swimming velocity (V, mm s^{-1}) was calculated for each individual track. V (at the scale of 1/30 s) was estimated as follows. At a generic time step t, V between two points in the 2D space was computed from the x and y coordinates using equation (4-4) (modified from Dur et al. 2011):

$$V = \sqrt{(x_{t+1} - x_t)^2 + (y_{t+1} - y_t)^2} \times \alpha \times p \qquad (4\text{-}4),$$

where (x_t, y_t) and (x_{t+1}, y_{t+1}) are the positions of the copepod at time t and $t + 1$, respectively, α is the distance of one pixel in frames ($\alpha = 0.0729$ mm px^{-1}), and p is the frame rate of the camera (30 frames s^{-1}).

2.6 Statistical analysis

Kaplan–Meier plots, produced with Prism 9 software (GraphPads 2021), were prepared to describe relationships between survival rates and exposure periods in different pH treatments. Paired log-rank tests were used to distinguish differences in survival curves in different pH treatments. LC_{50} values (where 50% of animals were dead after 24 h exposure) and 95% confidence intervals were calculated by Probit analysis (Finney 1971). The differences of pH ranges projected by the Probit analysis for estimating LC_{50} between 10 and 20 ºC treatments were tested using a generalized linear model (GLM), to evaluate how temperature affects acidity stress for each taxon.

Differences in $R*$ values among pH and temperature treatments were tested by two-way analysis of variance (ANOVA). Differences in V values for *E. japonicus* among pH treatments were tested by one-way ANOVA. The post-hoc Tukey–Kramer

tests were also conducted when the ANOVA revealed significantly different. Linear regression analysis between R^* and V for *E. japonicus* female was performed using a least-squares method. All statistical analyses were performed with SPSS software (IBM 2015) at a significance level of $P < 0.05$.

Table 4-1. The pH in experiments on survival and metabolic rates in *Daphnia pulicaria*, *Eodiaptomus japonicus* and Cyclopoida spp. at 10 and 20 ºC, and swimming behavior in *E.*

Temp	Taxa	pHs tested in survival exp.	pHs tested in respiration exp.	pHs tested in swimming behavior exp.
20 ℃	*D. pulicaria*	4.0, 4.2, 4.4, 4.6, 4.8, 5.0	4.6, 5.0, 6.0, 7.0, 8.0	—
	E. japonicus	4.8, 5.0, 5.2, 5.4, 5.6, 5.8, 6.0	5.4, 6.0, 7.0, 8.0	5.4, 6.0, 7.0, 8.0
	Cyclopoida spp.	4.0, 4.2, 4.4, 4.6, 4.8, 5.0	4.6, 5.0, 6.0, 7.0, 8.0	—
10 ℃	*D. pulicaria*	4.0, 4.2, 4.4, 4.6, 4.8, 5.0	4.8, 5.0, 6.0, 7.0, 8.0	—
	E. japonicus	4.8, 5.0, 5.2, 5.4, 5.6, 5.8, 6.0	—	—
	Cyclopoida spp.	4.0, 4.2, 4.4, 4.6, 4.8, 5.0	—	—

3. Results

3.1 Survival

Survival rates of all three taxa decreased with incubation period at both temperatures below pH 4.6, though the pH is slightly higher in *E. japonicus* (Fig. 4-1). After 24 h exposure, all *D. pulicaria* had died at pH < 4.2 at both temperatures, and all *E. japonicus* had died at pH < 4.8; whereas 16% of Cyclopoida spp. individuals survived even at pH 4.0 in 20 ºC.

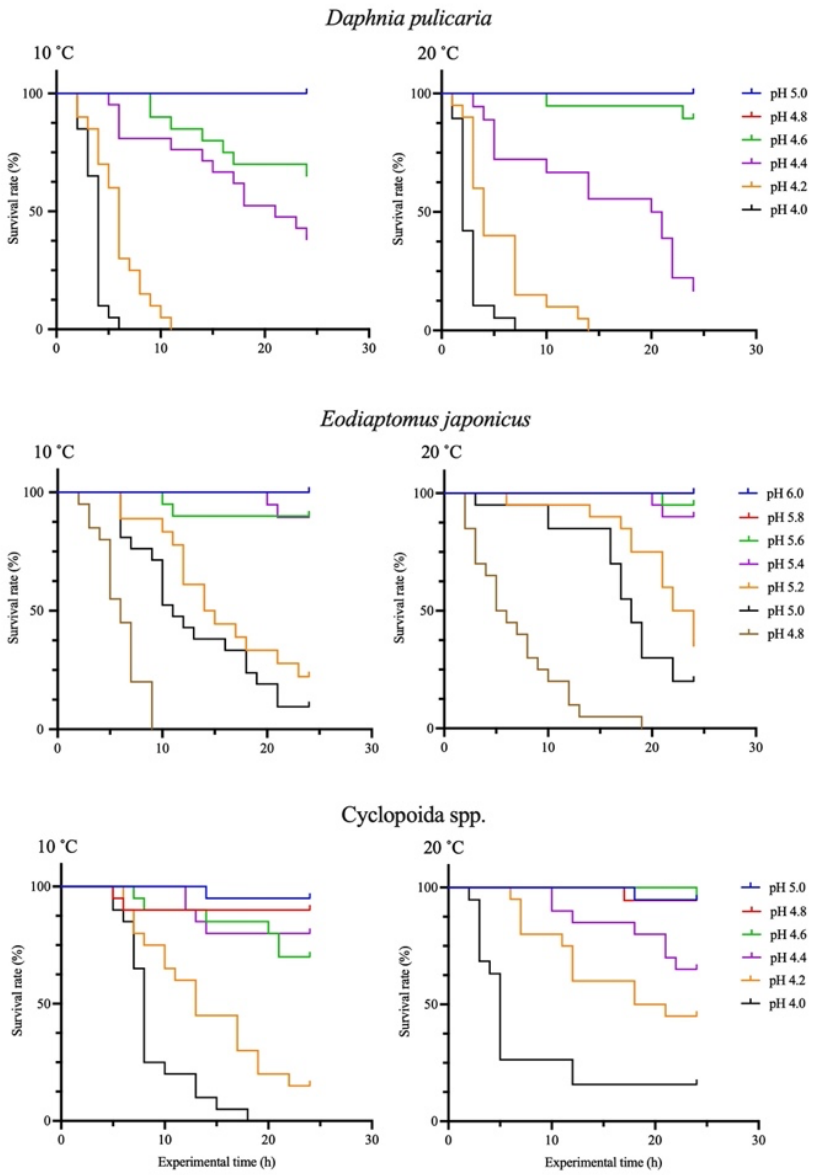

Fig 4-1. Kaplan-Meier plots of survival rate (%) of *Daphnia pulicaria*, *Eodiaptomus japonicus*, and Cyclopoida spp. over 24 h at various pH levels at 10 and 20 ˚C.

Log-rank tests revealed significant differences in survival curves for *D. pulicaria* between pH 4.6 and 4.8 at 10 ºC, and between pH 4.4 and 4.6 at 20 ºC (Table 4-2), for *E. japonicus* between pH 5.2 and 5.4 at both 10 and 20 ºC (Table 4-3), and for Cyclopoida spp. between pH 4.2 and 4.4 at 10 ºC, and between pH 4.4 and 4.6 at 20 ºC (Table 4-4).

Table 4-2. Pairwise comparisons of survival curves for each pH treatment in *Daphnia pulicaria* at 10 and 20 ºC. Data show chi-square values; asterisks represent significant differences between each of any two pH values. NA = not applicable for the analysis because all animals survived to the end of the experiment.

Species	Temp.	pH	5.0	4.8	4.6	4.4	4.2
D. pulicaria	10 ºC	4.8	NA				
		4.6	8.322*	7.909*			
		4.4	18.105*	17.224*	2.704		
		4.2	45.498*	43.637*	40.582*	29.532*	
		4.0	41.793*	40.255*	41.793*	39.676*	14.069*
	20 ºC	4.8	NA				
		4.6	1.839	2.055			
		4.4	25.300*	28.04*	21.352*		
		4.2	39.444*	43.072*	40.886*	18.502*	
		4.0	38.329*	41.618*	41.618*	30.652*	12.431*

Table 4-3. Pairwise comparisons of survival curves for each pH treatment in *Eodiaptomus japonicus* at 10 and 20 ºC. Data show the chi-square values; asterisks represent significant differences between each of any two pH values. NA = not applicable for the analysis because all animals survived to the end of the experiment.

Species	Temp.	pH	6.0	5.8	5.6	5.4	5.2	5.0
E. japonicus	10 ºC	5.8	NA					
		5.6	1.950	1.847				
		5.4	2.055	1.947	< 0.001			
		5.2	26.614*	25.298*	18.559*	21.626*		
		5.0	34.563*	32.929*	27.329*	30.353*	2.076	
		4.8	40.746*	38.942*	42.542*	40.746*	33.252*	23.732*
	20 ºC	5.8	NA					
		5.6	1.000	1.000				
		5.4	2.052	2.052	0.375			
		5.2	19.189*	19.189*	15.676*	12.368*		
		5.0	27.475*	27.475*	24.779*	22.292*	3.441	
		4.8	46.237*	46.237*	46.237*	46.237*	37.635*	25.886*

Table 4-4. Pairwise comparisons of survival curves for each pH treatment in Cyclopoida spp. at 10 and 20 ºC. Data show the chi-square values; asterisks represent significant differences between each of any two treatments. NA = not applicable for the analysis because all animals survived to the end of the experiment.

Species	Temp.	pH	5.0	4.8	4.6	4.4	4.2
Cyclopoida spp.	10 ºC	4.8	NA				
		4.6	4.182*	2.060			
		4.4	2.119	0.606	0.425		
		4.2	27.191*	20.449*	14.456*	16.805*	
		4.0	43.109*	31.501*	33.231*	35.196*	10.207*
	20 ºC	4.8	NA				
		4.6	< 0.001	0.003			
		4.4	5.137*	4.701*	5.479*		
		4.2	11.571*	10.638*	11.853*	2.275	
		4.0	26.263*	24.983*	26.263*	16.852*	10.459*

Probit curves revealed LC_{50} values with 95% confidential intervals for *D. pulicaria* to occur at pH 4.5 ± 0.1 at both two temperatures, for *E. japonicus* at pH 5.3 ± 0.1 and pH 5.2 ± 0.1, and Cyclopoida spp. at 4.4 ± 0.1 and 4.2 ± 0.1 at 10 and 20 ºC, respectively (Table 4-5).

Table 4-5. LC_{50} (pH) values and its 95% confidence intervals in *Daphnia pulicaria*, *Eodiaptomus japonicus* and Cyclopoida spp. for 24 hours exposure in acidic condition at two different temperatures (10 and 20 ºC).

Taxa	Temp (ºC)	24-h LC_{50} (pH)	95% confidence interval (pH)
D. pulicaria	10	4.5	4.39−4.61
	20	4.5	4.42−4.57
E. japonicus	10	5.3	5.21−5.39
	20	5.2	5.14−5.34
Cyclopoida spp.	10	4.4	4.27−4.46
	20	4.2	4.15−4.32

The order of LC_{50} of pH in three taxa is *E. japonicus* > *D. pulicaria* > Cyclopoida spp., and obviously different (Fig. 4-2). Of these taxa, *E. japonicus* is the most vulnerable to acidification at both temperatures, and Cyclopoida spp. is the least. The GLM showed significantly different (n = 70, df = 1, chi-square = 5.342, P = 0.021) in the estimated pH at which the LC_{50} was reached at 10 and 20 ºC for Cyclopoida spp., but no significant differences were found for *E. japonicus* (n = 70, df = 1, chi-square = 1.374, P = 0.241), and *D. pulicaria* (n = 70, df = 1, chi-square = 0.007, P = 0.932).

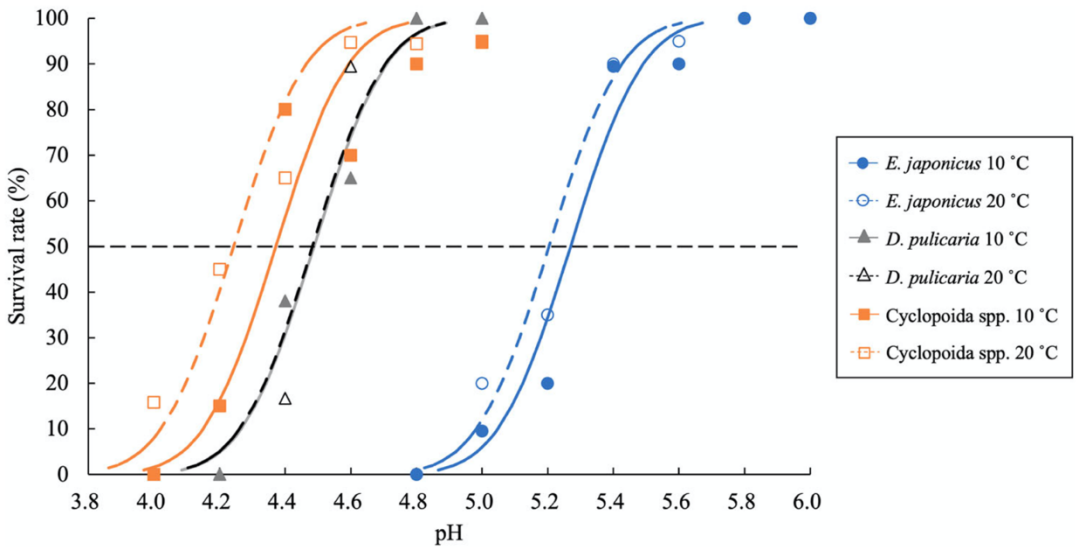

Fig 4-2. Survival rates (%) after 24 h exposure in *Daphnia pulicaria*, *Eodiaptomus japonicus*, and Cyclopoida spp. at various pH levels at 10 and 20 ºC. Curves are fitted using *Probit* analysis.

However, survivals of *E. japonicus* declined more rapidly at pH < 5.2, and that of *D. pulicaria* at pH < 4.6 at 10 ºC than did at 20 (Fig. 4-1). This reflected to the result of log-rank test (Table 4-2, 4-3). Cold water slightly seemed to increase the acidic detriment in all tested zooplankton taxa.

3.2 Respiration rate

Mean *R** values for *D. pulicaria* were relatively stable among the pH treatments at both 10 and 20 ºC, and varied 2.9−3.4 μLO_2 mg-dry-weight^{-1} h^{-1} in pH 4.8−8.0 at 10 ºC and 5.2−5.8 μLO_2 mg-dry-weight^{-1} h^{-1} in pH 4.6−8.0 at 20 ºC (Fig. 4-3a). Two-way ANOVA reveals that the *R** values of *D. pulicaria* did not differ significantly ($P > 0.05$) among pH treatments, but that they did differ significantly ($P < 0.05$) between temperatures, without an interaction effect ($P > 0.05$) (Table 4-6). The Q_{10} of *R** for *D. pulicaria* is 1.77 when temperature increased from 10 to 20 ºC.

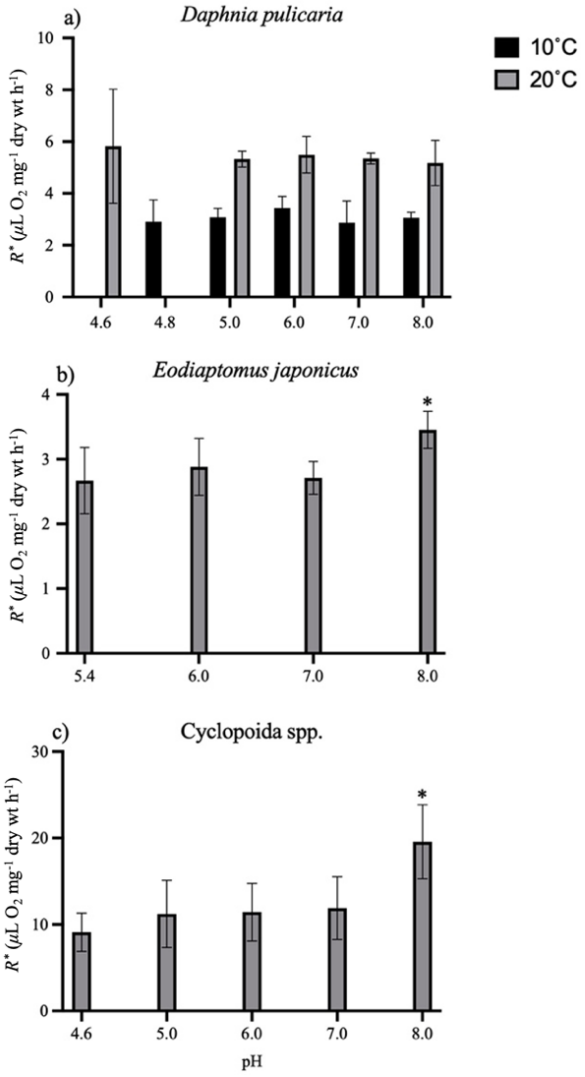

Fig.4-3. Average weight-specific respiration rate (*R**, μLO_2 mg-dry-weight^{-1} h^{-1}) in *Daphnia pulicaria* (a), *Eodiaptomus japonicus* (b), and Cyclopoida spp. (c) at various pH for 10 and 20 ºC. Vertical bars represent standard deviation. Asterisks indicate significant differences at $P < 0.05$.

For copepod taxa, mean R^* values at 20 ºC were relatively stable below pH 7.0, at 2.8 μLO_2 mg-dry-weight^{-1} h^{-1} (*E. japonicus*) and 10.9 μLO_2 mg-dry-weight^{-1} h^{-1} (Cyclopoida spp.), and showed no significant difference among pH treatments ≤ 7 (post-hoc Tukey–Kramer test, $df = 29$ (*E. japonicus*) and 40 (Cyclopoida spp.), both $P > 0.05$), while the R^* values were significantly higher at pH 8.0, at 3.5 μLO_2 mg-dry-weight^{-1} h^{-1} (*E. japonicus*) and 19.6 μLO_2 mg-dry-weight^{-1} h^{-1} (Cyclopoida spp.), respectively (one-way ANOVA, $df = 3$ and 29, $F = 7.088$, $P = 0.001$ (*E. japonicus*), and $df = 4$ and 40, $F = 12.495$, $P < 0.001$ (Cyclopoida spp.), both at 20 ºC) (Fig. 4-3b, c).

Table 4-6. Results of two-way ANOVA on effects of pH and temperature (Temp) on weight-specific respiration rate (R^*) in *Daphnia pulicaria*, and one-way ANOVA on the effect of pH on R^* in *Eodiaptomus japonicus* and Cyclopoida spp.

Taxa	Parameter	*df*	*F*	*P*
D. pulicaria	pH	5	0.737	0.598
	Temp	1	86.331	< 0.001
	pH × Temp	3	0.148	0.931
E. japonicus	pH	3	7.088	0.001
Cyclopoida spp.	pH	4	12.495	< 0.001

df, degrees of freedom.

3.3 Swimming behavior

During survival experiments, the swimming behaviors appeared to differ between pH 7.0 and 8.0 for both *E. japonicus* and Cyclopoida spp., but not for *D. pulicaria*, possibly because of different responses in R between the daphnid and copepods.

Therefore, we compared relationships between R^* and V for *E. japonicus* as a representative copepod. Mean V values for *E. japonicus* were also stable and did not differ significantly (post-hoc Tukey–Kramer test, $df = 32$, $P > 0.05$) at pH ≤ 7.0, being 0.713 mm s^{-1} on average, but they were significantly higher (one-way ANOVA, $df = 3$ and 32, $F = 5.341$, $P = 0.004$) at pH 8.0 compared with those at lower pHs, which were 1.417 mm s^{-1} on average (Fig. 4-4a). Linear regression analysis revealed R^* significantly increased with V ($n = 36$, $r^2 = 0.986$, $t = 11.8$, $P < 0.05$) (Fig. 4-4b).

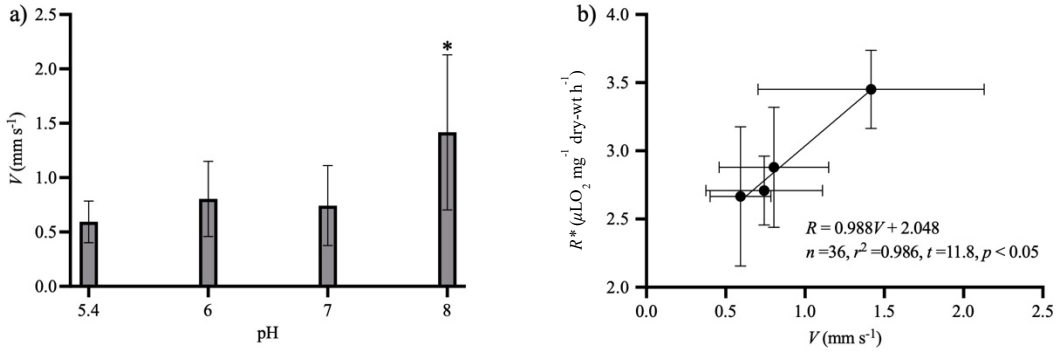

Fig 4-4. Average instantaneous swimming velocity (V, mm s^{-1}) of *Eodiaptomus japonicus* adult females at four different pH levels (a), and relationship between weight-specific respiration rate (R^*, μLO_2 mg-dry-weight^{-1} h^{-1}) and V in adult female *E. japonicus* adult females. Regression line equation $R^* = 0.988V + 2.048$ ($n = 36$, $r^2 = 0.986$, $P < 0.05$). Vertical and horizontal bar represent standard deviation. The asterisk represents a significant difference at $P < 0.05$.

C

4. Discussion

In this study, most test individuals of each taxon survived for 24 h even at a low pH of 4.6, although *E. japonicus* was more vulnerable to low pH. Similar tolerance to acidic stress has been reported for many zooplankton species. For example, Ghazy et al. (2011) reported that all *Daphnia magna* to survive at pH 4.66 over 24 h exposure.

Havas and Hutchinson (1982) found most individuals survived at pH 4.5 in both *Daphnia middendorffiana* and *Diaptomus arcticus* after 12 h exposure. Price and Swift (1985) showed > 80% of the *Daphnia pulex* experimentally exposed to pH 5.2, *Daphnia galeata mendotae* to pH 5.0, and *Mesocyclops edax* to pH 4.4, survived after 48 h exposure.

High tolerance to low pH in zooplankton may be related to physiological compensatory mechanisms involving high proton tolerance (Rosseland 1994). Weber and Pirow (2009) reported *D. pulex* to resist acidic stress through an acid–base balance mechanism reaction ($H^+ + HCO_3^- = H_2O + CO_2$) to maintain a relatively stable pH (8.10−8.33) in its hemolymph even at a low ambient pH of 5.5. When the proton concentration exceeds the tolerance limit for the compensatory mechanism, excess protons may decrease the pH of *in vivo* hemolymph (e.g., the pH of hemolymph of *D. pulex* rapidly decreased from pH 8.3 to < 7.8 within 1 h when transferred them from pH of 7.0 to 3.0) (Weber and Pirow 2009).

Depressing the pH in hemolymph may cause significant ammonia accumulation in zooplankton (Schründer et al. 2013), which is generally toxic for most aquatic organisms including zooplankton (Weihrauch et al. 2004). Carbonic anhydrase plays an important role in zooplankton acid–base regulation because it can efficiently produce bicarbonate through hydration of carbon dioxide (Whiteley et al. 1999; Culver and Morton 2015). High concentrations of protons in hemolymph may inhibit the activity of many enzymes including carbonic anhydrase (Roughton and Booth 1946; Solgaard et al. 2007), and even may damage the shape or configuration of some proteins because

of the structures of them are partly shaped by hydrogen bonds (Grabowski 2006; Han and Zhao 2011). Consequently, excess protons induce interlamellar mucous clogging in crustaceans (Havas and Hutchinson 1983), swelling of heart muscle in *D. magna* and *D. pulex* (Brett 1989), and gill tissue damages in *Daphnia ambigua* (Zimmer 1987). Because these organs have important physiological functions, such as normal circulation of body fluid, respiration, ion and osmoregulation, acid–base regulation, and nitrogen excretion, any breakdown in function may cause death of the animals (Brett 1989).

Of the three taxa studied, Cyclopoida spp. are the most tolerant to acidic stress, and *E. japonicus* is the most vulnerable. Cladocerans are reputedly susceptible to acidic stress (based on LC_{50} over 48 h exposure), followed by *Mesocyclops* and *Chaoborus* larvae (Price and Swift 1985). Tolerance to acidification varies among species (Havas and Hutchinson 1982; Bulkowski et al. 1985; Price and Swift 1985), possibly because of species-specific water permeability — the water exchange rate between the zooplankton body and surrounding waters. Net daily osmotic flux is equivalent to 5–10% per day of the total body water in water mites and *Chaoborus* spp., and 480–600% per day in daphnids; a cyclopoid copepod took at least one day to replace its entire body water (Nilssen et al. 1984). Information on whole-body water permeability of freshwater calanid copepods is lacking (Krogh 1939; Rasmussen and Andersen 1996). High-water permeability in zooplankton may easily induce lowered body fluid pH under acidic conditions.

In the present study, acidic detriment was slightly intensified at low temperature.

Lowered temperature decreases zooplankton metabolic rates (Heinle 1969; Liu and Ban 2017), reducing their physiological activity (Heinle 1969), including resistance to acidic stress. For example, proteolytic activity in *Calanus finmarchicus* (Solgaard et al. 2007), α-amylase activity in *Heliodiaptomus viduus* (Dutta et al. 2006), and carbonic anhydrase activity in crustaceans (Henry and Cameron 1982), are all inhibited by low temperature. These enzyme activities play important roles in maintaining physiological processes and/or *in vivo* pH balances in zooplankton (Whiteley et al. 1999; Knotz et al. 2006).

Respiration rates of the copepods, *E. japonicus* and Cyclopoida spp. decreased in acidic conditions (pH $\leq$ 7.0), but not so those in daphnid. This difference may be attributed to indirect effects of lowering pH on swimming behavior. Because respiration rates of *E. japonicus* correlated positively with its swimming activity, lowering pH may decrease swimming activity, thereby reducing respiration (Vlymen 1970; Seuront 2010). Chen et al. (2012) reported that behavior strengths (including swimming velocity, movement frequency, and movement extent) in *D. magna* did not change significantly even at pH 5.0 after 8 h of exposure but declined thereafter. Since we conducted both respiration and swimming behavior experiments for a 6 h period only, the lack of any apparent response in respiration rate was found because acidic stress in *D. pulicaria* may be an artefact of the relatively short exposure duration.

In Lake Biwa, *E. japonicus* was the dominant species which accounts for 65% of the total biomass of crustacean zooplankton over four decades (1971−2010), followed by *Daphnia* spp. at 17% and Cyclopoida spp. at 15% (Liu et al. 2020). Short-term acid

stress caused by acid rain may also affected the community structure of zooplankton. Vulnerability for acidity was different among the zooplankton taxa; *E. japonicus* was the most vulnerable species, followed by *D. pulicaria* and Cyclopoida spp. Population dynamics of these three taxa may be directly or indirectly interrelated with each other in the food web because *D. pulicaria* is herbivore, *E. japonicus* is omnivore, and Cyclopoida spp. is carnivore and ingests small cladocerans and juveniles of *E. japonicus* (Kawabata et al. 2006; Liu et al. 2021). Especially, cold waters in winter may induce a depression of the ability for acidic tolerance of the zooplankton under a potential threat on low pH of snowmelt water from the catchment area into Lake Biwa (Fushimi et al. 1994). Almer et al (1974) investigated nearly 400 lakes in the Swedish west coast region and found most species of diatoms and green algae disappeared in the acidified lakes at pH < 5.8. Wang et al. (2011) reported the growth of algae *Microsystis aruginosa* was significantly inhibited by acidic condition (pH < 6.5), even occurred death at pH < 6.0. Therefore, the acidic stress may also potentially affect zooplankton community through the food web.

General Discussion

Organisms take up energetic resources from surrounding environments and convert them into other forms within their bodies and allocate the converted materials to enhance the fitness processes of production. The overall rate of these processes, i.e. the metabolic rate, determines the biological activities (Brown 2004). Zooplankton is poikilotherm and contributes most of secondary production in aquatic ecosystem. Because they are very sensitive to environmental changes and can be used as an environmental indicator, it is important to evaluate how the zooplankton production changes in a chaotic aquatic environment, to clarify the environmental stresses affects the biological processes in zooplankton.

Temperature and body size are the main two abiotic and biotic factors that regulate metabolism of aquatic organisms (Mauchline 1998; Brown 2004). Food condition, population density and acidic stress can be considered as other crucial factors largely influencing the metabolic processes (Alibone and Fair 1981; Kiørboe 1985; DeLong et al. 2014). However, it was difficult to determine metabolic loss as respiration rates in mesozooplankton due to low oxygen consumption of small body mass until recently. The measurements of respiration rates in such a small animal largely depended on the methodology including measuring procedure and the precision of measurements in the equipment uesed (Devol, 1979; Williams and Jenkinson, 1982; Ploug et al., 2008). Therefore, it was difficult to conclude how zooplankton dynamics through biological processes responded to environmental changes in the previous studies. Recently, a

contactless optical spot-fiber oxygen sensor was developed to measure dissolved oxygen concentration in water (Bode et al., 2013; Teuber et al., 2013). Liu and Ban (2017) developed a method for measuring the respiration rates at high precision (detection limit: 10^{-4} μLO_2 ind^{-1} h^{-1}) for a single zooplankton in body size of 1 mm within just several hours using such a contactless oxygen sensor. Benefit of this method is that zooplankton metabolic responses to the environmental stresses can be measured more conveniently and precisely.

In this study, I newly found that metabolic response to starvation in juvenile daphnids responded relatively short period starvation of > 13 h compared to those in adults (> 3 days). This suggests that the juvenile stages exhibit more sensitive biological response compared to adult. Generally, juvenile zooplankton exhibit high mortality under not only the starved conditions (Tsuda 1994; Liu et al. 2015) but also the sufficient food conditions (Liu et al. 2014) due to its low feeding ability (Berggreen et al. 1988; Merrell and Stoecker, 1998). It has been known that metabolic maintenance in zooplankton is supported by the energy source stores (Bradley et al. 1991), and that juvenile zooplankton does not accumulate lipid stores even in the presence of excess food condition (Håkanson, 1984). According to the present study, juvenile daphnids reduced the metabolic loss just at a half day starvation. The rapid response to starvation might benefit its survival and consequently the population growth. Whereas, adult daphnids can maintain a constant metabolism until 3 days starvation due to greater energy source stores (Tessier et al. 1983).

Food shortage for zooplankton is more common in nature due to nutritional

limitation and competitive interactions, and generally cannot reach its potential growth in the field (Liu et al. 2018; Liu et al. 2021). In this study, under food shortage, growth indicators such as both size and body weight decreased in adult daphnids but not in juveniles. This difference in somatic growth might be induced by different biological responses to food conditions between adults and juveniles. It has been known that adult individuals need more energy to mature their reproductive organs (Devreker et al. 2007). In the present study, *D. magna* adult almost ceased egg production under food shortage. This indicated that most energy allocated to maintain survival rather than reproduction. Net growth efficiencies in the adults were also higher in food shortage than those supplied sufficient food, suggesting that the adult daphnids might adapt food shortage and reduce metabolic loss to increase its net production. These results suggests that food shortage mainly influences adult stages rather than juveniles, and the population growth might be obviously depressed due to lowering reproduction under severe food shortage.

Population density has been shown as another important biotic factor regulating zooplankton metabolism. However, the crowding effect on zooplankton remains unclear because different responses were confirmed even for the same species probably due to the different determining procedures (Yashchenko et al. 2016) as I mentioned in Chapter 3. In the present study, metabolic rate of *D. magna* showed negative response to crowding. The similar response has been shown in copepods which also determined by using the same contactless oxygen sensor (Takayama et al. 2020). Relationship between metabolic loss and crowding might be associated with lowering specific

dynamic action (SDA) due to density-mediated reduction of ingestion rate (Helgen 1987; Ban et al. 2008). I also found the interaction effect between density and temperature on metabolic rate of zooplankton. This implies that the density-mediated response on metabolic loss may be decelerated at lower temperature.

Acidification of aquatic ecosystem induced by human activities such as increasing CO_2, SO_x and NO_x emissions to the atmosphere during the past half century (IPCC 2019). Acidic stress can be considered as one of influential abiotic factor, and potentially regulate zooplankton population dynamics because animals are highly sensitive to acidic stress through an acid-base balance (Hamm et al. 2015). It has been known that zooplankton life history traits and metabolism response to acidic stress and that the responses vary among species (Alibone and Fair 1981; Bailey et al. 2017). Concentration of bicarbonate ions plays an important role for regulating acid-base balance in the haemolymph and intracellular compartments of crustacean zooplankton (Egginton et al. 2004). The elevation in bicarbonate ions is insufficient to compensate for the metabolic acidosis (Taylor and Innes 1988), and consequently causes increasing mortality. In the present study, three dominant crustaceans from Lake Biwa showed stronger acidic tolerance compared to those in marine species (Cripps et al 2015; Bailey et al. 2017), because freshwater environments show large pH variance due to the low ironic concentrations. In the present study, copepods were more sensitive to acidification than cladocerans. Since copepods are the most abundant secondary producers in aquatic ecosystem, acidification therefore may threaten secondary production.

Summary

1. Chapter 1

Respiration rates of well-fed *Daphnia magna* were measured after starvation from 2 h to 7 days and determined a threshold period for starvation. Respiration rates of *D. magna* decreased under starvation, but the metabolic response was different among ages. Small sized individuals maintained relative constant metabolic rates until 12 h starvation and decreased after, whereas the large sized individuals maintained constant metabolic rate until 3 days starvation and start decreasing. These results indicated that starvation resistance ability largely depends on body size, i.e., small animals can tolerant starvation only in several hours, but large animals can tolerant starvation at least several days. We also found that weight-specific respiration rates of *D. magna* decreased with body size with the interaction effect of body weight and starvation, indicated that starvation induced metabolic rate depression may change with body weight. We finally concluded that mature zooplankton may tend to maintain their basal metabolism to keep survival.

2. Chapter 2

In the oligo- and mesotrophic lakes, pelagic zooplankton are often faced to food shortage, and consequently the physiological activities of zooplankton was affected. Therefore, the physiological activates of *Daphnia magna* such as carapace length, body dry-weight, clutch size and respiration rate at food shortage (2.5×10^3 cells mL^{-1} of *C.*

reinhardtii) and well-fed (5×10^5 cells mL^{-1} of *C. reinhardtii*) conditions had been monitored from 2 to 18 days old. The results shown that food shortage significantly reduced the carapace length, body dry-weight, cumulative number and clutch size of *D. magna* at adult stage, while no significantly affect those at juvenile stage. The metabolic rate of *D. magna* was depressed by food shortage compared with those in well-fed, and the respiration rate of *D. magna* in food shortage increased with body dry-weight was more slowly compared with those in well-fed condition.

3. Chapter 3

Physiological responses of zooplankton to crowding may be fundamentally related to metabolic functions, but remain unclear on complexities with species specific responses, measurement conditions, and synergistic effects with temperature. In this study, we determined respiration rates of cladoceran *Daphnia magna* in three different densities (1, 10, and 20 ind. 50-mL^{-1}) at two temperatures (10 and 20 ºC) using a high accurate optical oxygen meter to clarify synergistic effects of crowding and temperature on metabolic rate. Respiration rates (R, μLO_2 ind^{-1} h^{-1}) of *D. magna* in the two crowded treatments were significantly lower than those in a single treatment at both temperatures with interaction effect of temperature, while not significantly different between the two crowding treatments. Q_{10} values varied from 1.63 at 20 ind. 50-mL^{-1} to 2.29 at 1 ind. 50-mL^{-1} treatment. The density mediated metabolic rate might be related to food uptake and that this relationship may be decelerated at low temperature.

4. Chapter 4

Acute acidic stress from sources such as acid rain might impact on lake ecosystems in many aspects. Because zooplankton is sensitive to environmental change, it could be a good biotic indicator of the effects of acidification. We investigated survival and metabolic rates of three dominant zooplankton taxa in Lake Biwa, the cladoceran *Daphnia pulicaria*, and the copepods *Eodiaptomus japonicus* and Cyclopoida spp., when subjected to different pH values (pH 4.0−8.0) at low (10 ºC) and high (20 ºC) temperatures. Because mortality of *D. pulicaria* and Cyclopoida spp. exceeded 50% over 24 h of incubation at pH < 4.6, but for *E. japonicus* this occurred at pH < 5.6, *E. japonicus* may be more vulnerable to acidic stress than other two taxa. This vulnerability to acidification slightly increased at 10 ºC for each taxon. Metabolic rates in *D. pulicaria* remained relatively constant over a wide pH range (4.6−8.0), but those of both copepods decreased at acidic conditions (pH 4.6−7.0). This decrease of metabolic rate might be related to their swimming activity that decreased obviously at acidic conditions.

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APPENDIX

Appendix 1: C media

Add the elements (total 20 mL) to a flask (1 L) with distilled water of 980 mL. Use HCL (1 mol/mL) to adjust the pH of medium to 7.5. Finally autoclaved at 120 ˚C for 20 min.

Elements	Stock solution	Quantity
$Ca(NO_3)_2 \cdot 4H_2O$	15 g / 100 mL	1 mL
KNO_3	10 g / 100 mL	1 mL
β-Na_2glycerophosphate・$5H_2O$ (Disodium β-Glycerophosphate)	5 g/ 100 mL	1 mL
$MgSO_4 \cdot 7H_2O$	4 g / 100 mL	1 mL
Vitamin B_{12}[b]	0.01mg / 100 mL	1 mL
Biotin[b]	0.01 mg / 100 mL	1 mL
Thiamine HCl	1 mg / 100 mL	1 mL
P IV metals[a]	————	3 mL
Tris (hydroxymethyI) aminomathane	50 g / 1000 mL	10 mL

[a] See P IV metals

[b] Store in refrigerator or freezer

Add 1.5 g agar to 100 mL of medium to give a solid medium.

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Appendix 2: VT media

Add the elements (total 20 mL) to a flask (1 L) with distilled water of 980 mL. Use NaOH (1 mol/mL) to adjust the pH of medium to 7.5. Finally autoclaved at 120 ˚C for 20 min.

Elements	Stock solution	Quantity
$Ca(NO_3)_2 \cdot 4H_2O$	11.78 g / 100 mL	1 mL
KCL	5 g / 100 mL	1 mL
β-Na_2glycerophosphate · $5H_2O$ (Disodium β-Glycerophosphate)	5 g/ 100 mL	1 mL
$MgSO_4 \cdot 7H_2O$	4 g / 100 mL	1 mL
Vitamin B_{12}[b]	0.01mg / 100 mL	1 mL
Biotin[b]	0.01 mg / 100 mL	1 mL
Thiamine HCl	1 mg / 100 mL	1 mL
P IV metals[a]	————	3 mL
Glycylglycine	50 g / 1000 mL	10 mL

[a] See P IV metals

[b] Store in refrigerator or freezer

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Appendix 3: P IV metals

Add the elements (total 5.5 mL) to a flask (500 mL) with distilled water of 494.5 mL.

Elements	Stock solution	Quantity
Na_2 EDTA・$2H_2O$	————	0.5 g
$FeCl_3$・$6H_2O$	1.96 g / 100 mL	5 mL
$MnCl_2$・$4H_2O$	0.36 g/ 100 mL	5 mL
$ZnSO_4$・$7H_2O$	0.22 g / 100 mL	5 mL
$CoCl_2$・$6H_2O$	0.04 g / 100 mL	5 mL
Na_2MoO_4・$2H_2O$	0.025 g / 100 mL	5 mL

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