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Physiological and molecular responses of the Antarctic harpacticoid copepod *Tigriopus kingsejongensis* to salinity fluctuations – A multigenerational study

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ABSTRACT

Since Antarctica and the surrounding Southern Ocean are facing global climate change, biota inhabiting those coastal regions is now challenged by environmental fluctuations including coastal freshening. In this study, the effects of salinity range of 0–75 (practical salinity unit, PSU) on the Antarctic harpacticoid copepod *Tigriopus kingsejongensis* was investigated by measurement of 96 h survival rate, lifespan, and sex ratio with further analysis of multigenerational growth parameters and mRNA expressions under salinity of 15–45. Different stages of the copepods (i.e., nauplius, male, and female) generally showed tolerance to hypo– and hypersalinity, wherein female copepods were more tolerant than males when exposed to salinity fluctuations. Lifespan was significantly shortened by hypo- and hypersalinity compared to control salinity (34), but there was no significant difference in the sex ratio between salinity treatments. Multigenerational experiments across five generations revealed that exposure to salinities of 15 and 45 reduced body length compared to that in control salinity and the first generation of each salinity group. Our results provide evidence regarding *T. kingsejongensis* on their preferred salinity ranges, physiological limit to salinity fluctuations, and population dynamics in future salinity.

1. Introduction

Antarctica, surrounded by the Southern Ocean, is one of the most extreme remote environment on earth and plays a crucial role in regulating the earth's climate system. Despite its geographic isolation and consistent environmental conditions (e.g., air/sea temperatures) during millions of years, rapid global warming in and around Antarctica is one of the greatest threats to its ice-based polar ecosystem (Cheng et al., 2019; Convey and Peck, 2019; Rogers et al., 2020). Since ectotherms living in the marine and terrestrial environments of Antarctica have adapted to these unique environmental conditions, they are likely to be susceptible to unpredictable environmental changes. Recently, it was reported that seawater temperature of the western Antarctic Peninsula has elevated by approximately 2 °C during the last half century (Meredith and King, 2005; Cook et al., 2016), leading to substantial glacier retreat and melting land ice. It is expected that a rapidly increased inflow of freshwater may have significantly changed salinity in western Antarctic coastal regions (Turner et al., 2005; Durack, 2015; Haumann et al., 2016; Li et al., 2020). In the Southern Ocean, continuous salinity change has considered one of the most prominent signals of climate change in the global oceans (Jacobs et al., 2002; Böning et al., 2008; Helm et al., 2010; Purkey and Johnson, 2013; de Lavergne et al., 2014). Previously, trends in the northward transport of Antarctic sea ice were suggested as a crucial contributor to salinity change with a freshening rate of -0.02 ± 0.01 g kg^{-1} per decade from 1982 to 2008 in the surface and intermediate waters (Haumann et al., 2016). This environmental change requires urgent attention and study of the response of Antarctic aquatic organisms to rapid salinity variation (Convey and

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Peck, 2019). However, to date, little is known about the behavioral, physiological, molecular, and biochemical responses of Antarctic aquatic organisms to salinity variation.

Salinity gradient is an important factor controlling the size, distribution, and composition of zooplankton communities (Cervetto et al., 1999; Kimmel and Roman, 2004; Svetlichny and Hubareva, 2014; Kayfetz and Kimmerer, 2017). Changes in the salinity range can fundamentally alter their environment in concert with increased temperature, altered sea ice, and ocean acidification (Convey and Peck, 2019; Sugie et al., 2020), by modulations of the vertical exchanges of heat, carbon, oxygen, chlorophyll, and other crucial components that required for animal homeostasis (Meredith and King, 2005). Copepod is a major zooplankton taxon with krill and slaps in the Antarctic Ocean (Atkinson et al., 1999). Estuarine and marine copepods have shown broad plasticity in response to salinity changes (Lance, 1963; Dutz and Christensen, 2018). Among copepods, the genus *Tigriopus* (order Harpacticoida) has continuously been studied with respect to tolerance and physiological responses against salinity fluctuations. For example, an exposure experiment lasting 15 days revealed that *T. fulvus* died at 84 h in distilled water and solutions of salinity (practical salinity unit, PSU) above 90, whereas they survived the entire exposure duration from salinity 4.2 to 90 (Ranade, 1957). Interestingly, when the copepods were immediately transferred to salinity 34 after exposure to above salinity 90, they regained normal activity, demonstrating their metabolic tolerance to salinity fluctuations during tidal and drought periods in intertidal rock pools (Ranade, 1957). Reproduction success in artificial seawater was observed in salinity of 5–21 (Huizinga, 1971) and active sodium regulatory metabolism in response to salinity range (McDonongh and Stiffier, 1981) was observed in *T. californicus*. Survival in extreme salinity (139 PSU) was also detected in *T. californicus* (Powlick, 1999). In this species, the accumulation of intracellular organic osmolytes such as proline, glycine, and alanine was identified as a physiological mechanism for maintaining homeostasis against hyperosmotic stress (Burton and Feldman, 1983). Subsequently, roles of the accumulation of certain amino acids in the regulation of salinity have been conducted in the genus *Tigriopus* (Goolish and Burton, 1988; McAllen, 2003). In *T. japonicus*, high tolerance to salinity of even 1.8 PSU (Lee and Hu, 1981) was observed with a wide range of tolerance (Kwok and Leung, 2005). *T. brevicornis* also showed tolerance to a broad range of salinity (5–60 PSU) and acclimation to high salinities enhanced its tolerance to high osmotic stress (45 and 60 PSU), while tolerance in low salinities (5 and 10 PSU) was hardly affected by salinity acclimation (Damgaard and Davenport, 1994). The most suitable salinity for *T. japonicus* exposed to different salinities (4, 8, 16, and 32 PSU) was 32 PSU for significant population growth, time to reach maturation stage, survival rate, and lifespan (Hagiwara et al., 1995). Recently, in *T. californicus*, noteworthy results on the salinity tolerance mechanism were obtained. Population-specific transcriptomic responses (Northern versus Southern California) were studied and the potential involvement of transcripts coding for “amino acid transport” and “ion transport” pathways was suggested under osmotic stress (DeBiasse et al., 2018). Greater resistance to environmental stressors, including hypersaline or hyposaline conditions was observed in females of *T. californicus*, although they lack the canonical sex chromosomes (Foley et al., 2019). A study applied common-garden experiments along with transcriptome analysis and investigated the significant effects of acute low salinity on mortality and increased molecular process of proline metabolism in low salinity, along with consideration of a wider variety of environmental conditions including salinity range for understanding local adaptation of the species (Lee et al., 2021). However, information regarding the response of the Antarctic *Tigriopus* to salinity fluctuation is still unavailable.

The morphological characteristics of the Antarctic-endemic copepod *Tigriopus kingsejongensis* (Harpacticoida; Harpacticidae) are well described (Park et al., 2014), which was first discovered at the coast of the Barton Peninsula in Maxwell Bay, King George Island. *T. kingsejongensis* is known to be a suitable indicator to study Antarctic ecological and environmental changes, due to distinguishable developmental stages and sexual dimorphism, transgenerational reproduction, and sensitivity to external forces. The additional advantage of *T. kingsejongensis* is the availability of reference genome information (Kang et al., 2017). In contrast to many marine copepods, the tolerance, physiological responses at the molecular/biochemical level, and multigenerational effects on salinity fluctuation are rudimentarily understood in *T. kingsejongensis*. In this study, as a first step towards understanding the adaptation and distribution of Antarctic harpacticoid copepods in response to Antarctic environmental changes, we investigated the responses of *T. kingsejongensis* in light of tolerance, physiology at the molecular/biochemical level, and multigenerational effect to salinity variation.

Main objectives of the present study, therefore, were to investigate the tolerance of *T. kingsejongensis* in response to salinity variations (acute to chronic), and in turn to identify the salinity tipping point that might modulate their reproduction and population dynamics, such as mortality and growth rate. This study will provide significant insight into the alteration of the Antarctic zooplankton community in response to rapid Antarctic environmental changes.

2. Materials and methods

2.1. Sampling and culture

The Antarctic harpacticoid copepod *T. kingsejongensis* was captured using a 100-μm plankton net at the surface water (<1 m in depth) from King Sejong station to the inner regions of Marina Cove in September 2016 (measured salinity on the sampling site: 34.04) (Fig. 1A and **B**). Copepods were not sampled in the tidal pool, as they can be stressed by environmental fluctuations (e.g., increased in salinity and temperature, desiccation, ultraviolet radiation). Relatively low salinity of approximately 31 was seasonally observed (Kim et al., 2020), and drastically lower salinity of approximately 20 was also measured at the sampling region (Sin et al., 2020).

The copepods were transferred to the Korea Polar Research Institute (Incheon, South Korea) using several thermos containers (10 L) filled with pre-collected filtered (0.45 μm pore size) seawater from the sampling site while maintaining a temperature of ~3–5 °C. They were continuously cultured at 3 ± 0.3 °C under the following culture conditions: 6.9–7.6 mg dissolved oxygen (DO) L^{-1} and a light:dark photoperiod of 14:10 h, in Panasonic MIR cooled incubators (MIR–554, 238 L; Panasonic®, Japan) using filtered artificial seawater (ASW; 34 PSU, pH: 7.8–8.1, TetraMarine Salt Pro, Tetra, Cincinnati, OH, USA) through 0.45-μm pore size cellulose nitrate membrane filter. Temperature (accuracy: 0.1°C), salinity (0.10 ppt), pH (0.002), and DO (0.01 ppm) were monitored daily using an Orion Star meter (520M-01A, Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with pH/DO/conductivity electrodes. The copepods were fed a mixture of fresh microalgae (*Chlorella* spp. and *Tetraselmis* spp. cultured in 3 and 15 PSU, respectively.) and the concentration was maintained at approximately 1×10^6 cells mL^{-1} in each aquarium rationed two times weekly. To prevent salinity fluctuations due to feeding, the microalgae solution was concentrated and dissolved into ASW (30 PSU) before feeding. ASW was replaced with pre-made filtered ASW adjusted to 3 °C. Fifty percent of ASW was replaced two times per week to maintain the water quality. All experiments were conducted with the tenth generation of cultured copepods in laboratory.

2.2. Acute salinity tolerance experiments

The control salinity was set as 34 based on the measured salinity during sampling (34.04 PSU) and our 10-year observation of the site from where the copepods were sampled. Surface salinity (average from 0 to 5 m depth) data were collected 76 times from March 2011 to

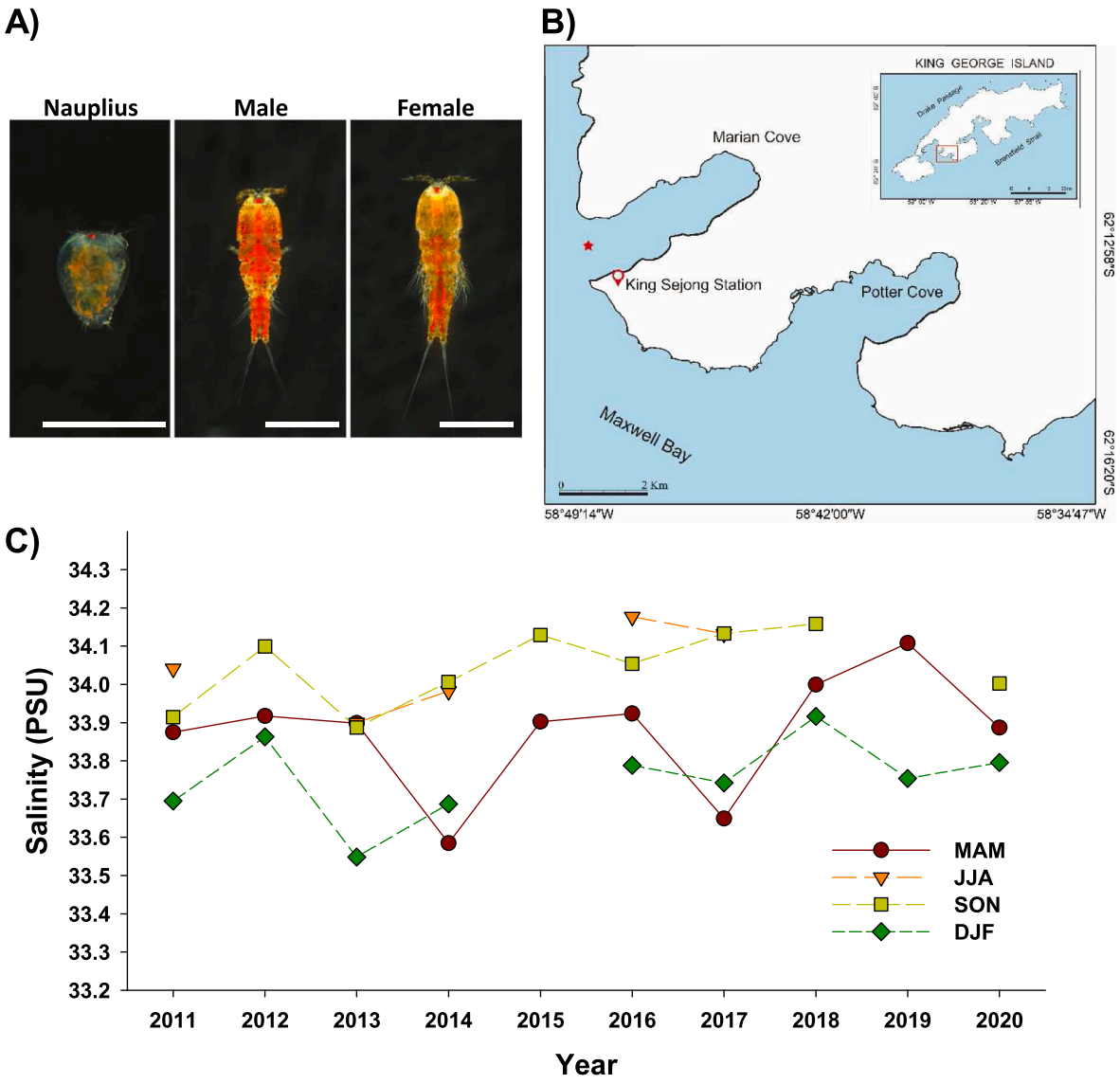

Fig. 1. A) Photographs of *T. kingsejongensis* nauplius, male, and female. The white bar on each figure represents 500 μm. B) Map of *T. kingsejongensis* sampling regions in the Marine Cove. C) Ten-year surface seawater salinity observation of the site from where the Antarctic harpacticoid copepod *T. kingsejongensis* was collected. MAM: March, April, May; JJA: June, July, August; SON: September, October, November; DJF: December, January, February.

December 2020 in Marian Cove (Marked the star in Fig. 1B) using the RBR XRX-620 CTD (Conductivity, Temperature, and Depth) and SeaBird 19 plus v2 CTD. The seasonally averaged surface salinity for each year ranged from 33.5 to 34.2. The average 10-year austral winter (June July August, JJA) and spring (September October November, SON) surface salinity was 34.05 and 34.04, respectively, which was relatively higher than that of 33.75 and 33.86 in the summer (December January February, DJF) and autumn (March April May, MAM) (Fig. 1C). Acute salinity tolerance experiments were conducted using different stages. Exposure to salinity was conducted in basic culture conditions as described. Ovigerous females possessing external egg sacs were sampled from the pool of cultured copepods in laboratory using a 1-mL Pasteur pipette into 6-well plates (an individual per well; SPL Life Science, South Korea) and distributed to different salinities before hatching of their egg sac to ensure that all nauplii had the same developmental conditions when exposed to salinity ranges. Nauplii hatched from a single ovigerous female were allowed to remain in the well, whereas the female was removed from the well immediately after hatching. Male and female copepods were randomly sampled from the pool of cultured copepods in laboratory based on their morphological characteristics (e.g., antennule structure) (Park et al., 2014) and mating behavior (Burton, 1985). The copepods were then transferred to a range of salinity [0 (distilled water, DW), 0 (dechlorinated tap water, DTW), 5, 10, 25, 34, 45, 60, and 75] prepared by dilution of pre-made filtered stock ASW (75 PSU; Tetra-Marine Salt Pro) with distilled water. Water conditions such as temperature, pH, and DO were adjusted with the value of original culture conditions before treatment. At each test concentration, three sets of 10 individuals each were used as triplicate. During the experiment, copepods were not fed. Mortality was inspected at 3, 6, 12, 24, 48, and 96 h under a Nikon SMZ25 stereomicroscope (Nikon, Japan). Copepods showing no movement of appendages were deemed dead. Since *Tigriopus* spp. can fall into a state of non-locomotion (e.g., temporal knockdown) upon exogenous environmental challenges, the specimens were further observed in the same experimental conditions for 24 h. Finally, dead copepods were transferred back to control ASW (34 PSU) and kept for 24 h before deciding if they were dead or alive.

2.3. Chronic exposure to different salinities

Effects of different salinities [5, 10, 25, 34 (control), 45, 60, and 75)]

on life parameters of individuals such as lifespan and sex ratio were measured. As we hypothesized that drastic freshening can be occurred in Antarctic coastal regions, three lower salinity ranges, 5, 10, and 25, were tested. Although higher salinity ranges such as 45, 60, and 75 are unrealistic to the actual environmental conditions of Antarctic coastal regions, we want to investigate tolerance and adaptability of *T. kingsejongensis* upon salinity increase and to compare the results with those reported in the genus *Tigriopus*. Exposure to these salinities was conducted in basic culture conditions as described. Entire exposures were conducted in Panasonic MIR cooled incubators (MIR–554, Panasonic®). Ovigerous females (30 individuals per salinity) were collected from the pool of cultured copepods in laboratory, moved in 500 mL glass beaker (Duran, Wertheim, Germany) filled with 100 mL ASW (34 PSU), and exposed to each salinity by adding of DW or ASW (100 PSU). Addition of DW was conducted with a rate of 1 PSU decrease per 10 min. Addition of ASW was performed with a rate of 1 PSU increase per 15 min. Final volume in each salinity exposure was 400 mL. The exposed ovigerous females distributed into 6-well plates (an individual per well; 15 mL; SPL Life Science) with different salinities before hatching of their egg sacs. Sixty nauplii (<6 h after hatching) for each salinity concentration were collected and distributed into 6-well plates (an individual per well; SPL Life Science). Half of the test solutions were changed every seven to ten days with the same concentrations of salinity and food source. To diminish potential effect during solution change, water conditions such as temperature, pH, and DO were adjusted with the values of the original culture conditions before treatment. Mortality was inspected every day under a Nikon SMZ25 stereomicroscope (Nikon). Dead copepods were fixed with 10% formalin and their sexes were inspected. Number of immature copepods was not counted.

2.4. Multigenerational experiment

Nauplii were developed as described. Four salinity ranges, 15, 25, 34 (control), and 45 were selected for multigenerational experiment, due to the significant mortality and decreased lifespan observed in salinity ranges, 5, 10, 60, and 75. Treatment groups for each salinity had 3 replicates with a total of 60 experimental units (20 individuals per replicate). Culture conditions for 20 weeks for each generation were identical to the basic culture conditions. Entire exposures were conducted in Panasonic MIR cooled incubators (MIR–554, Panasonic®). Half of the test solution was changed every seven to ten days with the same concentrations of salinity and food source. To diminish potential effect during solution change, water conditions such as temperature, pH, and DO were adjusted with the values of original culture conditions before treatment. Copepods were monitored under a Nikon SMZ25 stereomicroscope (Nikon) and their movements were recorded as a video file with a high-resolution camera linked to the stereomicroscope. Body length from the eye spot to caudal ramus was measured using the measurement tools of the Nikon imaging software (NIS–Element Analysis D 4.20.00). To diminish the potential effect of increased temperature during video recording, the entire monitoring was conducted in a cold room maintained at 5 ± 1 °C. As several copepods were dead for 20 weeks in each replicate, measurements were made only for the copepods surviving at week 20. More than 15 individuals per replicate survived at week 20. To obtain the next generation for each salinity, 30 females and 20 males were placed in a 200 mL glass beaker and 10 pairs of clasping copepods were selected from the single batch culture of each salinity. Sixty nauplii were randomly collected from the offspring of the 10 pairs of clasping copepods and their growth was measured for 20 weeks.

2.5. Quantitative real-time PCR

The transcriptional responses of genes involved in osmoregulation (Na^+/K^+–ATPase alpha and beta subunit, *NKAα* and *NKAβ*; H^+–ATPase, *HAT*; and $Na^+/K^+/2Cl^-$ cotransporter, *NKCC*), stress response (heat shock protein 20, *Hsp20*; *Hsp40*; *Hsp60*; *Hsp70*; and *Hsp90*), and antioxidant defense system (catalase, *CAT*; manganese superoxide dismutase, *MnSOD*; copper/zinc SOD, *CuZnSOD*; glutathione reductase, *GR*; and glutathione peroxidase, *GPx*) were examined using quantitative real-time PCR (qRT-PCR). The specific gene information and details were retrieved from the genome database of *T. kingsejongensis* (Kang et al., 2017).

To determine the time-course effects of instantaneous salinity change on the mRNA expressions of the genes, *T. kingsejongensis* were exposed to different salinities (15, 25, 34, and 45) for 96 h in static culture. The copepod samples (approximately 150 nauplii for each concentration level replicate) were homogenized in three volumes of TRI Reagent (Molecular Research Center Inc., Cincinnati, OH, USA) with a tissue grinder. Total RNA was isolated according to the manufacturer's instructions and genomic DNA was removed using DNase I (Sigma-Aldrich, Inc., St. Louis, MO, USA) by incubation at 36 °C for 30 min. Total RNA was quantified by the absorption of light at A260 and was quality-checked by analyzing the ratios A230/260 and A260/280 using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). A single-stranded cDNA was synthesized from the total RNA using an oligo(dT)20 primer for reverse transcription (SuperScript III RT Kit, Invitrogen, Carlsbad, CA, USA). qRT-PCR was performed with the SYBR Green Master Mix (Bio-Rad, Hercules, CA, USA). The reactions were conducted in the master mix (25 μL) containing 9.5 μL nuclease-free water, 12.5 μL SYBR Green buffer, 1 μL each of the forward and reverse primer (250 nM), and 1 μL cDNA (total RNA equivalent). The amplification was performed under conditions of 95 °C for 5 min; 40 cycles of 95 °C for 20 s, 55 °C for 30 s, and 72 °C for 40 s. The application was detected (SYBR Green-labeled amplicons) using CFX96 real-time PCR system (Bio-Rad). All the RT-PCR tests were conducted in unskirted low 96-well clear plates (Bio-Rad). Conditions of 95 °C for 1 min, 55 °C for 1 min, and 55 °C for 10 s (80 cycles) with a 0.5 °C increase per cycle were continued to achieve the melting curve. Expression of target genes was determined using the $2^{-\Delta\Delta CT}$ method. Heat map studies were performed using the MeV software (ver. 7.4; Dana-Farber Cancer Institute, Boston, MA, USA) to determine the transcript profiles.

2.6. Statistical analysis

All data were analyzed in the statistical software package SPSS (ver. 17.0, SPSS Inc., Chicago IL, USA) and are presented as the mean ± standard deviation (S.D.). The raw data were square root transformed and the assumptions of normality and variance homogeneity were tested by the Barlett test. The lifespan data was analyzed by one-way analysis of variance (ANOVA) and differences were analyzed a posteriori using the Fisher's Least Significant Difference (LSD) test. When the data did not follow ANOVA assumptions, the data were analyzed by a non-parametric test, Kruskal-Wallis. Significant differences between mRNA expression were determined using two-ways ANOVA, followed by HDS Tukey *post hoc* test. A type I error probability of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Salinity tolerance

T. kingsejongensis were tested at various salinities for 96 h and the results showed that different stages were tolerant to a wide range of salinity (Fig. 2). Food deprivation was not critical for mortality, as all stages showed 100% survival rate at salinities, 25 and 34. Exposure to DTW decreased the survival rate of all stages in a time-dependent manner, wherein the nauplii showed the highest sensitivity with approximately 50% individuals dead at 5 h and 100% mortality observed at 24 h (Fig. 2A). Overall, female copepods were more tolerant than males when exposed to hyposalinity. Male copepods showed 43, 57, and 66% mortality in DTW at 24, 48, and 96 h, respectively (Fig. 2B), whereas 30, 37, and 47% of females were dead at 24, 48, and 96 h,

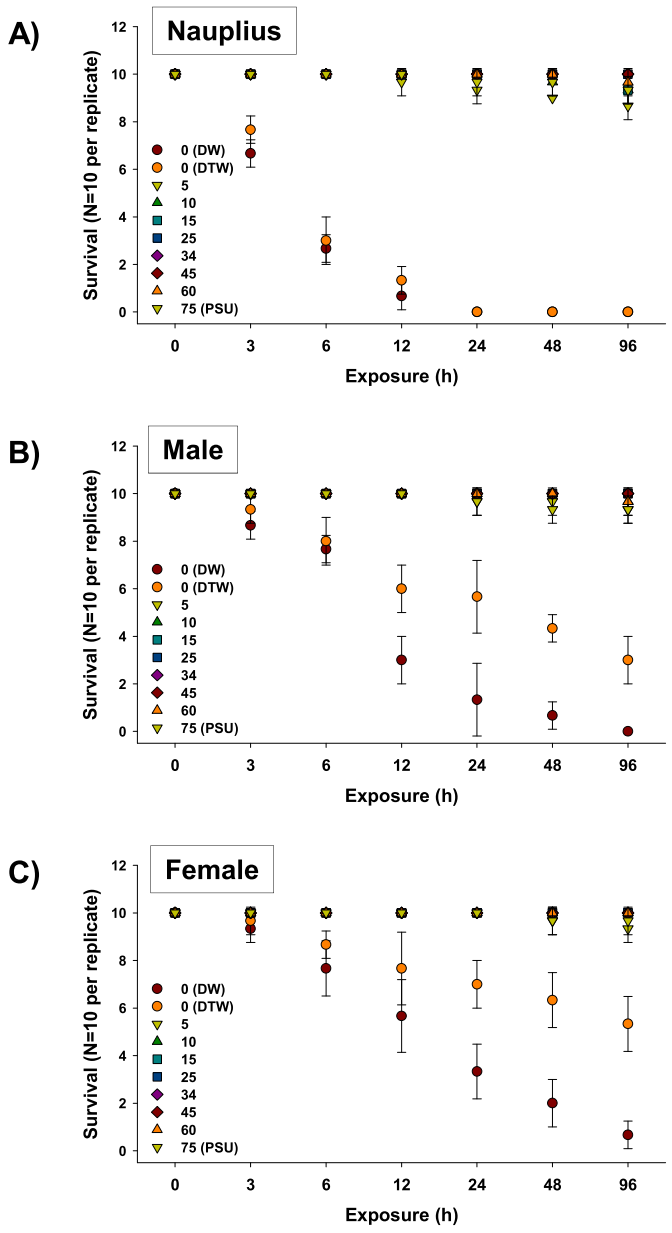

Fig. 2. Results of acute salinity tolerance experiments conducted with different stages of the Antarctic harpacticoid copepod *T. kingsejongensis*. Measurement of 96 h survival rate of A) nauplius, B) male, and C) female copepods in response to different concentrations of salinity [0 (distilled water, DW), 0 (dechlorinated tap water, DTW), 5, 10, 25, 34, 45, 60, and 75]. Data are presented as the mean ± standard deviation of the three replicates (n = 10 per replicate).

respectively (Fig. 2C). In the case of females, a few copepods showed response to exogenous stimuli even after 1 week of exposure to DTW (data are not shown). Although DW is irrelevant to the conditions of actual environmental freshwater, higher tolerance was observed in female copepods than in males in DW too.

Nauplii showed mortality in response to salinities, 5, 10, and 15 for 12, 48, and 96 h, respectively, with gradual decreases during the experiment. Mortality was observed in male copepods exposed to salinities, 5 and 10 for 24 and 96 h, respectively, while females showed mortality in response to salinity 5 for 48 h. In the case of hypersalinity, mortality was observed in response to salinities, 60 and 75 in nauplii and male copepods, whereas it was detected in females only after being exposed to salinity 75.

3.2. Chronic effects on life parameters

Lifespan significantly varied due to salinities (1–91 days for 5; 4–138 days for 10; 9–159 days for 15; 14–184 days for 25; 19–206 days for 34; 15–182 days for 45; 4–85 days for 60; and 1–68 days for 75) ($P < 0.05$) (Fig. 3A). The average lifespan of 30 individuals for each salinity was measured as 45 ± 26 days for 5, 77 ± 36 days for 10, 96 ± 41 days for 15, 109 ± 47 days for 25, 119 ± 48 days for 34, 97 ± 44 days for 45, 39 ± 24 days for 60, and 23 ± 18 days for 75. The lifespans of the 5, 10, 60, and 75 PSU groups showed no significant difference compared to that of the 34 PSU-treated group ($P > 0.05$).

The average percentage of male copepods ranged from 36.9% to 44% (40.4% for 5; 38.7% for 10; 40% for 15; 44% for 25; 43% for 34; 40.9% for 45; 38.4% for 60; and 36.9% for 75; Fig. 3B). There was no significant difference between salinities with respect to the sex ratio ($P > 0.05$). Mating behavior and spawning were observed in all the salinity concentrations.

3.3. Multigenerational experiment

The 34 PSU-exposed group was set as the control treatment. Overall,

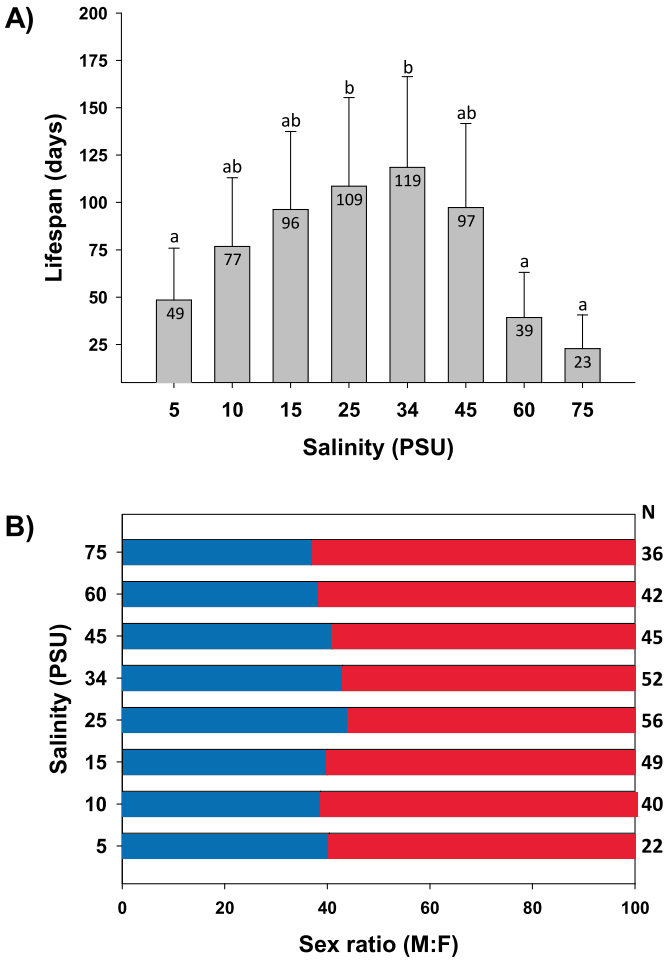

Fig. 3. A) Results of chronic exposure to different salinities (5, 10, 25, 34, 45, 60, and 75) on lifespan of the Antarctic harpacticoid copepod *T. kingsejongensis*. Data are presented as the mean ± standard deviation of 20 individuals in each salinity. Different letters above each error bar indicate statistically significant differences compared to control salinity (34) at $P < 0.05$. When exposed groups do not share a letter, the mean difference is statistically significant. B) Effects of different salinities (5, 10, 25, 34, 45, 60, and 75) on sex ratio of the copepod *T. kingsejongensis*. Data are presented as the mean ± standard deviation of individuals, which are represented in the right axis of the figure.

exposure to salinities, 15 and 45 reduced body length (Fig. 4), as indicated by the average body length of five specimens sampled from each generation at week 20 (840 ± 40 μm for 15; 1009 ± 45 μm for 25; 1017 ± 47 μm for 34; 945 ± 42 μm for 45). The effects of relatively low salinity on body length grew stronger across generations, especially in F4 and F5 (Fig. 4A). In detail, the average body length of F1 of 15 PSU-exposed copepods was 924 ± 43 μm at week 20, whereas it was measured as 804 ± 38 μm and 759 ± 38 μm for F4 and F5, respectively. There was no drastic difference between generations in the 25 PSU and 34 PSU-treated groups (Fig. 4B and C). In the case of 45 PSU-treated groups, body length was reduced at F2 (913 ± 43 μm) compared to F1 (978 ± 41 μm) at week 20, but body lengths similar to that of control were recorded in F3 (970 ± 41 μm) and F4 (979 ± 44 μm) at week 20 (Fig. 4D). However, the body length was reduced at F5 (875 ± 40 μm) at week 20. No morphological differences were observed between salinities as well as generations.

3.4. Transcript profile under instantaneous salinity change

To understand early transcriptional response to salinity changes, mRNA expressions of genes involved in osmoregulation (*NKAα*, *NKAβ*, *HAT*, and *NKCC*), stress response (*Hsp20*, *Hsp40*, *Hsp60*, *Hsp70*, and *Hsp90*), and antioxidant defense system (*CAT*, *MnSOD*, *CuZnSOD*, *GR*, and *GPx*) were measured for 96 h (Fig. 5). Acute exposure to 15 PSU significantly decreased mRNA expressions of *NKAα*, *NKAβ*, and *HAT* for 12 h, whereas their expressions were increased from 24 to 96 h with upregulation of *NKCC* transcript measured at 48 h ($P < 0.05$) (Fig. 5A). Among *Hsps*, mRNA expressions of *Hsp20* and *Hsp70* were highly upregulated for 96 h ($P < 0.05$). Overall transcriptional profiles of genes involved in antioxidant defense system showed decreased patterns with statistical significances for 48 h ($P < 0.05$). Similar transcriptional responses of genes involved in osmoregulation and stress response were observed in the 25 PSU-treated copepods (Fig. 5B). However, transcriptional upregulation of genes involved in antioxidant defense system showed significant upregulations from 48 to 96 h ($P < 0.05$). Although mRNA expressions of two *Hsps*, *Hsp20* and *Hsp70* showed significant upregulations at 3 h and/or 6 h ($P < 0.05$), there were no drastic modulations in the expression of other genes observed for 96 h ($P > 0.05$) (Fig. 5C). In the case of 45 PSU treatment, mRNA expressions of genes involved in osmoregulation (*NKAα*, *NKAβ*, and *HAT*) showed significant downregulation up to 24 h, and all osmoregulation genes showed significant increases from 48 to 96 h ($P < 0.05$) (Fig. 5D). Significant transcriptional downregulation of *Hsp40* and *Hsp60* was detected at 6 and 3 h, respectively ($P < 0.05$), while mRNA expressions of most *Hsps* showed upregulation from 12 to 96 h. Overall, transcripts involved in antioxidant defense system showed downregulated patterns for 96 h, except for *MnSOD*.

4. Discussion

Although the tolerance and adaptation to salinity fluctuation appear species-specific in copepods, spatiotemporal variations in salinity range is one of the crucial environmental factors determining their distribution and population dynamics (Dutz and Christensen, 2018). While the habitat of *T. kingsejongensis* in the coastal regions of Antarctica suggests a broad salinity tolerance, direct information on their salinity preferences and critical salinity levels is still scarce. In addition, there is no data on life span and sex ratio of *T. kingsejongensis* in natural population due to limited sampling and harsh environmental conditions for monitoring in Antarctica. Therefore, we investigated the acute and chronic effects of

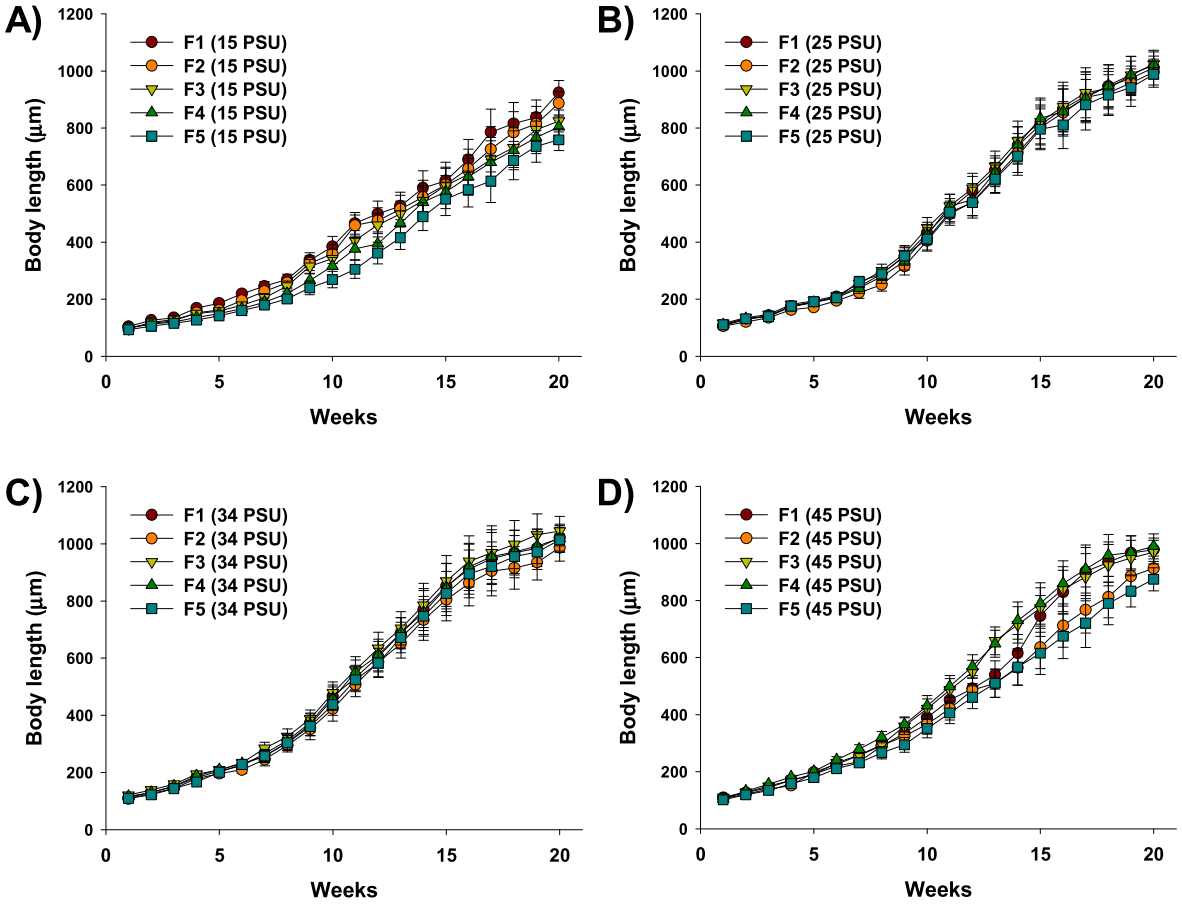

Fig. 4. Measurement of body growth rate (μm) of the Antarctic harpacticoid copepod *T. kingsejongensis* for 20 weeks in response to variable salinities: A) 15, B) 25, C) 34 (control), and D) 45 through five generations. Data are presented as the mean ± standard deviation of individuals, ranging from 22 to 30 copepods, for each day.

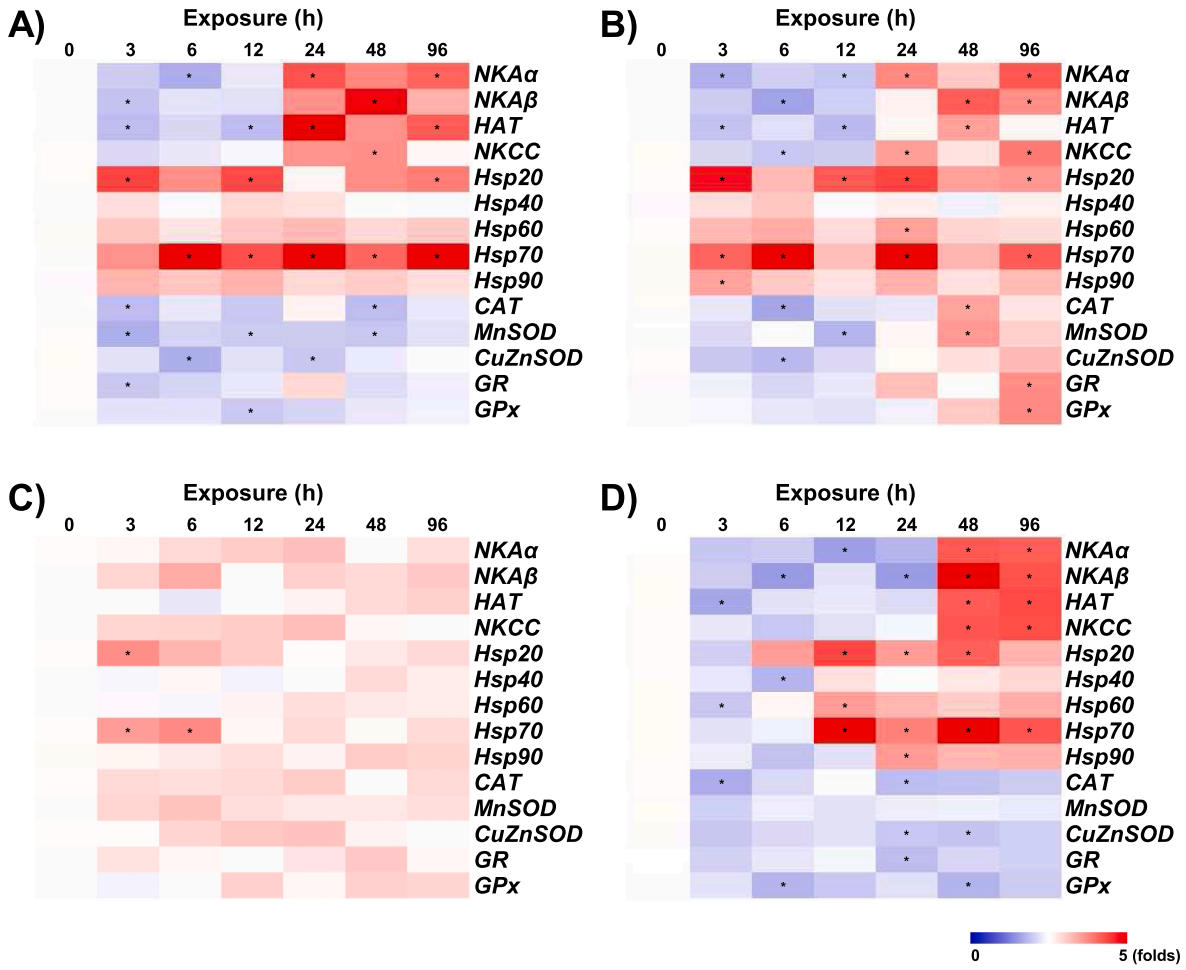

Fig. 5. Transcriptional profile of the Antarctic harpacticoid copepod *T. kingsejongensis* in response to variable salinities: A) 15, B) 25, C) 34 (control), and D) 45 for 96 h exposure, represented by a heat map. Data are expressed as the mean of three replicates. Asterisk (*) indicates significant difference at $P < 0.05$.

hypo- and hypersalinity to evaluate how salinity changes affect molecular components, physiology, and population. In line with several examples of responses of *Tigriopus* spp. to salinity ranges, our results reflect the broad tolerance range of *T. kingsejongensis* to salinities. Tolerance of *T. kingsejongensis* to salinity fluctuation for 96 h suggests its ability to regulate acute osmotic stress. Even though all nauplii died in DTW within 24 h, adults showed tolerance for 96 h. Generally, early life stages are often regarded to be more susceptible to environmental changes than adult stages in aquatic invertebrates (Gosselin and Qian, 1997; Pechenik, 1999; Charmartier et al., 2001; Lee et al., 2020; Paiva et al., 2020). Salinity fluctuation also induces more severe detrimental effects on early life stages than adults in copepod species (Lance, 1963; Nagaraj, 1988; Cervetto et al., 1999; Lee et al., 2007), possibly due to different osmoregulation capacities between stages and later onset of resilience metabolism against osmotic stress. Although no evidence was obtained in this study regarding whether nauplii are osmoconformers or actively control the acute salinity stress as osmoregulators, it could be attributed to different osmoregulation abilities between stages, as shown in the whole life cycle of Homarid lobsters (Charmartier et al., 2001).

The higher survival of *T. kingsejongensis* females compared to that of males represented their ability to cope well with instantaneous hyposalinity. The tolerance of females against salinity fluctuations is in line with a study that showed superior tolerance of *T. californicus* to a range of osmotic stress (Foley et al., 2019). Differences in sensitivity or tolerance upon environmental stressors between sexes are a common phenomenon as reported in invertebrates (Foley et al., 2019; Tower et al., 2020). However, the contributing factors to the sexual differences are still unclear. Higher tolerance of copepod females exposed to environmental stressors than that of males has explained with different body size and energy expenditure rate between sexes, as mass-specific energy expenditure rate declines by size and surface-volume ratio with a power of −1⁄4 (Kiørboe and Hirst, 2014; Holm et al., 2018). Males are generally smaller than females in copepods. Thus, copepod males might have a higher mass-specific metabolic rates than females (Almeda et al., 2011). In addition, copepod males commonly use more energy due to active swimming for female-searching behavior (Ohtsuka and Huys, 2001) and multiple insemination with females (Burton, 1985; Foley et al., 2019). It was suggested in fruit fly that male has deleterious mutations in mitochondria and this sex-specific mutation may contribute sex-specific longevity in fruit fly (Camus et al., 2015). High activity with energy expenditure may decrease the adaptation capacity to salinity fluctuation in copepod males, but more studies should be conducted to clarify in this species.

Species-specific tolerance or osmoregulatory ability against acute exposure to salinity fluctuations was observed in the genus *Tigriopus*, while there is a consensus on their general tolerance to a broad range of salinities. In *T. japonicas*, five out of ten ovigerous females died in DW in 10 h and all of them died at 20 h (Lee and Hu, 1981). In the case of *T. fulvus*, 100% mortality was observed at 84 h when cultured in DW (age and sex not described; Ranade, 1957). Significantly higher tolerance to acute salinity fluctuation observed in *T. kingsejongensis* could be explained by comparison of their habitat and the surrounding environment. Although coastal regions in temperate zones often meet with freshwater through runoff and heavy rainfall which subsequently result

in lowered salinity, a significant increase in salinity due to evaporation is generally observed in intertidal zones including beaches or rocky tidal pools (Robinson et al., 2007; Geng et al., 2016). In comparison with temperate zones, Antarctic copepods can encounter seasonal low salinity through melting of sea ice, land-terminating glaciers, and increased terrestrial runoff of meltwater (Dierssen et al., 2002; Mortensen et al., 2013). Since there is no specific report on the salinity range of tidal pools in Antarctic coastal regions, we were not able to speculate whether *T. kingsejongensis* frequently encounters hypersalinity. Even though a drastic decrease in salinity is uncommon in Antarctic coastal regions, *T. kingsejongensis* seems capable of developing adaptive metabolism for low salinity.

Measurement of lifespan is an essential parameter for estimating future population dynamics in copepods (Peterson, 2001). *T. kingsejongensis* showed considerable tolerance to low salinity during its entire lifespan. Overall lifespan patterns in response to low salinities were largely consistent with those of the temperate copepod *T. japonicus*, whose average lifespan was measured as 56 ± 11 days for 4 parts per thousand (ppt) (n = 9), 63 ± 23 days for 8 ppt (n = 9), 61 ± 24 days for 16 ppt (n = 9), and 101 ± 50 days for 32 ppt (n = 7) (Hagiwara et al., 1995). A rather narrow range of lifespan was observed in *T. kingsejongensis* exposed to hypersalinity compared to hyposalinity treatment.

Exposure of *T. kingsejongensis* to salinities, 15 and 45 affected its growth throughout the life cycle. Similarly, suboptimal salinity range retarded post-embryonic development of *Eurytemora affinis* (Devreker et al., 2007). Retarded development rate under osmotic stress correlated with an increase in mortality in copepods (Devreker et al., 2007). The modulations in body length under salinities, 15 and 45 can be attributed to the allocation of energy from growth to osmoregulation and maintenance of homeostasis against salinity stress. In fact, osmoregulation requires high energetic cost in copepods. For example, approximately 12% energetic cost was theoretically calculated for maintaining homeostasis under hyperosmotic stress in the total daily energy budget of *T. californicus* (Goolish and Burton, 1989). This cost for osmoregulation would be used for functions such as amino acid metabolism (Farmer and Reeve, 1978; Burton, 1991). The composition of amino acids in the amino acid pool is important for adapting to salinity fluctuation in copepods (Farmer and Reeve, 1978; Burton, 1991). A considerable reduction in metabolic rate and activity was observed in *T. brevicornis* in response to salinities, 10 and 90 P(McAllen and Taylor, 2001). Therefore, more energy might have been expended on osmoregulation and consequently, lesser energy was available for development and growth, leading to reduced body length. Although it was not tested in this study, feeding rate was also significantly lowered by chronic exposure to osmotic stress in copepods (Calliari et al., 2008; Dutz and Christensen, 2018). Limited ingestion could directly affect growth by lowering the available energy and nutrients.

The relatively high standard deviation observed in growth rate as well as in lifespan could be potentially explained by individual variability to osmotic stress, as suggested in copepods (Lee et al., 2003; Devreker et al., 2007). Even though offspring are generated from a single brood, some individuals can grow faster, whereas some individuals can be better adapted to environmental fluctuations. This variability among the *T. kingsejongensis* population could be helpful in adapting to local environmental changes in the Antarctic coastal regions. Detailed studies focusing on *T. kingsejongensis* should be conducted by application of reliable life cycle parameters to verify this theory. The sex ratio was unaffected by salinity range and was largely consistent with the results of similar studies on *T. japonicus* (Hagiwara et al., 1995) and other copepods (Ohs et al., 2010; Wilson et al., 2021).

Because stress tolerance and molecular responsive capacity are strongly linked, mRNA expressions of several genes involved in different metabolic activities were analyzed in response to instantaneous salinity fluctuations in *T. kingsejongensis*. These molecular signals can be useful ecological indicators, as suboptimal osmotic stress strongly modulated protein expressions in *T. californicus* by synthesis of osmoregulatory proteins or degradation of non-essential proteins (Kimmel and Bradley, 2001). Na^+/K^+-ATPase is a ubiquitously expressed membrane-bound enzyme acting as a key energy-consuming pump responsible for maintaining ionic and osmotic equilibrium. The plasma membrane H^+-ATPase has a crucial role in the generation of an electrochemical proton gradient by extruding protons from cells. $Na^+/K^+/2Cl^-$ cotransporter controls the precisely coupled movement of the three ionic species, $1Na^+$, $1K^+$, and $2Cl^-$ across the plasma membrane of cells for maintaining electrochemical potential equilibrium by accumulation of chloride in the cells. These proteins are involved in the adaptation of aquatic crustaceans to environmental stresses, such as salinity (Lucu and Towle, 2003). The decreased expressions of these transcripts measured at 0–12 h to salinities, 15, 25, and 45 indicate strong osmotic stress initially. Their transcriptional expressions were elevated at 48–96 h, suggesting recovery capacity and activity for adapting to osmotic stress by maintaining ionic homeostasis and osmotic balance. The response of the Hsp family is normally highlighted as one of the most sensitive biomarkers against a wide variety of exogenous stressful conditions in copepods (Rhee et al., 2009). In the present study, transcriptional levels of *Hsp20* and *Hsp70* were highly modulated in response to salinities, 15, 25, and 45. In *Tigriopus* spp., members of Hsp20 and Hsp70 families have been considered as strong inducible biomarkers to assess homeostasis in response to environmental fluctuations (Rhee et al., 2009; Kim et al., 2014). Based on the whole transcriptional profiles, the salinities seemed to be strong stressors in *T. kingsejongensis* and increased *Hsp* transcripts generated under salinity fluctuations confer cell protection, enabling the copepods to survive osmotic stress. As CAT and SOD enzymes are the first line of defense against oxidative stress and major components of glutathione metabolism, wherein GR and GPx contribute to cellular antioxidant protection by catalyzing the reduction of H_2O_2 and lipid peroxides (Lesser, 2006), the observed downregulations in *CAT* and *SOD* transcripts at salinities, 15 and 45 for 96 h indicate a possible oxidative stress and inhibition of antioxidative responses in *T. kingsejongensis* during early exposure to osmotic stress. Salinity change has been shown to modulate major components of the antioxidant defense system in copepods (Cailleaud et al., 2007; Glippa et al., 2018; Martínez et al., 2020). The energetic costs of osmoregulation are strongly associated with oxidative stress, as the mechanism requires ATP production for fueling osmoregulation, and subsequently, reactive oxygen and nitrogen species are accumulated in the mitochondria. In the case of salinity 25, their mRNA expressions were downregulated for 12 h and increased at 48 or 96 h, indicating that *T. kingsejongensis* strive to maintain homeostasis in response to osmotic stress. If the key responsive functional genes can be identified in copepods, the same molecular approach would be greatly applicable in understanding the adaptation potential for hyposalinity and hypersalinity.

Regarding the importance of *T. kingsejongensis* in the Antarctic food web, meltwater-driven freshening or stratification may directly impact primary producers (e.g., phytoplankton) as crucial preys and subsequently affect higher tropic levels including zooplankton populations. Although our results were based on controlled laboratory experimental conditions with consistent supply of food sources, synergetic detrimental effects would be induced by lowered salinity and modulation in dominance of phytoplankton. Copepods are major food sources for numerous aquatic animals from low trophic levels to fish larvae. Thus, negative effects of salinity fluctuations on copepods population dynamics would induce a knock-on effect on other major components of the Southern Ocean food web. Analyzing the salinity responses of Antarctic copepods will be particularly relevant for future estimation of population dynamics and food web modulation under global environmental changes. Furthermore, it is important to study the population dynamics of *T. kingsejongensis* for understanding how the Southern Ocean ecosystem functions.

5. Conclusions

This is the first study on the physiological and molecular responses of *T. kingsejongensis* to osmotic stress under controlled laboratory conditions assessed using several parameters which have been routinely studied in copepods. Although there are many essential parameters already applied and determined in copepods, and only partial measurements were conducted in this study, we expect further expansion in the application of this methodology in the future for better understanding their in-depth response, based on this primitive study. Results of *T. kingsejongensis* lifespan to different salinities showed their preferred habitat and also suggest that they will be well adapted to relatively low salinity, along with surviving at higher salinities too. The strong tolerance and plasticity of *T. kingsejongensis* in response to instantaneous salinity fluctuations clearly indicate that the copepod possesses efficient molecular and physiological mechanisms allowing adaptation to changing salinity. Gradual change in salinity was less stressful for survival and growth of copepods than instantaneous fluctuations (Lee and Peterson, 2003). Thus, *T. kingsejongensis* might persist in the Antarctic coastal regions, which is indicated by the maintenance of generations at relatively low and high salinity ranges compared to current salinity. However, future studies should include measurement of consistent mating potential, the maternal effect on offspring, and molecular adaptation to a wide range of salinity, in addition to our experimental conditions to estimate population dynamics against extreme salinity fluctuations. Unfavorable global climate changes such as warming and ocean acidification will synchronously modulate the sensitivity of *T. kingsejongensis* to salinity fluctuations.

Author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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