Coping with salinity change

How does the cyclopoid copepod Apocyclops royi (Lindberg 1940) do it?

Jepsen, Per M.; Dinsen, Cæcilie H.; Øllgaard, Esther S.H.; Jedal, Jonathan Y.B.; Aggerholm, Lasse; Salomonsen, Tor; Ramløv, Hans

Published in:
Comparative Biochemistry and Physiology -Part A : Molecular and Integrative Physiology

DOI:
10.1016/j.cbpa.2024.111794

Publication date:
2025

Document Version
Publisher's PDF, also known as Version of record

Citation for published version (APA):
Jepsen, P. M., Dinsen, C. H., Øllgaard, E. S. H., Jedal, J. Y. B., Aggerholm, L., Salomonsen, T., & Ramløv, H. (2025). Coping with salinity change: How does the cyclopoid copepod *Apocyclops royi* (Lindberg 1940) do it? *Comparative Biochemistry and Physiology -Part A : Molecular and Integrative Physiology*, *301*, Article 111794. https://doi.org/10.1016/j.cbpa.2024.111794

Comparative Biochemistry and Physiology, Part A 301 (2025) 111794

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Coping with salinity change: How does the cyclopoid copepod *Apocyclops royi* (Lindberg 1940) do it?

Per M. Jepsen *, Cæcilie H. Dinsen, Esther S.H. Øllgaard, Jonathan Y.B. Jedal, Lasse Aggerholm, Tor Salomonsen, Hans Ramløv

Department of Science and Environment, Roskilde University, Roskilde, Denmark

ARTICLE INFO

Edited by Michael Hedrick

Keywords:
Aquaculture
Zooplankton
Osmoregulation
Behaviour
Survival

ABSTRACT

The cyclopoid copepod species *Apocyclops royi* has attracted significant attention due to its importance in marine food webs and its role as a vital food source for many marine organisms, particularly marine fish larvae. This study aims to understand the activity patterns, osmoregulation mechanisms, and physiological adaptations of *A. royi* in response to acute decreasing salinities. In total three experiments were conducted. The first two experiments both investigated behavioural change and survival as a function of acute decreasing salinities in the range from 32 to 0, with steps of salinity reductions of five. The third experiment investigated the correlation between internal and external osmolality in *A. royi*, by using a novel method developed for the experiment.

The first experiment indicated that *A. royi* behaviour and survival were not affected at salinities from 20 and higher. Surprisingly, some copepods were able to survive an acute decrease in salinity from 32 to 0.

The second experiment utilized, for the first time for this copepod species, an in situ Multispecies Freshwater Biomonitoring system, to further observe *A. royi's* behaviour. The results showed that the system was able to monitor *A. royi* activity level. The system both documented that *A. royi* exhibit a statistically significant increase in activity levels in response to light. Furthermore, it provided knowledge about the temporal activity level of *A. royi* as a function of acute decreases in salinities, providing insights into that *A. royi* has an ~3 h acclimatization time to an acute decrease from 32 to 0 salinity.

In the third experiment, the osmolality of the copepods' body fluids with relation to external osmolality was examined using a vapor pressure osmometer. In this context a new method to extract body fluids from *A. royi* was developed. The body fluid osmolality of copepods exposed to three different salinities 10, 20 and 32 was examined. The results showed that *A. royi* is an osmoconformer at a higher salinity 32 but initiates hyperregulation at a lower salinity 10. Furthermore, it was observed that when copepods were exposed to a salinity of 10, 1000 individuals (stage: C5 or adults) were needed to obtain one sample of body fluid (10 μL) whereas when exposed to a salinity of 32, 3000 individuals were required to extract the same amount of body fluid.

Overall, the findings demonstrated that *A. royi* has a high tolerance for acute decreases in salinity, showcasing behavioural adaptations and osmoregulatory capabilities, at extreme salinities. These results contribute to our understanding of copepod physiology and their ability to thrive in various habitats. Further research is needed to fully comprehend the physiological mechanisms underlying *A. royi's* adaptation abilities to acute decreases in salinity.

1. Introduction

Copepods are widely recognized as one of the most abundant multicellular organisms in the marine pelagic environment. They play a vital role in the marine food web, serving as an essential food source for many marine organisms, particularly marine fish larvae (Kiørboe et al., 2018; Mellak et al., 2024). The evolutionary adaptation of marine fish larvae to feed on copepods and their nauplii has been extensively studied (Bron et al., 2011; Tocher, 2010; Williamson et al., 2021). Oceanic copepods often experience a stable abiotic environment,

* Corresponding author at: Department of Science and Environment, Roskilde University, Universitetsvej 1, Building 12.2, Postbox 260, DK-4000 Roskilde, Denmark.

E-mail address: pmjepsen@ruc.dk (P.M. Jepsen).

https://doi.org/10.1016/j.cbpa.2024.111794
Received 16 May 2024; Received in revised form 28 November 2024; Accepted 17 December 2024
Available online 19 December 2024

whereas nearshore copepods, are challenged by large temporal variations in the abiotic environment (Hansen et al., 2017), for example in salinity (Rivera-Ingraham and Lignot, 2017; van Someren Gréve et al., 2020). Zooplankton spatio-temporal distribution in coastal ecosystem depends on their physiological adaptations to fluctuating salinity. Copepods have developed different physiological strategies to counteract environmental changes in osmolality and ion concentrations. Most copepods do not strictly conform to rapid changing salinity, but have various degrees of osmoregulatory capabilities, depending on their physiology (Hauton, 2016; Ibrahim et al., 2016). It has been indicated that in the orders of Calanoida, Cyclopoida and Harpactoidathey employ active ion regulation andbody density processes to cope with salinity change (Bayly, 1969; Farmer, 1980; Roddie et al., 1984; McAllen et al., 1998; Svetlichny et al., 2021).

The genus *Apocyclops* includes species that has been reported from marine, brackish and freshwater, and are often recognized as a ubiquitous genus (Reid et al., 2002). The cyclopoid copepod species *Apocyclops royi*, inhabiting the area from the Bay of Bengal to the South Chinese Sea, has recently gained attention due to its ability to survive the harsh environments of Taiwanese outdoor aquaculture ponds (Blanda et al., 2015, 2017; Liao et al., 2001; Palanichamy et al., 2022; Su et al., 1997). As highlighted by Blanda et al. (2015, 2017), these ponds are characterized by a low-quality food supply and highly fluctuating abiotic conditions, including pH, temperature, oxygen, and salinity. Due to *A. royi* potential as a universal live feed product for fish larvae, its abiotic tolerance has been widely studied (Hansen et al., 2022). *A. royi* has been found to exhibit a high tolerance for low oxygen levels, allowing it to survive in severely hypoxic waters(Blanda et al., 2015). Additionally, *A. royi* has a wide temperature tolerance, ranging from 16 to 36 °C, with higher lethal temperatures occurring at 40 °C within 48 h (Blanda et al., 2015; Hansen, 2023; Lee and Park, 2005; Lee et al., 2005). The pH tolerance of *A. royi* is relatively unstudied, but it has been reported to thrive within a pH range of 7.5 to 8.7 (Blanda et al., 2015; Muthupriaya and Altaff, 2009). Furthermore, *A. royi* has been found to exhibit extraordinary plasticity in terms of salinity tolerance, being able to survive in salinity levels ranging from 0 to 60 (Hansen et al., 2022; Muthupriaya and Altaff, 2009; Su et al., 2005). Further it has been reported to cope with a rapid drop from 15.3 to 9.0 in surface salinity during a typhoon event (Blanda et al., 2015). Hansen et al. (2022) showed that *A. royi* living at salinity 20 can be adapted to 0 salinity by eight day stepwise salinity decrease. In addition, *A. royi* are spatially distributed in subtropical and tropical regions, where intense rainfall and associated freshwater runoff occurs. Hence the natural habitat of *A. royi* in brackish and nearshore environments has the potential for exposure to acute decreasing salinities.

Reports have documented that copepods exhibit different strategies to cope with acute changes in salinity (Hauton, 2016; Ibrahim et al., 2016). Few are found to be osmoconformers by passive exchange of their body fluid with the surrounding waters and thereby obtaining similar osmolarities (Svetlichny et al., 2012). Whiles others are osmoregulators, with an active ion exchange with the environment obtaining a body fluid osmolality different form the environment (Bayly and Boxshall, 2009; Farmer, 1980). Copepods that osmoregulate are often intertidal or euryhaline species, e.g. *Acartia tonsa*, *Eurytemora affinis*, or *Tigriopus californicus* (Farmer, 1980; Goolish and Burton, 1989; Johnson et al., 2014).

The present study investigates the behavioural and physiological responses of *A. royi* to variations in salinity in the range 0 to 32, to establish this species osmoregulatory capabilities and if it is an osmoconformer or an osmoregulatory within the tested salinity range. The findings from this study will enhance our understanding of copepod physiology and their ability to adapt to acute decrease in salinity.

2. Material and methods

2.1. Culture

The *Apocyclops royi* culture at Roskilde University originate from aquaculture ponds from the Donggang province in Taiwan (Blanda et al., 2015). Originally the species was taken into the aquaculture ponds, from the Kaoping estuary, a part of the South China Sea (Blanda et al., 2015).

The *A. royi* culture used in this study was taken into stock in 2016 at Roskilde University, Denmark, through collaborators in LOG-Marine Station of Wimereux in France (Pan et al., 2016). According to Yen-Ju Pan, the culture was transferred from Taiwan to France in the early 2010ies (personal comment).

Since 2016 the culture has been kept in a dark 25 °C temperature-controlled room. The stock cultures were kept in 0.2 μm filtered seawater with a salinity of 20, as recommended by Pan et al. (2016), and fed the microalga *Rhodomonas salina* in excess (950 μg C L^{-1}, Berggreen et al., 1988). *Rhodomonas salina* were kept under culture conditions described in Thoisen et al. (2018). The used seawater in all experiments is originated from 30 m depth in Kattegat (57°N, 10°E) Denmark (for analytical details of the water, see Jepsen et al., 2019; Hansen et al., 2022).

To study the effect of acute decreasing salinities, from a gradient starting with oceanic salinity conditions, *A. royi* was isolated from the stock culture and a new separate culture was established. The culture was kept at same conditions as previously described, except that the salinity was changed to 0.2 μm filtered 32 ± 2 seawater. *A. royi* were cultured for more than 10 generations before initiating the experiments.

2.2. Experiment one: acute decreasing salinities

To test acute decreasing salinity effects on *A. royi*, late stage copepodites and adults were gently transferred from the stock salinity 32 culture and into a 1 L glass beaker by using a 250 μm sieve. The isolated copepods were kept under the same conditions as the original culture, with 25 °C and salinity a of 32. Individual *A. royi*, at copepodite stage 5 or adults, were, under a dissection microscope (SZ40 Olympus Optical GmBH, Germany at 20× magnification), gently collected with a 2 ml plastic pipette and randomly distributed, into a 12 well Multiwell^{tm} plate (Falcon®, Tissue Culture Plate). Each well in the Multiwell plate contained 5 ml of preprepared seawater in eight different salinities being 0, 5, 10, 15, 20, 25, 30 and 32. Salinity 32 was considered the control treatment since this salinity was similar to the stock culture conditions of *A. royi* used in this study. The eight salinities were prepared by diluting natural seawater with deionized water. For each salinity, a Multiwell plate were prepared with one *A. royi* in each well, resulting in eight Multiwell plates with 12 *A. royi* in each. An in vivo dose response experiment was performed by having 100 % survival at time 0 and observing each of the 12 wells in each of the eight Multiwell plates every five minutes, to estimate survival rates. The observation of *A, royi* behaviour were conducted under a dissection microscope (20× magnification) as a structured observation where each individuals behaviour was categorized into either active, passive, or motionless. A predefined protocol was used, were each Multiwell plate was observed for 5 min and the 12 copepods in one Multiwell plate was categorized into one of the three different behaviours. Thereafter the observations were restarted and the same Multiwell plate was observed again for 5 min. Active *A. royi* were defined as swimming freely around, passive was defined if swimming was only observed when *A. royi* was touched with a dissection needle. Further to be categorized as passive, the dissection microscope was zoomed to maximum magnification (20×) on each individual *A. royi*, to check for movement of any mouth appendages, swimming legs, urosome, antenna or peristaltic gut movements. *A. royi* was categorized as motionless when none of the above-described activities was observed (Method modified from Jepsen et al., 2015). If all *A. royi* in a given Multiwell plate were categorized with the same

behaviour during an hour of observation, hence twelve similar observations in a row, the observation stopped, and the Multiwell plate was left until a final observation was conducted (after 24 h exposure).

2.3. Experiment two: multispecies freshwater biomonitor

For measuring the activity level of *A. royi* the Multispecie Freshwater Biomonitor® (MFB, LimCo Internationals GmbH, Germnay) was used. The system consists of 16 chambers (length 8 cm, diameter 2 cm) and is an automated biomonitor system based on quadrupole impedance technology (Gerhardt et al., 1994; Rastetter and Gerhardt, 2018). The chambers are water-filled flow-through chambers wherein an organism can move freely. To keep *A. royi* stage C5 or adults inside the chambers, they were enclosed with a 120 μm nylon filter in each end of the chambers. Before using the system, its ability to measure activity of *A. royi* was tested by placing different numbers of *A. royi* (stages: C5 and adults) into the chambers. The MFB could record activity of one *A. royi*, hence one *A. royi* in each chamber was used in the subsequent experiments (Data not shown). The MFB can be used both in fresh- and saltwater, but one needs to increase the voltage setting when used in freshwater as it contains less ions when compared to saltwater. For freshwater the voltage was 1, in salinity 5 the voltage was 0.5, in salinities 10 and 15 the voltages were 0.2 and in salinities 20, 25, 30 and 32 the voltages were 0.1. The measurement of activity was recorded in 10-min cycles, consisting of four minutes of recording, followed by a six-minute interval where the system was adjusted (Rastetter and Gerhardt, 2018). The raw monitoring signals were analysed with the Fourier frequency transformation to obtain the abundance of different frequencies between 0 and 8.5 Hz within the 4-min recording time (17 frequency intervals in steps of 0.5 Hz) (Gerhardt et al., 1998). To convert into an activity level between 0 and 100 % activity, the "activity level" in the blank chamber without animals were withdrawn from the activity level in the chambers with animals. Secondly, the obtained frequencies were converted to % activity by finding the maximum value of each of the measured frequencies and using that as a reference for maximum activity = 100 % activity. This reference point is considered full behavioural strength, which is an integrated reflection of all instantaneous motions of *A. royi* (Ren et al., 2007). These settings were used for all experiments conducted in the MFB.

2.3.1. Monitoring activity of A. royi in relation to light conditions

The copepods behaviour and activity of copepods in nature is often dependent on a day/night cycle. However, the diel behaviour of *A. royi* is unknown and this could potentially influence the experimental results (Roman et al., 1988). Hence, to observe any difference in *A. royi* activity between cultures in light or darkness, two 50 L aquariums were set up with a salinity of 32 and 25 °C. In each aquarium, eight chambers, identical to the ones described earlier, each containing one *A. royi* and connected to the MFB, (length 8 cm, diameter 2 cm) were placed. Thereafter one aquarium was covered with dark plastic to ensure darkness and the other received constant light from a 28 W LED light (Philips, GreenPower LED production module deep red/whiter 120), and their activity levels was monitored over 24 h, using the MFB settings earlier described.

2.3.2. Monitoring activity levels of A. royi when exposed to different salinities

To establish the activity levels and the behavioural responses in relation to different salinities, eight chambers, containing one copepod each, were placed in an aquarium with a specific salinity of 0, 5, 10, 15, 20, 25, 30, and 32, respectively. The copepods were transferred directly from the control salinity of 32 into any of the above given salinities or directly to freshwater. The MFB had 16 chambers in total, thus, by using two 50 L aquariums with 8 chambers in each, both with full light (24 L:0D) and 25 °C, two different salinities could be tested simultaneously. The activity levels of the copepods were measured for 24 h. Due to differences in operating voltage, the different salinities had to be run separately, resulting in an experimental span of five days in total.

2.4. Experiment three: determination of body fluid osmolality in A. royi at different environmental salinities

The effect of different rearing salinities (10, 20 and 32) on the body fluid osmolality of *A. royi* was investigated. To acclimatize *A. royi* to lower salinities, *A. royi* from the control salinity of 32 was used to establish a new "salinity" 20 culture, a month in advance to the experiment. Two weeks prior to the experiment another new salinity 10 culture was established, by transferring copepods from the 20 salinity culture. Hence, all cultures had at least two generations in any of the given salinity conditions, before they were used for experimental work. Twelve hours prior to an experiment, copepodites (stage C5) and adults were isolated from each of the established control cultures salinities 10, 20 and 32 using a 250 μm sieve, and transferred to a 4 L aquarium for depuration. The aquarium was located in the same temperature-controlled room as the stock cultures. Air was gently supplied to the aquarium with a Eheim 400 air pump with a connected air stone. To avoid pollution, uneaten food, faecal pellets, and other residuals at the bottom of the aquariums were manually removed using plastic Pasteur pipettes (Frisenette, stilk diameter 5.0 mm, bold volume 3.1 ml). Thereafter, the copepods (C5 and adults) were gently transferred with a 250 μm sieve into a 1 L polycarbonate bottle (Nalgene®) and three subsamples were poured into three 10 × 20 ml Petri dishes using a 10 ml kip-automate (NS 29.2/32; Buch & Holm, Witeg, Germany), fixed with 1 % acid Lugol, and counted using a dissection microscope (Olympus SZ 40; Olympus Optical (Europe) GmBH, Hamburg, Germany). By knowing the subsample volume (10 ml) and number of copepod in a subsample, and the total bottle volume (1 L), then the total number of copepods could be calculated. The sampled *A. royi* were concentrated, by vacuum filtration on a 120 μm nylon filter mesh, with a 1.28 cm filter area (Millipore Vacuum pump, 200 mm hg vacuum). To ensure all copepods were sampled, the vacuum filter setup was flushed with 10 ml of saltwater with salinity10, 20 or 32 according to the experiment. Thereafter, to remove salts from the exterior carapace of *A. royi*, they were washed briefly with 1 ml of milliQ water, and then excess external moisture was removed by leaving them on the filter for 20 sec. The 120 μm nylon filter mesh was removed, and the *A. royi* were scraped off, using a sterile scalpel, and transferred to a 1.5 ml Eppendorf tube containing 0.25 ml paraffin oil. The copepods were crushed and homogenised for two minutes using disposable micro-pestles and centrifuged for three minutes at 6,8 Relative Centrifugal Force (RFC). The centrifuged homogenate and oil mixture was transferred to a Spin-X cellulose acetate internal filter (0.22 um) fitted in a 2 ml Eppendorf tube and centrifuged for another three minutes at 6,8 RFC, thereby separating cell debris and the copepods exoskeletons from body fluids. Due to the difference in density of body fluid and the paraffin oil, the body fluid was located below the paraffin oil after centrifugation, thus preventing evaporation. The filter was removed, and the tube, containing body fluids and paraffin oil phases, were stored at −20 °C until further analysis.

A vapor pressure osmometer (VAPRO 5520, WESCOR, Elitech group (US)) were calibrated with two-point calibration using 10 μl of WESCOR OPTI-MOLE osmolarity standards (290 mOsmol/kg and 1000 mOsmol/kg standards). A Finn pipette (2–20 μl, ThermoScientific) was used to extract 10 μl of the body fluid phase and subsequently loaded into the osmometer. Each tube was continuously sampled until all the body fluids were used. When the volume was less than 10 μl, the remaining body fluids were sampled in 2 μl increments. The later, to obtain the total extracted body fluid volume and use to relate to the earlier amount of subsampled copepods. Thereby the average amount of body fluid sample could be calculated for each of the three salinity treatments. To extract a 10 μl sample volume of body fluids, minimum 1000 individuals of *A. royi* acclimated in salinity 10 were required, whereas it required minimum 3000 individuals to extract the same amount of body fluids if

A. royi was acclimated to salinity 32. The osmolarity of the external seawater media were tested by sampling the culture seawater and treating the samples identical to the copepod samples. Between each measurement at the osmometer, the sample holder was cleaned with 2 drops of WESCOR deionized water (SS-006) and wiped with lens paper.

2.5. Data analysis

The model used for experiment one, to analyse the mortality effects on *A. royi,* was fitted with 'Log (agonist) vs. response – Find EC anything'. The Top and Bottom plateau's have been constrained to 0 and 100, since the mortality ranges from 0 % to 100 % and LC50 ± 95 % CL extracted (GraphPad Prism version 5.04). Statistical analysis was performed in JASP Team (Version 0.17.3). Normality and equality of variance was performed with Shapiro-Wilks test and Levene's test, followed by students *t*-test (activity in light or darkness) or Kruskal-Wallis test for non-parametric data. A significance level of $\alpha = 0.05$ were used. If statistically significant differences were found, Dunns post hoc comparison test were conducted, to show statistically significant differences among samples at the $\alpha = 0.05$ level (Kasuya, 2001). All percentage data were arcsin transformed before further statistical comparison were performed.

3. Results

3.1. Experiment one: acute decreasing salinities

In the acute decreasing salinities experiment a statistically significant differences as a function decreasing salinities was found, resulting in increasing mortality of *A. royi.*

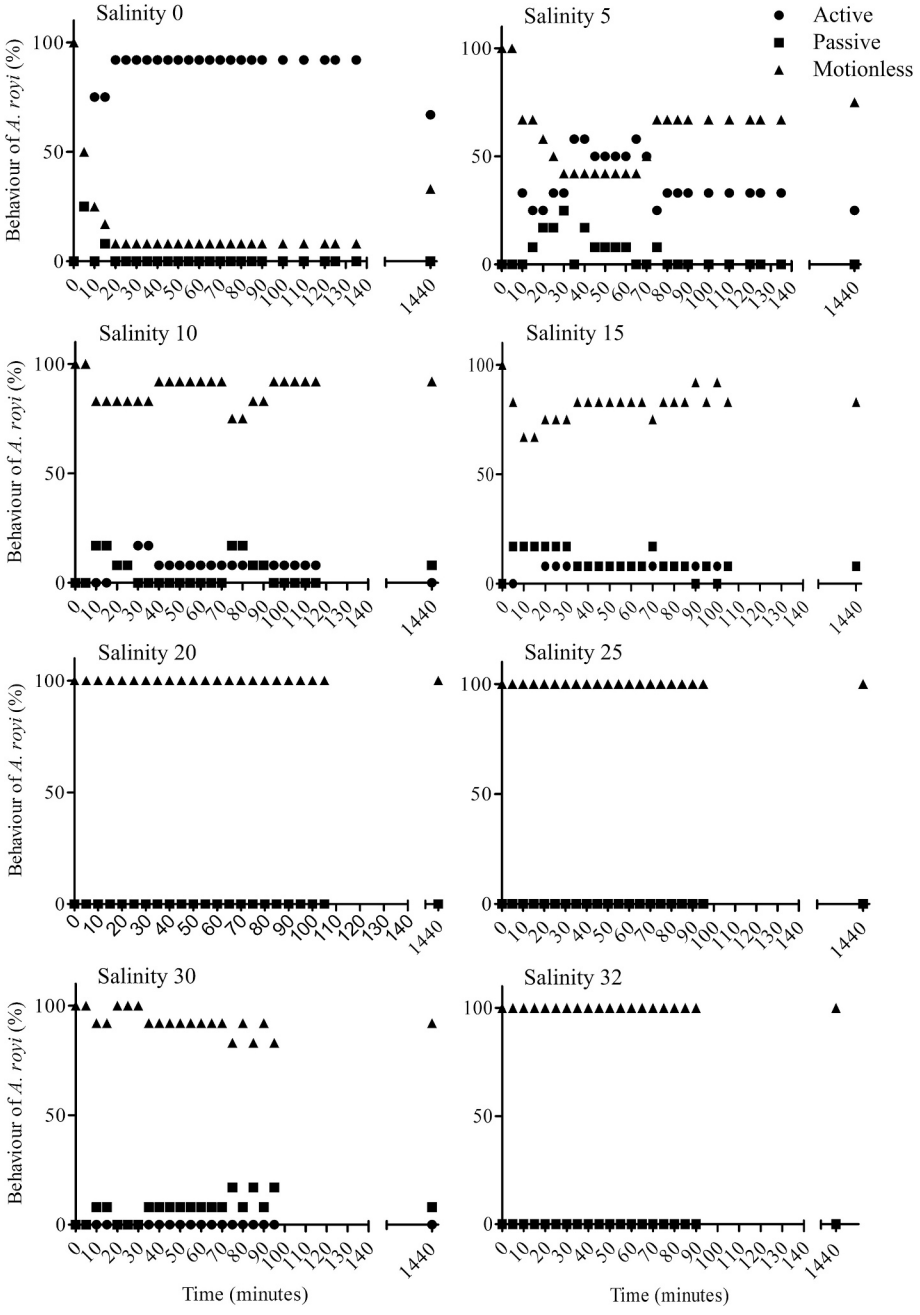

Fig. 1. Shows the three different observed behaviours of *A royi* when exposed to salinities in the range from 0 to 32 as a function of time. Note that the last time point at the x axis is not numerical spaced. The behaviours are active, passive, and motionless. Each dot represents the mean of an observation (dots are mean, $n = 12$).

Experiment one shows the response of *A. royi* when transferred from a salinity of 32 to lower salinities ranging from 0 to salinity 32 (see Fig. 1). A salinity of 32 was considered the control treatment. The copepods were observed and categorized into three groups: active, passive, and motionless. Interestingly, some *A. royi* survived all 24 h even in salinity 0. At salinity 5, a large number of *A. royi* were initially categorized as passive or motionless, but over time they were recategorized into active. Likewise, in salinity 10, 15 and 30 a few percentages of *A. royi* were initially categorized as passive or motionless, though at the end of the experiment only passive and active *A. royi* were observed. In salinities 20, 25 and 32 only active *A. royi* were observed throughout the entire span of the experiment.

At Fig. 2 it is observed that no mortality occurred after exposure to salinities at 10 and above, after 24 h. The LC_{50} were found to be salinity 2.1 (1.53–2.67, 95 % CL). The model parameter is hillslope −6.67, r^2 = 0.98 (n = 8).

The differences in mortality of *A. royi* exposed to decreasing salinities were found to be statistically significant different (Kruskal-Wallis, H = 47.043, df = 7 $p < 0.01$). A subsequent post hoc test (Dunns test) showed that all salinity treatments were significantly different from salinity 0 ($p < 0.001$). Though salinity 5 was not statistically significant different from any other treatments it was considered as the Lowest Observed Effect Concentration after 24 h ($LOEC_{t24}$). Despite an increase in mortality was observed at salinity 5, all *A. royi* survived at salinities of 10 and higher. Hence, No Observed Effect Concentration after 24 h ($NOEC_{t24}$) was determined to be salinity 10 (Fig. 2).

3.2. Experiment 2: multispecies freshwater biomonitor

3.2.1. Monitoring activity of A. royi in relation to light conditions

In general, it was observed that activity was almost four fold higher in the light treatments (19.4 %) when compared to the dark treatment (5.6 %) (Fig. 3). A statistically significant difference was observed between the dark and light treatment (Students *t*-test, U = 413,790, df = 1, p < 0.001).

3.2.2. Monitoring activity levels of A. royi when exposed to different salinities

When transferred acutely from salinity 32 to salinity 0 seven out of eight *A. royi* showed no activity during the 24 h experiment. At salinity 0, the first activity of *A. royi* was noticed after 180 min. At salinity 5, six out of eight *A. royi* were active during the entire exposure time, whereas two *A. royi* did not show any activity and were found motionless at the end of the experiment (Fig. 4). Initially at salinity 5 the highest activity level was observed during the first 20 min. After 330 min the activity level decreased but was observed to resume to the initial higher activity level after 1030 min. At salinity 10 we observed a similar mean activity

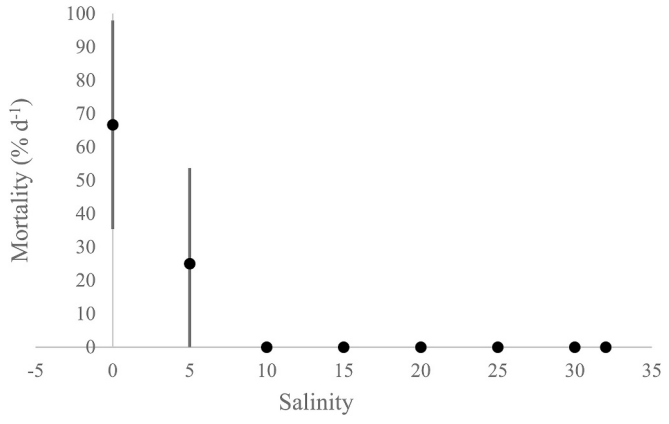

Fig. 2. Shows the 24 h mortality of *A. royi* exposed to salinities from 0 to 32, dots are mean ± 95 % CL (n = 12).

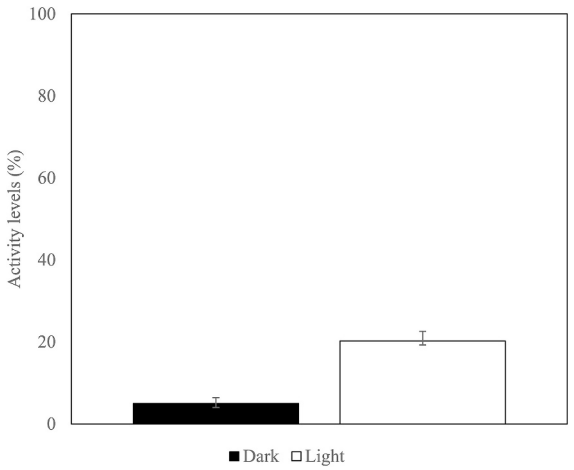

Fig. 3. Shows the mean percentage of activity levels as a function of light conditions of *A. royi*. The black bar is the mean percentage of activity monitored in full darkness (24 h no light). The white bar is the activity monitored in full light conditions 24 h full light, 28 W LED light). Bars are mean ± s.d (n = 8).

level though with a high variation. Salinity 15 showed a similar activity trend as salinity 5. Overall, the highest activity levels were observed in salinities 5, 15, 25 and 32 throughout the experiment. Exposure to salinity 20 and 30 resulted in the lowest mean activity, with least overall variances within the observed activity level (Fig. 4).

The differences in activity level of *A. royi* when exposed to different salinities was found to be statistically significant (Kruskal-Wallis, H = 33.473, df = 7 $p < 0.01$). The lowest activity levels as a function of salinities was in salinity 0. Activity levels peaked within the salinity range of 5 to 15 whereas salinities from 20 and above led to a new minimum. A pairwise comparisons using Dunn's test indicated that the low activity level in salinity 0 was statistically significant differences, from all other treatments ($p < 0.05$). Dunn's test also showed that the activity level of salinity 5 was statistically significant different from all salinities in the range from 0 to 20 (p < 0.05). The activity levels of salinities 10 and 15 was statistically significant different from all other salinities except between themselves (Dunn's test, p < 0.05). The activity level of salinity 20 was statistically significant different from all salinities except from salinity 30 (Dunn's test, p < 0.05). The activity levels of salinities 25, 30 and 32 were not statistically significant different from each other, but from all other salinities, except other mentioned before (Dunn's test, p < 0.05) (Fig. 5).

3.3. Experiment three: determination of body fluid osmolality in A. royi in relation to different salinities

Internal body fluid osmolality in *A. royi* as a function of the surrounding external salinity change is presented in Fig. 6. At the low salinity (~10) the body fluid osmolality in *A. royi* was ~547 mOsm/kg this increased to approximate 680 mOsm/kg at salinity ~20. From salinity ~25 and at higher salinity concentrations, an isosmotic relationship between inner body fluids and the outer environment, was observed.

4. Discussion

The aim of this study was to investigate *A. royi* survival, behavioural response and variations in body fluid osmolality as a response to acute decreasing salinities. Variations in body fluid osmolality was investigated using a novel developed method, using a vapor pressure osmometer. We found that *A. royi* survives acute changing salinities from salinity 5 to 32, with following different behavioural responses as an effect of salinity exposure, e.g. different recovery phases and activity

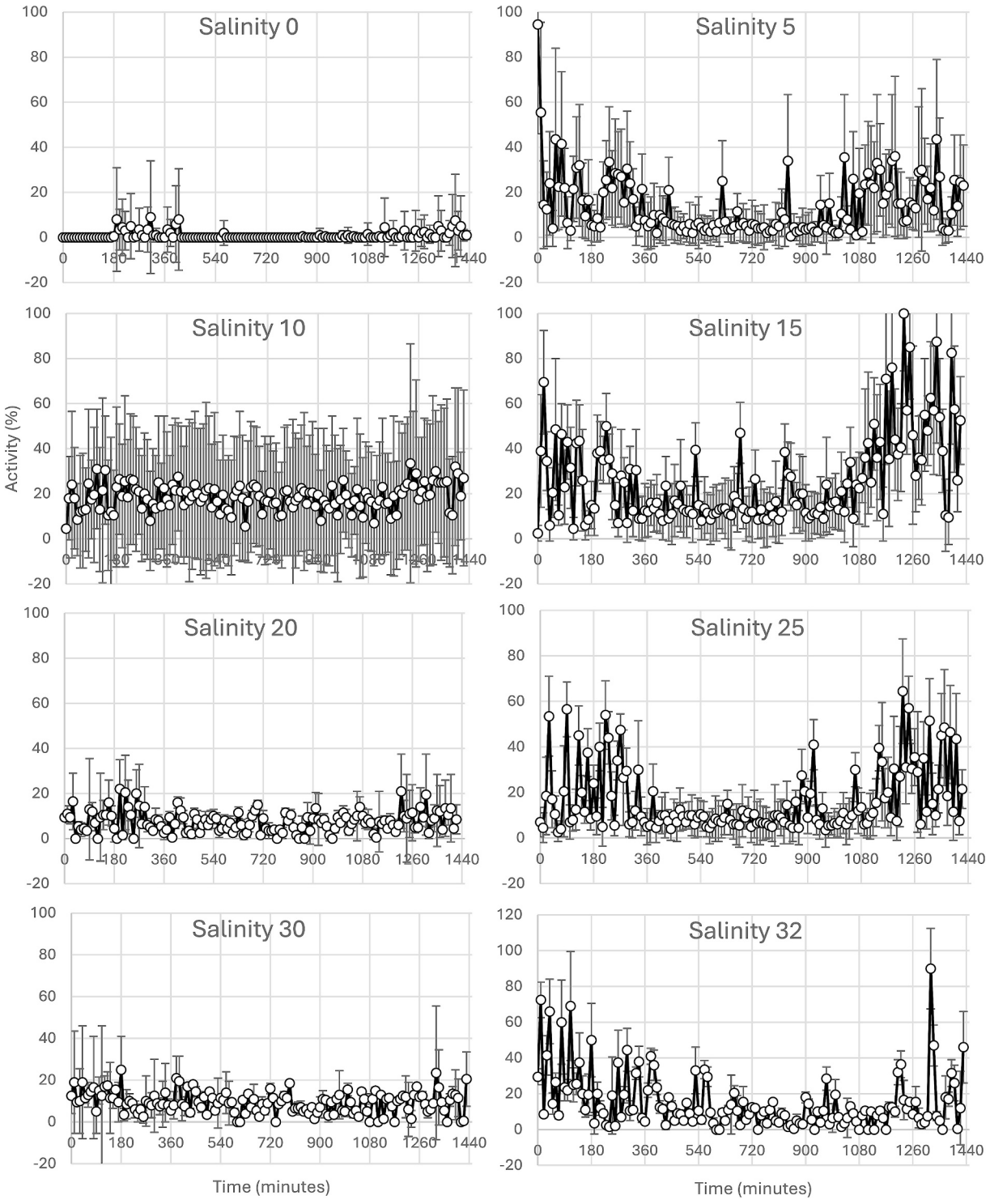

Fig. 4. Shows the monitored mean percentage of activity levels for *A. royi* exposed to salinities ranging from 0 to 32 for 1440 min (24-h) when monitored with a Multispecies Freshwater Biomonitoring system. Dots are means ± s.d. (n = 8).

levels.

The first experiment involved a 24 h acute dose-response study, where individuals of *A. royi* were exposed to an acute decrease in salinity in the range from 32 (control salinity) to 0. The results showed that *A. royi* exhibited a high tolerance for acute decreasing salinities as there was no increase mortality occurring after acute exposure to salinities of 10 and above. Pan et al. (2016) investigated female fecundity of *A. royi,* and did not find any effects of salinities from 10 to 30. Our results showthat survival is not affected in the same salinity ranges as in Pan et al. (2016). When exposed to salinities below 10, *A. royi* initially became passive or motionless, but had regained activity when observed 24 h later. Hansen et al. (2022) showed, similar results with survival of *A. royi* (24 and 48 h), even in salinity 0. Hansen et al. (2022) further showed that when acclimated to salinity 1 over an 8 day course, they could keep the cultures for 71 days but eventually with a loss of fecundity. This led to the next experiment, monitoring the in-situ behaviour of *A. royi* to investigate the timespan of the "newfound" recovery phase.

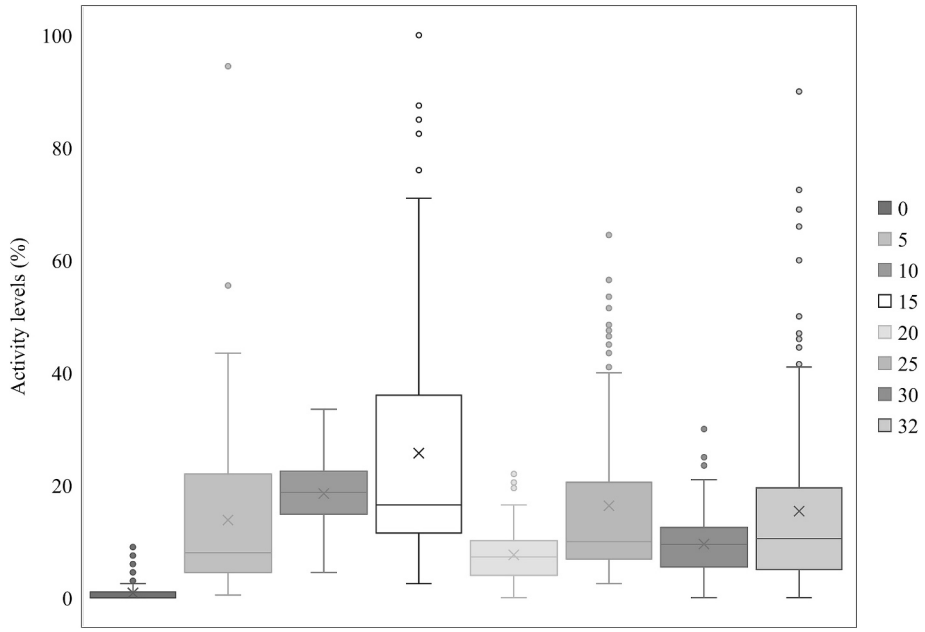

Fig. 5. Shows boxplots of the different levels of activity levels related to the exposed salinities. The boxplots are aligned from left to right ranging from salinity 0 to 32, respectively. The activity is monitored with a Freshwater Biomonitoring system over 24 h. Boxes are the interquartile range (IQR), the line is the median (second quartile), the x are the mean value, the whiskers extend from the box to the highest and lowest values within 1.5 IQR from the Q1 and Q3, the dots are outliers (n = 8).

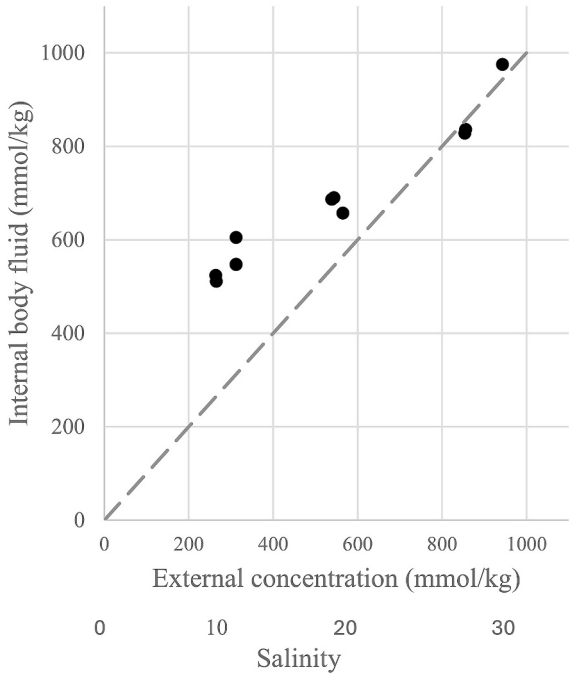

Fig. 6. The body fluid osmolality of *A. royi* as a function of the environmental salinity of the seawater in which the copepods have been reared. The punctuated line represents an isosmotic relationship, and the dots are single observations ($n = 11$).

In nature many factors influence copepods behaviour, including predator risk, food availability, temperature, salinity, and light (Almeda et al., 2017; Durbin et al., 1990; Falkenhaug et al., 1997; Isari et al., 2013; Roman et al., 1988). Another known factor, especially in copepod feeding experiments, is bottle effects, which could influence the results (Takahashi et al., 2023). In this study one individual was placed in a five ml well, which is the recommended maximum stocking density of *Acartia tonsa* by the International Standard (ISO, 1999). *A. royi* is a smaller copepod than *A. tonsa* so theoretically it can be stocked denser. Further Jepsen et al. (2021), observed no density limitation on ovigerous females of *A. royi* at 10 individuals pr. ml. Thus, it seems safe to believe that a bottle effect did not influence the present results.

In this study, salinity was the changing factor, however, it was also investigated whether activity levels did dependent on the experienced light environment. Light is a natural cue for many copepods to initiate the daily migration, hence many copepods exhibit different behaviour and activity level dependent on the surrounding light environment (Martynova and Gordeeva, 2010; Stearns and Forward, 1984). On the other hand, loss of a diel feeding rhythm is known to occur in laboratory cultured copepods (Tiselius et al., 1995). Hence to avoid effects from light, a comparative study with light and dark as the changing factor was conducted. The results showed a statistical significantly higher activity level in the light treatment compared to the dark treatment, which suggests that *A. royi* is more active in the presence of light. This can partly be explained since *A. royi* has been shown to have higher filtration and grazing rates in light when compared to darkness (Zhen et al., 2013). However, the possibility that *A. royi* may still exhibit endogenous rhythms cannot be ruled out, and further studies is needed to investigate this. Therefore experiment two were conducted in full light, to simulate a scenario with highest possible activity in *A. royi*. In this way an environment stimulating a constant activity level was ensured before individuals of *A, royi* were exposed to an acute decrease in salinity. In the second experiment it was found that a single *A. royi* was able to sustain and recover after ~3 h from an acute decrease in salinity from 32 to 0. In the first experiment observations were done with a dissection microscope, every five minute as a minimum for the first 2-h, and then again after 24-h. Hence, the end of the recovery phase, where motionless *A. royi* again became active, in the lower salinities e.g. 0 or 5 was never achieved. This recovery was however detected by the in situ MFB, as it continuously monitored *A. royi* activity levels during 24 h in experiment two. When exposed to salinity of 5 activity levels went to zero for two out of the initial eight copepods within the first 20 min in situ monitoring. In the same 20 min the remaining six copepods exhibited the highest activity levels monitored. Similar, high activity levels were

observed when *A. royi* were exposed to salinities of 10 and 15. Thus, it was speculated if the enhanced activity level in *A. royi* in these three salinities reflected an active regulation of ions. Lance (1965) demonstrated that the internal osmotic concentration of *A. tonsa* rapidly decrease when transferred from a salinity of 36.4 to 18.2 but stabilized within an hour. Future studies could investigate if *A. royi* has a similar response, but as the MFB system showed no activity for ~3 h, one could speculate that the acclimation period is longer. A series of experiments by van Someren Gréve et al. (2020), showed that the highest mortality rate of *A. royi* was observed directly after one hour acclimatization to a salinity of 5, with a lower mortality rate observed during the following 24 h exposure, regardless of absence or presence of algal food (van Someren Gréve et al., 2020). As demonstrated by experiment one and two of this study, accessing the mortality during severe salinity stress in *A. royi* after one hour may result in misleading results, due to the motionless nature of the aforementioned recovery phase. Thus, one could speculate if van Someren Gréve et al. (2020) would have concluded differently had they waited longer than one hour before assessing the mortality rate. Hansen et al. (2022) suggested a strong and fast-negative physiologic response to acute salinity changes. The observations in this study are in support of this statement. In general, lower activity levels in the salinity range from 20 to 32 was observed. The statistical test partly confirmed this trend with no statistical significance differences in the salinity range from 25 to 32. It seems plausible that these activity levels reflect a normal activity level of non-stressed *A. royi*. In future studies, longer exposure times could validate if the activity levels in salinities lower than 25 are related to *A. royi* salinity coping strategy, or a general picture of the activity level in these salinities.

In addition to behavioural observations, this study also examined the body fluid osmolality of *A. royi* reared at three different salinities. A new method to extract body fluid from copepods was developed for this purpose. The results of the extraction method were validated by measuring the osmolality of saline samples, with a known osmolality, with and without paraffin oil, and the result showed maximum 8 % difference between samples ($n = 8$, data not shown). The newly developed method has the advantage of giving a quick and a direct measurement on the vapor pressure osmometer. This is in contrast to present methods like NMR or HPLC, that requires further sample preparation and analysis, We have reasons to believe that both the extraction and osmometer methods can be widely applied to other copepod species, and potentially other planktonic crustaceans. The disadvantage of the method is that one need bulk numbers of copepods. Despite being isotonic to the surroundings at higher salinities, the inner body fluid of *A. royi* becomes hypertonic when exposed to lower external salinities. This indicates that *A. royi* is capable of osmoregulation, adjusting its internal osmotic concentration to cope with lower external salinity. When acclimating copepods to the different salinities, there is always the possibility that one indirectly selects on a salinity robust geno- or phenotypes (Lee and Petersen (2002). It is known that *A. royi* can be selectively breed for a specific trait within only one generation Pan et al. (2017). Thus, it is possible that salinity tolerance exhibited by the *A. royi* used in this study differ from those found in the wild.

Regarding the number of copepods used for extraction of body fluids, it was observed that *A. royi* exposed to salinity 10 had a higher amount of body fluid, than those exposed to salinity 32. This indicates that when exposed to hypoosmotic conditions water from the surrounding medium is taken up by *A. royi*. A volume increase at lower salinities has also been observed in eggs of the marine calanoid copepod *A. tonsa*, however, in this case the osmolality of the eggs followed the molality of the surrounding water (Hansen et al., 2012). Thus, it seems reasonable to conclude that *A. royi* are unable to completely counteract the osmotic driven influx of water. However, they appear to function normally despite the increased amount of body fluid. Further studies are needed to determine whether the motionless behaviour observed in the first two experiments is related to this presumed influx of water. One of the remaining questions is how *A. royi* osmoregulatory functions works? The Na^+/K^+-ATPase activity of *A. royi* would be a naturally next step to investigate, like it has been done for the euryhaline *Psedodiatoptomus richardi* (Kaminski et al., 2014). In some osmoregulating copepods synthesis and increased concentration of free amino acids (alanine, proline and glycine) during acclimation to high salinity are often observed (Burton, 1991; van der Meeren et al., 2008; Lindley et al., 2011). But also, other types of metabolites potentially upregulated as osmolytes are observed and present in copepods, e.g. glycine betaine increase in lactate (Goolish and Burton, 1989; Hansen et al., 2022). Shadrin and Anufriieva (2013) suggested that the osmoconforming copepod *Arctodiaptomus salinus* depends on consuming exoosmolyte through its diet. Thus, one could speculate that lack of food could have influenced the results. However, since the experiments conducted in this study are acute and copepods general utilized the food they consumed the previous day, it seems safe to assume that this was not the case (Berggreen et al., 1988). Furthermore, the results by van Someren Gréve et al. (2020) shows that the survival of *A. royi* was not affected by presence or absence of food, when exposed to acute decreases in salinity from 32 to 5. They do however emphasize that active ion regulation leaves less energy for basic physiological functions in copepods like reproduction (Chen et al., 2006; van Someren Gréve et al., 2020). Hence in a stock culture condition one should keep *A. royi* salinity constant for them to conserve energy (Bradly, 2009; Hand and Hardewig, 1996). Based on the summarized results from all three experiments salinity range of 25 to 32 would be ideal conditions for cultures of *A. royi*.

Further understanding how *A. royi* copes with acute decreasing salinity is crucial for comprehending its ecological adaptation and survival strategies. The result in this study indicates that *A. royi* are thriving in salinities from 25 and higher, but can sustain and survive lower salinities, down to freshwater conditions. The ability of *A. royi* to physiologically being able to cope with acute decreasing salinity, and understanding the mechanism behind, can contribute to our understanding of copepod physiology and their ability to thrive in various environments. Further the ability of low-salinity tolerance of *A. royi,* could potentially allow saline populations to invade freshwater habitats, like observed for *Eurytemora affinis* (Lee, 2016; Roddie et al., 1984).

5. Conclusions

The cyclopoid copepod *A. royi* exhibited a high tolerance to acute decreasing salinities from 32 to 0. Moreover, this species were observed to become motionless when transferred from salinity 32 to 5 or 0, but after ~3 h were are able to regain activity. Further investigations showed that at low salinities *A. royi* are hyperregulating their body fluid osmolality whereas at higher salinities they osmoconform. Further studies are needed to elucidate the exact mechanisms by which osmohyperregulation takes place as well as the energetic cost of such hyperregulation. Such information will be of importance when assessing *A. royi* capability to invade freshwater habitats.

CRediT authorship contribution statement

Per M. Jepsen: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Cæcilie H. Dinsen:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Esther S.H. Øllgaard:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jonathan Y.B. Jedal:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Lasse Aggerholm:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation,

Formal analysis, Data curation, Conceptualization. **Tor Salomonsen:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Hans Ramløv:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

Statement: During the preparation of this work the author(s) used Avidnote (Version 2.0) [Computer software]. Retrieved from https://www.avidnote.com/ in order to improve readability and language. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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.

Data availability

Data will be made available on request.

Acknowledgements

The research was a part of the project ORACLE-FISH (J. nbr. 34,009–22-2029) that is part of the Organic RDD 8 programme, which is coordinated by International Centre for Research in Organic Food Systems (ICROFS). It has received grants from the Green Growth and Development programme (GUDP) under the Danish Ministry of Food, Agriculture and Fisheries.

References

Almeda, R., van Someren Gréve, H., Kiørboe, T., 2017. Behavior is a major determinant of predation risk in zooplankton. Ecosphere 8 (2), e01668. https://doi.org/10.1002/ecs2.1668.

Bayly, I.A., Boxshall, G.A., 2009. An all-conquering ecological journey: from the sea, calanoid copepods mastered brackish, fresh, and athalassic saline waters. Hydrobiologia 630, 39–47. https://doi.org/10.1007/s10750-009-9797-6.

Bayly, I.A.E., 1969. The body fluids of some centropagid copepods: total concentration and amounts of sodium and magnesium. Comp. Biochem. Physiol. 28, 1403–1409. https://doi.org/10.1016/0010-406X(69)90577-5.

Berggreen, U., Hansen, B., Kiørboe, T., 1988. Food size spectra, ingestion and growth of the copepod *Acartia tonsa* during development: implications for determination of copepod production. Mar. Biol. 99 (3), 341–352. https://doi.org/10.1007/s11160-010-9169-3.

Blanda, E., Drillet, G., Huang, C.C., Hwang, J.S., Jakobsen, H.H., Rayner, T.A., Su, H.-M., Wu, C.-H., Hansen, B.W., 2015. Trophic interactions and productivity of copepods as live feed from tropical Taiwanese outdoor aquaculture ponds. Aquaculture 445, 11–21. https://doi.org/10.1016/j.aquaculture.2015.04.003.

Blanda, E., Drillet, G., Huang, C.C., Hwang, J.S., Højgaard, J.K., Jakobsen, H.H., Rayner, T.A., Su, H.-M., Hansen, B.W., 2017. An analysis of how to improve production of copepods as live feed from tropical Taiwanese outdoor aquaculture ponds. Aquaculture 479, 432–441. https://doi.org/10.1016/j.aquaculture.2017.06.018.

Bradly, T., 2009. Animal Osmoregulation (Oxford Animal Biology Series)(pp. 184). Oxford University Press. ISBN 13: 978–0198569961.

Bron, J.E., Frisch, D., Goetze, E., Johnson, S.C., Lee, C.E., Wyngaard, G.A., 2011. Observing copepods through a genomic lens. Front. Zool. 8 (1), 1–15. https://doi.org/10.1186/1742-9994-8-22.

Burton, R.S., 1991. Regulation of proline synthesis during osmotic stress in the copepod *Tigriopus californicus*. J. Exp. Zool. 259 (2), 166–173. https://doi.org/10.1002/jez.1402590204.

Chen, Q., Sheng, J., Lin, Q., Gao, Y., Lv, J., 2006. Effect of salinity on reproduction and survival of the copepod *Pseudodiaptomus annandalei* Sewell, 1919. Aquaculture 258, 575–582. https://doi.org/10.1016/j.aquaculture.2006.04.032.

Durbin, A.G., Durbin, E.G., Wlodarczyk, E., 1990. Diel feeding behavior in the marine copepod *Acartia tonsa* in relation to food availability. Mar. Ecol. Prog. Ser. 23–45. http://www.jstor.org/stable/44634875.

Falkenhaug, T., Tande, K.S., Semenova, T., 1997. Diel, seasonal and ontogenetic variations in the vertical distributions of four marine copepods. Mar. Ecol. Prog. Ser. 149, 105–119. https://doi.org/10.3354/meps149105.

Farmer, L., 1980. Evidence for hyporegulation in the calanoid copepod *Acartia tonsa*. Compar. Biochem. Physiol. A 65, 359–362. https://doi.org/10.1016/0300-9629(80)90043-2.

Gerhardt, A., Svensson, E., Clostermann, M., Fridlund, B., 1994. Monitoring of behavioral patterns of aquatic organisms with an impedance conversion technique. Environ. Int. 20 (2), 209–219. https://doi.org/10.1016/0160-4120(94)90138-4.

Gerhardt, A., Carlsson, A., Ressemann, C., Stich, K.P., 1998. New online biomonitoring system for *Gammarus pulex* (L.)(Crustacea): in situ test below a copper effluent in south Sweden. Environ. Sci. Technol. 32 (1), 150–156. https://doi.org/10.1021/es970442j.

Goolish, E.M., Burton, R.S., 1989. Energetics of osmoregulation in an intertidal copepod: effects of anoxia and lipid reserves on the pattern of free amino accumulation. Funct. Ecol. 81-89. https://doi.org/10.2307/2389678.

Hand, S.C., Hardewig, I., 1996. Downregulation of cellular metabolism during environmental stress: mechanisms and implications. Annu. Rev. Physiol. 58, 539–563. https://doi.org/10.1146/annur ev.ph.58.030196.002543.

Hansen, B.W., 2023. Two tropical marine copepods demonstrate physiological properties needed for mass production. Rev. Fisher. Sci. Aquac. 31 (1), 141–159. https://doi.org/10.1080/23308249.2022.2095198.

Hansen, B.W., Drillet, G., Pedersen, M.F., Sjøgreen, K.P., Vismann, B., 2012. Do *Acartia tonsa* (Dana) eggs regulate their volume and osmolality as salinity changes? J. Comp. Physiol. B. 182, 613–623. https://doi.org/10.1007/s00360-012-0646-y.

Hansen, B.W., Hansen, P.J., Nielsen, T.G., Jepsen, P.M., 2017. Effects of elevated pH on marine copepods in mass cultivation systems: practical implications. J. Plankton Res. 39 (6), 984–993. https://doi.org/10.1093/plankt/fbx032.

Hansen, B.W., Ciappini, G., Malmendal, A., Rayner, T.A., 2022. Can we adapt a marine cyclopoid copepod to freshwater? – first step towards a 'universal' live feed product for fish larvae. Aquac. Res. 53 (1), 178–190. https://doi.org/10.1111/are.15563.

Hauton, C., 2016. Effects of salinity as a stressor to aquatic invertebrates. In: Solan, M., Whitley, N.M. (Eds.), Stressors in the Marine Environment. Physiological and Ecological Responses; Societal Implications. Oxford University Press, Oxford, pp. 3–24. ISBN: 978–0–19-871882-6.

Ibrahim, A., Souissi, A., Leray, A., Heliot, L., Vandenbunder, B., Souissi, S., 2016. Myofibril changes in the copepod Pseudodiaptomus marinus exposed to haline and thermal stresses. PLoS One 11 (11), e0164770.

Isari, S., Antó, M., Saiz, E., 2013. Copepod foraging on the basis of food nutritional quality: can copepods really choose? PLoS One 8 (12), e84742. https://doi.org/10.1371/journal.pone.0084742.

ISO, 1999. Water quality – Determination of acute lethal toxicity to marine copepods (*Copepoda, Crustacea*). International Standard ISO14669:1999(E).

Jepsen, P.M., Andersen, C.V., Schjelde, J., Hansen, B.W., 2015. Tolerance of un-ionized ammonia in live feed cultures of the calanoid copepod *Acartia tonsa* Dana. Aquac. Res. 46 (2), 420–431. https://doi.org/10.1111/are.12190.

Jepsen, P.M., Thoisen, C.V., Carron-Cabaret, T., Pinyol-Gallemí, A., Nielsen, S.L., Hansen, B.W., 2019. Effects of salinity, commercial salts, and water type on cultivation of the cryptophyte microalgae Rhodomonas salina and the calanoid copepod *Acartia tonsa*. J. World Aquacult. Soc. 50 (1), 104–118. https://doi.org/10.1111/jwas.12508.

Jepsen, P.M., van Someren Gréve, H., Jørgensen, K.N., Kjær, K.G., Hansen, B.W., 2021. Evaluation of high-density tank cultivation of the live-feed cyclopoid copepod *Apocyclops royi* (Lindberg 1940). Aquaculture 533, 736125. https://doi.org/10.1016/j.aquaculture.2020.736125.

Johnson, K.E., Perreau, L., Charmantier, G., Charmantier-Daures, M., Lee, C.E., 2014. Without gills: localization of osmoregulatory function in the copepod *Eurytemora affinis*. Physiol. Biochem. Zool. 87 (2), 310–324. https://doi.org/10.1086/674319.

Kaminski, S.M., Bersano, J.G., Freire, C.A., 2014. Euryhalinity of the estuarine copepod *Pseudodiaptomus richardi* and its high potential to be employed as live food in aquaculture. Aquaculture 424, 63–70. https://doi.org/10.1016/j.aquaculture.2013.12.034.

Kasuya, E., 2001. Mann-Whitney U test when variances are unequal. Anim. Behav. 61, 1247–1249. https://doi.org/10.1006/anbe.2001.1691.

Kiørboe, T., Visser, A., Andersen, K.H., 2018. A trait-based approach to ocean ecology. ICES J. Mar. Sci. 75 (6), 1849–1863. https://doi.org/10.1093/icesjms/fsy090.

Lance, J., 1965. Respiration and osmotic behaviour of the copepod *Acartia tonsa* in diluted sea water. Comp. Biochem. Physiol. 14 (1), 155–165. https://doi.org/10.1016/0010-406X(65)90016-2.

Lee, C.E., 2016. Evolutionary mechanisms of habitat invasions, using the copepod *Eurytemora affinis* as a model system. Evol. Appl. 9 (1), 248–270. https://doi.org/10.1111/eva.12334.

Lee, C.E., Petersen, C.H., 2002. Genotype-by-environment interaction for salinity tolerance in the freshwater-invading copepod *Eurytemora affinis*. Physiol. Biochem. Zool. 75 (4), 335–344. https://doi.org/10.1086/343138.

Lee, K.W., Park, G.H., 2005. Effects of temperature and salinity on productivity and growth of five copepod species. J. Korean Fisher. Soc. 38 (1), 12–19. https://doi.org/10.5657/kfas.2005.38.1.012.

Lee, K.W., Kwon, O.-N., Park, H.G., 2005. Effects of temperature, salinity and diet on the productivity of the cyclopoid copepod, *Apocyclops royi*. J. Aquac. 18 (1), 52–59.

Liao, I.C., Su, H.M., Chang, E.Y., 2001. Techniques in finfish larviculture in Taiwan. Aquaculture 200 (1–2), 1–31. https://doi.org/10.1016/S0044-8486(01)00692-5.

Lindley, L.C., Phelps, R.P., Davis, D.A., Cummins, K.A., 2011. Salinity acclimation and free amino acid enrichment of copepod nauplii for first-feeding of larval marine fish. Aquaculture 318, 402–406. https://doi.org/10.1016/j.aquac ulture.2011.05.050.

Martynova, D.M., Gordeeva, A.V., 2010. Light-dependent behavior of abundant zooplankton species in the White Sea. J. Plankton Res. 32 (4), 441–456. https://doi.org/10.1093/plankt/fbp144.

McAllen, R.J., Taylor, A.C., Davenport, J., 1998. Osmotic and body density response in the harpacticoid copepod *Tigriopus brevicornis* in supralittoral rock pools. J. Mar. Biol. Assoc. U. K. 78 (4), 1143–1153. https://doi.org/10.1017/S0025315400044386.

van der Meeren, T., Olsen, R.E., Hamre, K., Fyhn, H.J., 2008. Biochemical composition of copepods for evaluation of feed quality in production of juvenile marine fish. Aquaculture 274, 375–397. https://doi.org/10.1016/j.aquaculture.2007.11.041.

Mellak, L., Hafferssas, A., Hamri, F., Alioua, Z., Khames, G.E.Y., 2024. Importance of copepods in the diet of Sardina pilchardus and Sardinella aurita: preliminary investigation in Bou Ismail Bay (Algerian Basin-SW Mediterranean Sea). Thalassas: Intern. J. Marine Sci. 40 (1), 607–623. https://doi.org/10.1007/s41208-023-00651-5.

Muthupriaya, P., Altaff, K., 2009. Effects of salinity and temperature on the reproduction of the estuarine copepod *Apocyclops royi* (Lindberg 1940). J. Exp. Zool. India 12, 103–106 (ISSN (Electronic): 0976-1780).

Palanichamy, M., Kandhasamy, S., Kareem, A., 2022. Insight into the reproductive biology of euryhaline cyclopoid copepods *Apocyclops dengizicus* and *Apocyclops royi*. Intern. J. Aquatic. Biol. 10 (4), 285–298. https://doi.org/10.22034/ijab.v10i4.1689.

Pan, Y.J., Souissi, A., Souissi, S., Hwang, J.S., 2016. Effects of salinity on the reproductive performance of *Apocyclops royi* (Copepoda, Cyclopoida). J. Exp. Mar. Biol. Ecol. 475, 108–113. https://doi.org/10.1016/j.jembe.2015.11.011.

Pan, Y.J., Souissi, A., Sadovskaya, I., Hansen, B.W., Hwang, J.S., Souissi, S., 2017. Effects of cold selective breeding on the body length, fatty acid content, and productivity of the tropical copepod *Apocyclops royi* (Cyclopoida, Copepoda). J. Plankton Res. 39 (6), 994–1003. https://doi.org/10.1111/anu.12633.

Rastetter, N., Gerhardt, A., 2018. Continuous monitoring of avoidance behaviour with the earthworm *Eisenia fetida*. J. Soils Sediments 18, 957–967. https://doi.org/10.1007/s11368-017-1791-4.

Reid, J.W., Hamilton IV, R., Duffield, R.M., 2002. First confirmed New World record of Apocyclops dengizicus (Lepeshkin), with a key to the species of Apocyclops in North America and the Caribbean region (Crustacea: Copepoda: Cyclopidae). Jeffersoniana 10, 1–25.

Ren, Z., Zha, J., Ma, M., Wang, Z., Gerhardt, A., 2007. The early warning of aquatic organophosphorus pesticide contamination by on-line monitoring behavioral changes of *Daphnia magna*. Environ. Monit. Assess. 134, 373–383. https://doi.org/10.1007/s10661-007-9629-y.

Rivera-Ingraham, G.A., Lignot, J.H., 2017. Osmoregulation, bioenergetics and oxidative stress in coastal marine invertebrates: raising the questions for future research. J. Exp. Biol. 220 (10), 1749–1760.

Roddie, B.D., Leakey, R.J.G., Berry, A.J., 1984. Salinity-temperature tolerance and osmoregulation in *Eurytemora affinis* (Poppe)(Copepoda: Calanoida) in relation to its distribution in the zooplankton of the upper reaches of the forth estuary. J. Exp. Mar. Biol. Ecol. 79 (2), 191–211. https://doi.org/10.1016/0022-0981(84)90219-3.

Roman, M.R., Ashton, K.A., Gauzens, A.L., 1988. Day/night differences in the grazing impact of marine copepods. Hydrobiologia 167, 21–30. https://doi.org/10.1007/BF00026291.

van Someren Gréve, H., Jepsen, P.M., Hansen, B.W., 2020. Does resource availability influence the vital rates of the tropical copepod *Apocyclops royi* (Lindberg, 1940) under changing salinities? J. Plankton Res. 42 (4), 467–478. https://doi.org/10.1093/plankt/fbaa031.

Shadrin, N.V., Anufriieva, E.V., 2013. Dependence of Arctodiaptomus salinus (Calanoida, Copepoda) halotolerance on exoosmolytes: new data and a hypothesis. J. Mediterranean Ecol. 12 (2013), 21–26.

Stearns, D.E., Forward, R.B., 1984. Copepod photobehavior in a simulated natural light environment and its relation to nocturnal vertical migration. Mar. Biol. 82, 91–100. https://doi.org/10.1007/BF00392767.

Su, H.M., Su, M.S., Liao, I.C., 1997. Collection and culture of live foods for aquaculture in Taiwan. Hydrobiologia 358 (1–3), 37–40. https://doi.org/10.1023/A:1003107701367.

Su, H.-M., Cheng, S.-H., Chen, T.-I., Su, M.-S., 2005. Culture of copepods and applications to marine finfish larval rearing in Taiwan. In: Lee, C.-S., O'Bryen, P.J., Marcus, N.H. (Eds.), Copepods in Aquaculture. Blackwell Publishing, Australia, pp. 183–195. https://doi.org/10.1002/9780470277522.ch18.

Svetlichny, L., Hubareva, E., Khanaychenko, A., 2012. *Calanipeda aquaedulcis* and *Arctodiaptomus salinus* are exceptionally euryhaline osmoconformers: evidence from mortality, oxygen consumption, and mass density patterns. Mar. Ecol. Prog. Ser. 470, 15–29. https://doi.org/10.3354/meps09907.

Svetlichny, L., Hubareva, E., Uttieri, M., 2021. Ecophysiological and behavioural responses to salinity and temperature stress in cyclopoid copepod *Oithona davisae* with comments on gender differences. Mediterr. Mar. Sci. 22 (1), 89–101. https://doi.org/10.12681/mms.22496.

Takahashi, K., Ichinomiya, M., Okazaki, Y., Nishibe, Y., 2023. Higher ingestion rates and importance of ciliates in the diet of a large, subarctic copepod revealed by larger volume incubations. Limnol. Oceanogr.y 68 (4), 790–802.

Thoisen, C., Vu, M.T.T., Carron-Cabaret, T., Jepsen, P.M., Nielsen, S.L., Hansen, B.W., 2018. Small-scale experiments aimed at optimization of large-scale production of the microalga *Rhodomonas salina*. J. Appl. Phycol. 30 (4), 2193–2202. https://doi.org/10.1007/s10811-018-1434-1.

Tiselius, P., Hansen, B., Jonsson, P., Kiørboe, T., Nielsen, T.G., Piontkovski, S., Saiz, E., 1995. Can we use laboratory-reared copepods for experiments? A comparison of feeding behaviour and reproduction between a field and a laboratory population of *Acartia tonsa*. ICES J. Mar. Sci. 52 (3–4), 369–376. https://doi.org/10.1016/1054-3139(95)80052-2.

Tocher, D.R., 2010. Fatty acid requirements in ontogeny of marine and freshwater fish. Aquac. Res. 41 (5), 717–732. https://doi.org/10.1111/j.1365-2109.2008.02150.x.

Williamson, A., Blandon, I., Scarpa, J., Vega, R., Siccardi, A., 2021. Copepod Propagation and Use as a Live Food for Fish Larviculture. Texas Parks & Wildlife, Coastal Fisheries Division.

Zhen, M., Guodong, W., Xinfu, L., Bin, L., Yodong, J., Ying, B., Hesen, Z., 2013. Effects of environmental factors on the feeding of *Apocyclops royi*. Mar. Sci. 37 (1), 81–86.