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The Effect of Density and Temperature on Survival and Fatty Acid Profiles of Copepods (*Thermocyclop* sp.)

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Abstract Cyclopoid copepods (*Thermocyclop* sp.) have the nutritional attributes of an ideal diet for fish larvae. However, long-term production and availability of copepods for feeding larval fish in hatcheries remain a challenge. The present study investigated the effect of density and temperature on survival and fatty acid profiles of *Thermocyclop* sp. at densities: 1 000, 3 000, and 5 000 individuals/L and temperatures: 4 °C, 8 °C, and 12 °C. A log-rank test showed a significant difference between the percentage survival of *Thermocyclop* sp. at 12 °C and 4 °C (P<0.001) and a significantly higher survival at 1 000 than at 5 000 individuals/L (P<0.001). Generally, saturated fatty acids (SFAs) were dominant compared to monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). For essential fatty acids, no significant differences were observed between storage temperatures on the 7th and 14th days of the experiment. The results demonstrated that at least a 50% survival rate is obtained when these organisms are stored at 12 °C with a density of 1 000 individuals/L for 14 days, with no significant changes in fatty acid profiles. Further studies are necessary to determine the effect of increased storage conditions, perhaps with aeration, on storage time.

Keywords *Thermocyclop* sp.; Density; Temperature; Survival; Fatty acids

Introduction

Over the last decades, climate change, illegal, unregulated, and unreported (IUU) fishing, and pollution have led to the depletion of fisheries resources (Nakiyende et al., 2023). For example, overfishing results in the removal of a large number of fish from the population. This leads to a decline in the overall size and abundance of the targeted fish species, making it difficult for the population to sustain itself (Kwikiriza et al., 2023a). Presently, there is an increased global investment in aquaculture, seen as an alternative for enhancing fish production to meet the demand-supply gap arising from the continual decline in capture fisheries (Abaho et al., 2016; FAO, 2022). By 2021, the leading African countries contributing to aquaculture production were Egypt (72.20%), followed by Nigeria (12.60%), and Uganda (6.33%) (FAO, 2022). Even though global aquaculture continues to increase, the African contribution remains low, estimated at 2.7% (Adeleke et al., 2020; FAO, 2022), and this is attributed to many factors, majorly; lack of access to quality and affordable feeds (Izaara et al., 2020) and poor-quality seed among others (Kwikiriza et al., 2023b). Poor-quality fish seed results in lower survival rates in fish farms, thus increasing financial losses for farmers who invest in fingerling production. To boost aquaculture, there is a need to invest in raising quality fish seed, and one way to achieve this is by enhancing larval nutrition. Larval nutrition poses a major bottleneck in aquaculture hatcheries worldwide, impeding the full commercialization of most domesticated fish species (Abate et al., 2016). Developing larvae are very small, fragile, and have poorly developed physiological systems, which limits their ability to efficiently utilize formulated feeds, resulting in poor growth and survival rates (Kimmerling et al., 2018). Presently, aquaculture production relies on live food organisms like Brine shrimp (*Artemia* sp.), rotifers, and copepods to meet the nutritional requirements of these

small larvae (Olivotto et al., 2010; Kimmerling et al., 2018). The availability of live starter organisms like Brine shrimp, copepods, and rotifers is thus vital for the successful fry production of different fish species in aquaculture (Abaho et al., 2016).

Amongst the live starter feeds, copepods are known to be nutritionally superior as they contain higher levels of Docosahexaenoic acid (DHA) and a protein content of 44%~52% with a suitable amino acid profile (Radhakrishnan et al., 2020). Additionally, the copepodites and adults have digestive enzymes required in the early life stages of fish and crustacean larvae (Alejos et al., 2022). The size suitability of nauplii and early copepodite stages of copepods are easily utilized by small-sized larval fish compared to Brine shrimp and rotifers during larval fish nutrition (Chepkwemoi et al., 2013).

Copepods classes include Calanoid, Harpacticoid, and Cyclopoid copepods. Among these classes, Cyclopoid copepods are advantageous in different ways; they are easy to culture and can be maintained in higher densities compared to Calanoids (Park et al., 2021). Also, the presence of the paired egg sacs attached to the female genital segment means that higher production of Cyclopoid copepods is achieved compared to Calanoids (Mironova and Pasternak, 2017). The short development times of 4-5 days to maturation for copepods make them ideal to use in the mass culture and subsequent feeding of the fish larvae (Chepkwemoi et al., 2013). Therefore, all these advantages make the cyclopoids better copepods for larval nutrition.

In the family Cyclopidae, *Thermocyclop sp.* is dominant. The species is distinguishable from other copepods by the first antennae, which are shorter than the combined length of the head and thorax, along with the uniramous second antennae (Chepkwemoi et al., 2013). *Thermocyclops* sp has a wide distribution in freshwater systems including lakes, rivers, and marshes (Jaime et al., 2021). The species is predominantly pelagic thrivings in littoral zones characterized by dense stands of immersed macrophytes. The *Thermocyclop sp.* also tolerate salinities of up to 7.2% and pH, ranging from 5.9 to 8.4. Ecologically, the species plays a pivotal role as the primary link connecting phytoplankton's primary production to higher predators, including shrimps and juvenile fish (Abaho et al., 2016). Besides, these organisms can elongate essential fatty acids to produce polyunsaturated fatty acids (DHA and EPA), which are required for the physiological functioning of fish (Chepkwemoi et al., 2013). These unique qualities provide a competitive advantage for *Thermocyclop sp.* as an ideal food source in larval fish culture.

Although copepods present a competitive advantage as crucial live starter feeds in aquaculture, their appropriate storage conditions have not been thoroughly explored (Chepkwemoi et al., 2013; Abaho et al., 2016; Beingana et al., 2016; Izaara et al.; 2020). For example, sustaining their availability for use in hatcheries is still challenged by inadequate information on ideal storage temperatures and densities in the Ugandan aquaculture industry. Therefore, the present study explored the effects of storage conditions (temperature and density) on the survival and fatty acid profiles of *Thermocyclop* sp. It was hypothesized that the manipulation of the storage density and temperature of Cyclopoid copepods (*Thermocyclop sp.*) results in variations in survival rates and fatty acid profiles of the copepods. The successful storage and packaging of live *Thermocyclop* sp. will enhance their accessibility and utilization in fish hatcheries as alternatives to the presently commonly used Artemia in Uganda. The results from this study will directly impact larval fish nutrition by providing insights into how storage conditions influence the fatty acid profiles of copepods. This information can be translated into practical recommendations for ensuring that larval fish receive the best possible nutrition during their critical early stages. Subsequently, interventions will bridge the supply gap to live starter feeds thus contributing to the growth of aquaculture in Uganda.

1 Results

1.1 Survival rates

Generally, there was a gradual decrease in the survival rate of *Thermocyclop* sp. at different temperatures with time. The percentage survival rates of *Thermocyclo*p sp. were significantly higher at 12 °C than at 4 °C (Figure 1) (2 = 9.9, df = 2, P = 0.007). Percentage survival after 14 days was 55.7 ± 0.9% (12 °C), 44.0 ± 1.5% (8 °C), and 30.7±1.2% (4 °C).

Percentage survival rates were also significantly higher at the lower packaging densities (1 000/L) than at higher packaging densities (5 000/L) (Figure 2) (2 = 8, df = 2, P = 0.02). Percentage survival after 14 days was 67.9 ± 1.0% (1 000/L), 50.2±0.6% (3 000/L) and 29.3 % (5 000/L).

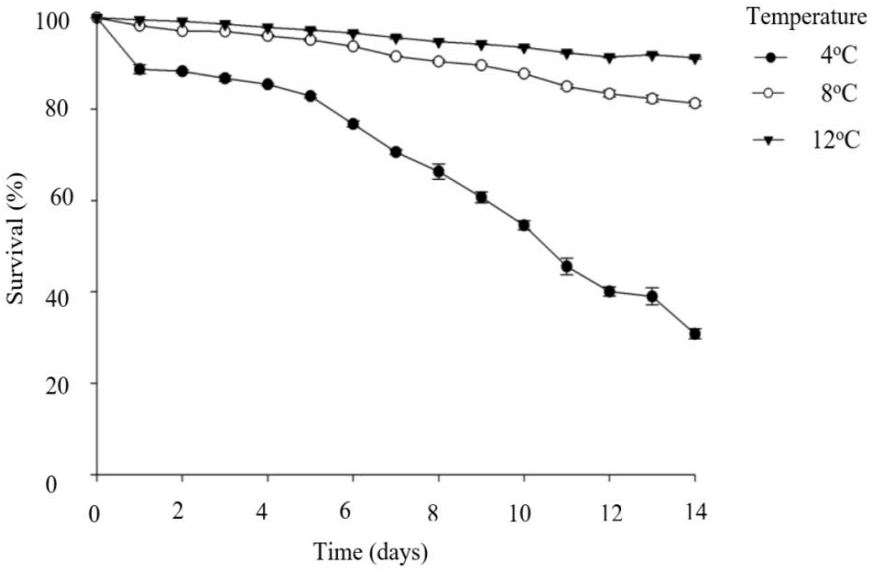

Figure 1 Percentage survival of *Thermocyclop* sp. with time at different temperatures

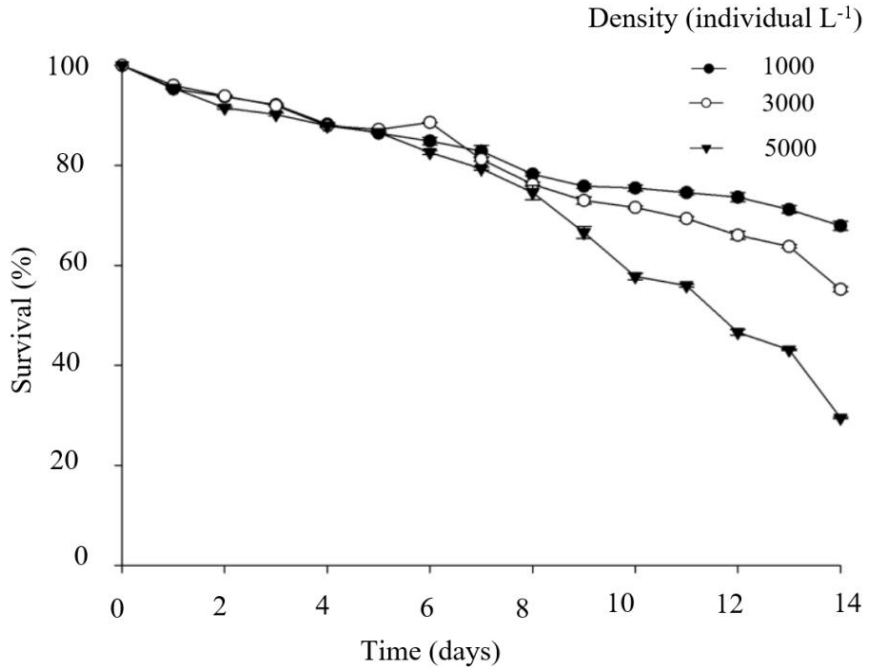

Figure 2 Percentage survival of *Thermocyclop* sp. with time at different storage densities

1.2 Fatty acid composition

Generally, the fatty acid (FA) composition was dominated by saturated fatty acids (SFAs) followed by monounsaturated fatty acids (MUFAs) while Polyunsaturated fatty acids (PUFAs) were the least dominant (Table 1). At 4 °C, SFAs increased from the 7th day (59.00%) to the 14th day (65.66%), while a decrease in MUFAs was observed from the 7th (32.99%) to the 14th day (25.8%) of the experiment. At 12 °C, total SFAs were highest on the 14th day (61.08%) compared to MUFAs and PUFAs at 33.31 ± 6.71% and 5.61 ± 1.62% respectively (Table 1). Amongst the SFAs at 4 °C, Palmitic acid (C16:0) and Stearic acid (18:0) were dominating. The highest composition of MUFAs was observed at 8 °C on the 14th day (34.71 ± 8.96) with Palmitoleic acid (16:1n7), Oleic acid (18:1n9), and vaccenic acid (18:1n7) dominating.

Table 1 Relative proportions of fatty acids (% of total FAs; mean ± SE, n=3) of the *Thermocyclop* sp. were stored at varying temperatures: 4, 8, and 12 °C after the 7th and 14th day of the experiment

Fatty Acids	4 °C		8 °C		12 °C	
	Day 7	Day 14	Day 7	Day 14	Day 7	Day 14
14:0	2.43±0.32	2.07±0.69	1.84±0.40	1.22±0.52	3.80±1.32	2.39±1.15
16:0	38.48±0.89	41.01±4.18	26.39±0.56	35.26±3.54	37.45±3.75	39.16±2.75
18:0	15.49±0.57	20.31±2.89	9.31±0.15	14.72±1.18	12.23±0.43	18.49±2.78
20:0	1.41±0.11	nd	0.61±0.14	nd	0.28±0.05	nd
22:00	0.47±0.13	0.95±0.18	nd	0.71±0.13	0.29±0.04	1.04±0.07
24:0	0.73±0.14	1.33±0.23	nd	nd	nd	nd
Total SFAs	59.00±2.16	65.66±8.18	38.15±1.25	51.92±5.37	54.04±5.58	61.08±6.76
14:1n5	2.11±0.75	2.98±1.91	0.74±0.24	0.91±0.27	0.88±0.17	1.81±1.27
16:1n7	8.20±0.09	5.61±1.20	9.72±0.24	7.18±1.75	9.40±1.47	6.79±1.67
18:1n9	5.07±0.56	5.35±0.28	11.45±0.71	7.44±2.22	5.65±0.58	5.18±0.81
18:1n7	4.94±0.24	6.48±0.93	7.63±0.00	6.84±2.15	5.35±0.24	6.12±1.11
20:1n9	0.67±0.07	nd	0.13±0.03	2.13±0.29	1.71±0.10	nd
22:1n9	0.41±0.14	0.73±0.06	nd	0.82±0.10	nd	1.39±0.12
24:1n9	1.60±0.21	2.92±0.72	nd	1.04±0.24	0.83±0.12	1.90±0.54
Total MUFAs	32.99±3.13	25.89±5.43	33.54±2.02	34.71±8.96	32.99±4.02	33.31±6.71
18:2n6	1.73±0.22	1.66±0.10	11.19±0.71	3.36±0.63	2.40±0.65	1.53±0.45
18:3n3	1.28±0.23	1.59±0.11	6.58±0.42	2.96±1.12	2.32±0.17	0.52±0.09
20:2n6	0.75±0.42	0.71±0.05	0.62±0.07	0.81±0.25	2.49±0.36	0.97±0.31
20:3n3	1.60±0.15	nd	2.53±0.28	0.76±0.07	nd	1.76±0.15
20:4n6	1.32±0.28	2.60±0.33	nd	2.40±0.15	3.43±0.61	nd
20:5n3	0.61±0.07	1.01±0.01	4.54±0.53	1.35±0.29	1.25±0.37	0.83±0.61
22:6n3	0.71±0.12	0.88±0.16	3.48±0.21	1.73±0.71	1.08±0.15	nd
Total PUFAs	8.01±1.48	8.45±0.76	28.95±2.21	13.38±3.21	12.97±2.31	5.61±1.62

Notes: Not detected (nd), Saturated fatty acids (SFAs), Monounsaturated fatty acids (MUFAs), and Polyunsaturated fatty acids (PUFAs)

The total PUFAs were highest on the 7th day at 8 °C (28.95 ± 2.21%). Among the total FAs, Linoleic acid (18:2n6) and Linolenic acid (18:3n3) were dominating. Notably, Arachidonic acid (20:4n6) and Docosahexaenoic acid (22:6n3) were not detected at 12 °C on the 14th day. Amongst the essential fatty acids: Arachidonic acid (AA; 20:4n6), Linoleic acid (LA;18:2n6), Linolenic acid (LNA;18:3n3), Eicosapentaenoic acid (EPA; 20:5n3) and Docosahexaenoic acid (DHA; 22:6n3), no significant difference was observed across the storage temperatures (P = 0.81) on the 7th and 14th day (P = 0.87). Additionally, there was no significant interaction effect of temperature and day on fatty acid composition (P = 0.88).

2 Discussion

Successful viable storage and packaging of live *Thermocyclop* sp. is an essential requirement for their utilization as alternatives to the presently used Artemia in fish hatcheries. Live feeds provide a more nutritionally rich food and one that can be utilized by small-mouthed fishes/ larvae. However, use of the live feeds is limited by many challenges in defining appropriate cultural and storage conditions. Therefore, the present study investigated the effect of storage conditions on the survival and fatty acids of Cyclopoid-copepods as a starter diet for the African catfish larvae.

2.1 Effects of storage temperatures on *Thermocyclop* sp.

Generally, there was a gradual decrease in the survival of *Thermocyclop* sp. with time at different temperatures. This decrease could be due to starvation since these organisms were not fed throughout the experimental period (Koussoroplis et al., 2014; Hansen et al., 2020). Starvation of organisms lowers the amount of energy required to keep them active, and this results in death (Hansen et al., 2020). Starvation in copepods results in more than 50% loss in body weight and a decrease in Polyunsaturated Fatty Acids, leading to death (Koussoroplis et al., 2014).

However, *Eudiaptomus gracilis* and *Calanus* sp. are able to regulate the fatty composition of their cell membranes to slow down metabolism and prolong their survival with time (Titocci and Fink, 2022) and this aligns with the current findings where more than 50% survival was observed after the 10th day of the experiment for all temperatures.

The significantly higher survival rate of the copepods at 12 °C and 8 °C could be attributed to the relatively higher temperatures that increase the enzymatic activity of the organisms (Koussoroplis et al., 2014; Bai and Wang, 2020). Additionally, moderate temperatures (6 °C~15 °C) increase the survival of Thermocyclop sp. since the energy reserve depletion related to oxygen consumption is decreased (Werbrouck et al., 2017). This survival pattern has also been seen in adult *Calanoid* sp. (Devreker et al., 2009) with higher survival at 10 °C compared to lower temperatures of 3 °C. This suggests that the survival of copepods rises with temperature until reaching a maximum threshold, beyond which survival decreases with further temperature increases (Van Dinh et al., 2019).

Thermocyclop sp. stored at 4 °C in the current study experienced higher mortalities than other temperatures. The decreased survival rate of *Thermocyclop* sp. at 4 °C may be associated with the impairment of their enzymatic activity, leading to mortality (Payne and Rippingale, 2001; Werbrouck et al., 2017). Temperature can directly affect the activity of enzymes by changing their physical structure and thereby changing catalytic efficiency (Cailleaud et al., 2007; Svetlichny et al., 2022). Moreover, various studies have found that different species of estuarine copepods reduce their metabolism to a minimum to sustain physiological activity in low temperatures and conserve energy in high temperatures (Koussoroplis et al., 2014; Werbrouck et al., 2017). These observations are consistent with the results obtained in the current study. Studies by Frisch and Santer (2004) observed higher mortality of *Cyclops strenuus* at 5 °C, and this is in line with the current findings where higher mortality was recorded at 4 °C. Therefore, the results of the present study demonstrate that the *Thermocyclop* sp. can be collected daily from the tank culture units. Copepods can be stored at 8 °C or 12 °C, and more than 50% of the copepods would be available after 10 days post-harvesting for use as a live starter feed for fish larvae.

2.2 Effect of different densities on survival of *Thermocyclop* sp.

The density of the copepod population can significantly impact their growth, survival, development, and fecundity (Frisch and Santer, 2004). The percentage survival of *Thermocyclop* sp. increased with a decrease in packaging densities. The lowest survival of the *Thermocyclop* sp. at 5000 individuals/L could be attributed to density-dependent mortality. High copepod packaging densities result in many types of stressors including limited food resources, oxygen depletion, accumulation of metabolic products, and physical interaction with other individuals (Jepsen et al., 2015; Rajkumar and Rahman, 2016; Punnarak et al., 2017). Therefore, the accumulation of such higher metabolic wastes and low oxygen levels could have resulted in higher mortalities at 5000 individuals/L. Densities ranging from 50 to 1000 mature Calanoid *Acartia tonsa* /L have shown little or no negative effects on the Cyclopoid copepods (Jepsen et al., 2015).

Higher density of adult copepods up to 6000 individuals/L has shown negative effects like higher mortalities as well as cannibalism for each other (Drillet et al., 2015; Franco et al., 2017). Different studies show that several omnivorous copepods become carnivores when phytoplankton concentration decreases in the culture medium (Drillet et al., 2015; Punnarak et al., 2017; Franco et al., 2017; Heneghan et al., 2023). In the current study, the copepods were starved throughout the experiment, which may have elicited cannibalism thus reducing the number of *Thermocyclop* sp. at higher densities. Similar rates of survival (67%) at 1000 individuals/L were reported for *Acartia tonsa* (Drillet et al., 2015). This confirms that it is possible to store copepods at 1,000 Individuals/L while keeping low mortality for 14 days without feeding and achieving a more than 50% survival rate.

2.3 Impacts of temperature and density on fatty acid profiles of *Thermocyclop* sp.

The general decrease of FA content with time in the present study could be due to the degradation of the cell organelles of the *Thermocyclop* sp. (Mäkinen et al., 2017; Werbrouck et al., 2017). In degradation processes, these organelles can undergo breakdown, releasing stored fatty acids into the cellular environment, thereby causing a decrease in the overall fatty acid levels (Meyers et al., 2019). Previous studies in copepods have shown an

increase in the number of intracellular membranes under cold conditions. This is believed to be a compensatory mechanism that reduces the diffusion path length of metabolites, thereby counteracting the reduced diffusivity constants (Mäkinen et al., 2017; Werbrouck et al., 2017). Ectotherms maintain physiological functions by restructuring the lipid composition of biological membranes when temperatures keep changing (Martin-Creuzburg et al., 2012;). When copepods are exposed to both starvation and heat, it is observed that their storage fatty acid (FA) pool loses its buffering function, leading to increased mortality rates (Werbrouck et al., 2017). The fatty acid profile of *Thermocyclop* sp. in the present study was dominated by SFAs and MUFAs with lower concentrations of PUFAs at different storage temperatures. Similar studies (McKinnon et al., 2015) observed that the FA composition of *Calanoid* copepods *Bestiolina similis* and *Parvocalanus crassirostris* were dominated by SFAs (16:0, 18:0, 14:0) and MUFAs (22:1, 20:1) and that the EFA, DHA, and EPA were present in smaller proportions and lipid peroxidation was identified as the main cause of this phenomenon (Camus et al., 2021). Among the various classes of fatty acids (FAs), polyunsaturated fatty acids (PUFAs) are particularly susceptible to lipid peroxidation. Once lipid peroxide radicals are formed, they can initiate an autocatalytic chain reaction of lipid peroxidation (Gladyshev et al., 2015; McKinnon et al., 2015). Additionally, the short-term storage minus feeding at 12 °C reduced SFA composition compared to 4 °C. Cold temperatures are also thought to increase the membrane lipid order but can be compensated by first and second cis-double bond insertions in the fatty composition of the cyclopoid membrane. Therefore, this might explain the higher levels of SFAs in the copepods under 4 °C in this study (Werbrouck et al., 2017). The composition of palmitic acid remained higher than other SFAs across all temperatures. As such, the storage of copepods at the study temperatures might have not compromised palmitic acid levels which is a key metabolite in fish growth (Osibona et al., 2006). Furthermore, the higher composition of PUFAs observed in this study at 8 °C and 12 °C storage temperatures demonstrate the viability of storing the copepods at these temperatures. Similar results were observed in copepod *Eudiaptomus gracilis*, with higher levels of PUFAs especially EPA and DHA (Koussoroplis et al., 2014). It is imperative to note that essential fatty acids like EPA and DHA in copepods form a cornerstone of the nutritional requirements for larval fish. The presence of these essential nutrients in copepod diets directly influences larval development, health, immune function, and overall success in both natural ecosystems and aquaculture settings. Besides these fatty acids are responsible for maintaining copepods' membrane fluidity (Gladyshev et al., 2015).

2.4 Implications of the study

The study offers a pathway towards optimizing copepod-based feeding strategies, a critical component in aquaculture. Given the essential role of copepods in the diets of fish larvae, understanding how storage conditions influence their fatty acid profiles allows fish farmers to make informed decisions. This optimization can result in healthier and faster-growing fish larvae, contributing to the success of aquaculture operations. By implementing optimal storage conditions for *Thermocyclop* sp., aquaculture operations align with the broader goals of sustainable aquaculture. The efficiency gained in live feed management not only improves economic viability but also reduces environmental impact and promotes the responsible use of resources, contributing to the long-term sustainability of the industry. Economically, implementing optimal storage conditions for copepods can have positive implications for aquaculture operations. Improved survival rates and enhanced nutritional quality may translate into more efficient and cost-effective practices, positively impacting the economic viability of the aquaculture industry.

3 Materials and Methods

3.1 Culture of Micro-algae

The culture of micro-algae was achieved following the protocol from (Izaara et al., 2020). Initial stock of the micro-algae was sourced from Sewerage treatment lagoons of the National Water and Sewerage Corporation (NWSC)-Entebbe. Serial dilution followed by multiple sub-cultures using Bold's Basal Medium (BBM) was used to achieve a pure stock of the micro-algae as modified by Izaara et al. (2020). Rectangular glass tanks (25 L) were utilized for the culture of the micro-algae. To facilitate maximum exposure of the micro-algae to light for better distribution and also keep the algae in constant circulation, continuous aeration through perforated air stones was provided. The micro-algae were raised on a combination of Diammonium phosphate (DAP) (15 g/L) and Urea (15

g/L) dissolved in 1.0 liter of de-chlorinated tap water. Algal culture conditions for the laboratory were maintained; temperature (25 ± 1.5) °C and pH were maintained at a range similar to that of the collection source (6.5 ~ 8.0). The cultures were also supplied with 24-hour constant lighting using a single 40 W (daylight) fluorescent tube (equivalent to 1 000 Lux). Counts of cells/mL were taken using a magnification of x200 on a Hund Wilovert Standard pH 20 inverted microscope (WILOVERT® series) and a Sedgwick-Rafter Cell counting chamber.The feeders allowed for an increase in the number of daily meals and for the extension of the feeding time throughout the day, but the rations must be fixed by the farmer, except when a demand feeder is used, as shrimp can then regulate their feed intake. Jescovitch et al. (2018) and Ullman et al. (2019b) reported a higher feed input with the sound feeder than manual feeding (181% and 171%, respectively), but feed input with the time feeder with respect to manual feeding was lower (112% and 118%, respectively), which could explain the high growth with an optimal FCR observed in sound feeding. In the study of Reis et al. (2020) the increment of 60% rations using a time feeder improved the yield, but it was lower than yield using sound feeder, due probably to feed input being higher with sound feeder, around 1 100 kg/ha more.

3.2 Copepod culture and estimation of survival

Mass cultures of freshwater *Thermocyclop* sp. in the laboratory were maintained on a diet of freshwater microalgae (*Chlorella* sp.) as adopted from Chepkwemoi et al. (2013). The initial stock of the live *Thermocyclop* sp. was sourced from the Umoja fish farm (0°24'59" N, 32°23'00" E) and then acclimatized to the laboratory conditions; room temperatures, and continuous lighting. In the laboratory, the *Thermocyclop sp.* were screened to isolate the size fraction containing predominantly adult cyclopoids and later-stage copepodites; this was achieved by coarse screening through 45, 100 and 200 µm mesh size planktons. Finally, uncontaminated cultures of *Thermocyclop* sp. were achieved using sucker pipettes (Huawei® PIPETTE H100) of 10~30-micron (µ) aperture, coupled with a Hund Wilovert Standard pH 20 inverted microscope (WILOVERT® series) set at a magnification of x40 to pick out adult individuals. The collected samples were thereafter placed in a 100 mL Erlenmeyer flask, where *Chlorella* sp. was introduced. The uncontaminated *Thermocyclop* sp. was used as a stock culture by introducing 10~15 *Thermocyclop* sp. per mL in 10 L algae (*Chlorella* sp.) at a density (18.43±6.0)×104 cells/mL obtained by day 14 of the algae culture. Laboratory culture conditions for the copepods were maintained at the recommended levels. 40-watt electric lighting tubes were used for illumination. Constraints due to culture density were monitored to avoid decreased fecundity due to overcrowding; by upscaling when the density reached 10 individuals/L. The density of *Thermocyclop* sp. (individuals/L) was determined at a magnification of x100 using a Hund Wilovert Standard pH 20 inverted microscope (WILOVERT® series) on a Sedgwick-Rafter Cell counting chamber to guide the packaging densities utilized in this study.

3.3 Experimental setup

Two sets of experiments (1 and 2) were run in series using the experimental facilities at the Department of Botany, Makerere University (0°20'09" N, 32° 33'56" E) with requisite storage conditions.

Experiment 1 focused on determining the effect of packaging density on the survival of the *Thermocyclop* sp. at a fixed temperature (12 °C). The experiment comprised a total of 42 one-liter glass jars for each storage density (N=42). The storage densities were varied at 1000, 3000, and 5000 individuals/L, and the glass jars were placed in refrigerators set at 12 °C at the Department of Botany, Makerere University. The density variations were chosen based on previous studies and species characteristics, which indicated that copepods can survive for at least 20 days at a density of 500 individuals per liter and recommended that under controlled conditions, the stocking density can be increased (Drillet et al., 2015; Franco et al., 2017; Punnarak et al., 2017). Micro-algae (18.43±6.0×104 cells/mL) with dissolved Oxygen levels (4.0±1.0 mg/L) were added to each storage bottle and sealed. The three treatments were replicated in a Complete Block Design and the experiment was run for 14 days. To monitor survival, three one-liter jars of each treatment were enumerated daily (9 total daily) to establish the percentage of live or dead copepods in the bottle.

Experiment 2 determined the effect of varying storage temperature on survival at a constant storage density for 14 days. The varying temperature settings were (4, 8 and 12 °C) with each treatment comprising 42 glass jars (N=42).

These temperature variations were chosen based on previous studies indicating that copepods can still survive at moderate temperatures (4 °C~15 °C) (Mäkinen et al., 2017; Werbrouck et al., 2017). The copepod density in all jars was 1 000 individuals/L. The jars were distributed in three separate fridges, each set at the target temperature. Each treatment had a total of three replicates and N=42.

3.4 Estimation of survival of *Thermocyclop* sp.

The culture media from each specimen bottle was stained using intra-vitam staining with neutral red stain, a method for separating live and dead copepods in natural samples. Live *Thermocyclop* sp. stain red by filtering and ingesting the dye, whereas dead copepods remain unstained as they don't ingest the dye (Drillet et al., 2015). Survival was estimated by computing the proportion of red-stained copepods (live) against the initial storage density and temperature in the preservation bottles over a 14 - day experimental cycle. When using intra-vitam staining with neutral red stain for separating live and dead copepods in natural samples, it's essential to take certain precautions to ensure the reliability and accuracy of the results. For example, it ensures the quality and freshness of the neutral red stain as it can degrade over time, potentially leading to variations in staining intensity and affecting the accuracy of live/dead differentiation.

3.5 Fatty acid composition

The fatty acid composition of the Cycloid copepods stored at 4, 8 and 12 °C was also analyzed. *Thermocyclop* sp. were collected on the 7th and 14th days of the experiment and stored at -80 °C for further analysis. Before extraction, individual *Thermocyclop* sp. were removed from the deep freezer (-80 °C) and thawed for at least one hour under standard laboratory conditions. Lipid extraction followed a protocol by (Evjemo et al., 2003). 0.5 ±0.1 g of each sample was weighed and then ground to get a homogeneous powder. It was then used for extraction with chloroform–methanol mixture (2:1, v/v). Each sample was then filtered and transferred into a separating funnel and added to a 0.2 volume of 0.9% sodium chloride. The samples were shaken to allow two distinct layers to separate; the lower chloroform phase was transferred into a pre-weighed tube and then evaporated using a stream of nitrogen gas until a constant weight of the lipid was achieved.

The fatty acid composition was determined using gas chromatography–mass spectrometry analysis following (Kwetegyeka et al., 2011). Approximately 10 mg of the lipid extract was transferred into a reaction vial containing an internal standard, nano decanoic acid (C19:0), and 1.0 mL of acidified methanol. The vials were securely closed with Teflon-lined screw caps and placed in an oven for two hours at 90 °C to allow complete methanolysis. Finally, the vials were removed from the oven and then allowed to cool at room temperature. Subsequently, methanol was evaporated to half its original volume by a stream of nitrogen to make methyl esters less soluble in the methanol phase. Then, 0.5 mL distilled water, followed by 1 mL hexane, was added to the methanolized lipid fraction. The tubes were capped tightly and shaken at least 4 times to allow mixing, followed by centrifugation to separate the phases. The upper hexane layer containing fatty acid methyl esters (FAMEs) was carefully transferred to the vial using the pipette. The water–methanol phase was extracted twice using 1.0 mL n-hexane.

The extracts were pooled and stored under refrigeration, awaiting GC–MS analysis. The samples were then quantitatively analyzed using GC–MS equipment (Agilent 6890-version N.05.05, GC-System, 5301 Stevens Creek Blvd, Santa Clara, CA 95051, USA) fitted with an electronic pressure control and mass selective detection (ionizing energy, 70 eV; source temperature, 250 °C). The injector temperature was set at 260 °C while the detector was set at 330 °C. The oven program was set at 90 °C for 4 min, 30 °C/min to 165 °C, then 3 °C/min to 225 °C where it was left to isothermal for 10.5 minutes before cooling for the next run. Fatty acids were quantitatively identified in the samples by means of the standard mixture and mass spectrometry, quantified using internal standard C19:0. The peaks were further integrated using Chemstation software (Thermo Lab Systems), and the contribution of each of the fatty acids was calculated based on their relative retention times and peak areas. The relative amount of each common fatty acid is expressed as a percentage of the total fatty acids.

3.6 Data analysis

The R statistical software (R Core Team, 2020) was used for data analysis. A log-rank test was used to compare the differences between the survival curves at different temperatures and densities. Comparisons were done for the time it took to attain 50% mortality. Regression analysis on log-transformed percent composition of essential fatty acids was performed to compare the differences between storage temperature and days.

4 Conclusion and Recommendation

The survival of the *Thermocyclop* sp. decreased with time and changes in temperatures. More than 50% survival rate can be achieved for copepods stored at 8 °C and 12 °C 10 days after harvesting and storage. Thus, the organisms can be harvested, stored, and used to start new cultures with minimal costs of isolating and identifying a pure stock from the wild. Although the survival of *Thermocyclop* sp. is inversely proportional to the storage densities, the present study results showed that it's possible to store copepods at 1000 individuals/L, with low mortality, for 14 days without feeding and achieve more than 50% survival rate. It is necessary to upscale such densities into the outdoor systems to maximize the production of the copepods while considering the quality of micro algae, water quality and aeration systems. The long-term exposure of *Thermocyclop* sp. to cold temperatures causes a significant increase in EPA. The HUFAs, such as AA and EPA, are highly conserved during starvation. This means the *Thermocyclop* sp. can be stored for future use, with no loss of their nutritional quality.

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