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Species Identification and Optimizing Salinity to Enhance Reproduction and Life Cycle in *Amphipoda* sp.

Mohamad Fadjar[1*], Sri Andayani[1], Cucun Herlina[1], Fariana Banyu Kartika[2], Riva Dianto[1], Fadila Nur Aziza[1], Josia Fernando Sihombing[1], Cyntia A. W. Lumban Batu[1], Luki Kristinawati[1], Hafizh Wijdan Wadha[1]

[1]Aquaculture Study Program, Department of Water Resources Management and Fisheries Science, Faculty of Fisheries and Marine Science, University of Brawijaya. Jl Veteran-Ketawanggede, Malang, East Java,Tel./Fax **(**0341**)** 553512

[2]Fisheries Training and Extension Center of Banyuwangi, Jl. Raya Situbondo No. Km 17, Bangsring, Banyuwangi, East Java, Tel/Fax **(**0333**)** 510688

[*]**Corresponding Author: f4dj4r_02@ub.ac.id**

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ABSTRACT

The sustainable production of high-quality live feed is essential for the success of modern aquaculture. Amphipods, particularly *Amphipoda* sp., offer considerable potential due to their high nutritional value, small body size, and adaptability to diverse aquatic environments. However, the availability of consistent cultured amphipod populations remains limited, largely due to reliance on wild-caught individuals and suboptimal rearing protocols. This study aimed to assess the effects of salinity on the reproduction, life cycle synchronization, and nutritional profile of *Amphipoda* sp. to support the development of standardized cultivation practices. Experimental treatments were conducted using three salinity levels (25, 30, and 35ppt) under controlled conditions. DNA barcoding confirmed the identity of *Amphipoda* sp. with 84.16% similarity to existing sequences in the NCBI database. Reproductive performance peaked at 30ppt with a production of 510 eggs/day, while lower and higher salinity levels led to reduced fecundity. Life cycle observations revealed clear morphological differentiation between juvenile and adult stages, including sexual dimorphism. Amino acid profiling through LC-MS/MS indicated that samples from Situbondo had higher concentrations of L-alanine, L-glutamic acid, glycine, L-isoleucine, and L-leucine compared to samples from Jepara, supporting their superior nutritional potential. These findings highlight the critical role of salinity in optimizing reproductive output and feed quality in *Amphipoda* sp., emphasizing the need for precise environmental management.

INTRODUCTION

The use of amphipod-based natural feed in aquaculture, particularly in Indonesia, remains limited due to continued dependence on wild-captured populations **(Yusuf *et al.*, 2020)**. This reliance poses sustainability challenges, especially in ensuring a stable and scalable supply of live feed. As aquaculture continues to expand, the availability of high-

quality natural feed has become an essential factor in optimizing larval development and overall culture success **(Nailulmuna *et al.*, 2020)**. Among amphipod species, *Amphipoda* sp. has shown significant potential due to its high nutritional value, small body size appropriate for larval fish and shrimp, and suitability for mass cultivation **(Pangestika *et al.*, 2020)**. Empirical studies support its efficacy as a live feed, showing superior growth rates in *Litopenaeus vannamei* fed with *Phronima* sp. compared to those fed with *Daphnia magna* **(Ramadhani *et al.*, 2017; Ratri *et al.*, 2020)**. The biological characteristics of *Phronima* sp., particularly the dominant role of females in egg protection and larval care, underscore its reproductive significance. As euryhaline organisms, amphipods exhibit high adaptability across diverse aquatic environments, tolerating a broad salinity range of 15–34 PSU **(Nailulmuna *et al.*, 2020)**. However, despite their adaptability, reproductive success is highly dependent on the optimization of environmental parameters, particularly salinity.

Salinity is a critical ecological variable influencing osmoregulation, metabolism, and reproductive dynamics in aquatic invertebrates, including amphipods **(Hirose *et al.*, 2005)**. Variations in salinity beyond optimal thresholds can disrupt the physiological balance of female amphipods, leading to reduced fecundity, lower egg viability, and ultimately diminished larval survival **(Rojano *et al.*, 2013; Seale *et al.*, 2024)**. For *Amphipoda* sp., reproductive performance is typically optimized within a salinity range of 15 to 30 PSU, though this may vary depending on developmental stage. Larvae are particularly vulnerable to salinity fluctuations and often require more stable conditions between 15–25 PSU to ensure successful development **(Nour *et al.*, 2021)**. Elevated salinity has been associated with delayed metamorphosis, reduced hatching success, and compromised early ontogeny in other aquatic organisms, such as *Artemia* and *Anopheles* spp. **(Nwaefuna *et al.*, 2019; Lyubomirova *et al.*, 2023)**. Moreover, salinity interacts synergistically with other abiotic factors, such as temperature and dissolved oxygen, which collectively modulate energy balance and reproductive output **(Chen *et al.*, 2022; Fan *et al.*, 2023)**. Under conditions of salinity-induced osmotic stress, amphipods divert substantial portions of metabolic energy toward maintaining homeostasis, often at the expense of gamete production **(Root & Kültz, 2023)**. This reallocation may result in lower reproductive efficiency, as supported by studies in crustaceans demonstrating trade-offs between survival and fecundity under environmental stress **(Shahba *et al.*, 2022)**. In extreme cases, prolonged stress can induce shifts in population sex ratios, skewing toward males due to higher female mortality, thereby threatening population sustainability **(Chelyadina *et al.*, 2021)**.

Recent research has suggested that the deliberate manipulation of salinity in controlled environments may serve as a biotechnological intervention to enhance reproductive efficiency and synchronize life cycles in amphipods **(Ávila-García *et al.*, 2023)**. Stable salinity conditions within the optimal range have been shown to reduce stress, improve egg quality, increase ovigerous rates, and support juvenile survival **(Chiba *et al.*, 2023)**.

Furthermore, by minimizing metabolic strain and stabilizing reproductive physiology, optimal salinity regimes can facilitate more balanced population structures, particularly with respect to maintaining higher proportions of reproductive females **(Ávila-García *et al.*, 2023)**. These conditions contribute to enhanced reproductive stability, which is crucial for consistent harvesting and the efficient supply of amphipods as live feed in aquaculture. Beyond reproductive metrics, synchronization of the reproductive cycle is essential for improving hatchery operations, especially in aligning egg harvesting and juvenile production with feeding schedules for other cultured species **(Dashinov & Uzunova, 2021)**. This study aimed to analyze the effects of salinity on the life cycle and reproductive performance of *Amphipoda* sp., as well as identifying the species accurately through DNA barcoding to support the development of optimized aquaculture protocols.

MATERIALS AND METHODS

Experimental design

The research was conducted using nine aquaria measuring 60 × 30 × 35cm as rearing media for *Amphipoda* sp. Each aquarium was filled with 15 liters of seawater sourced from the Bali Strait, obtained from the Marine and Fisheries Training and Extension Center (BPPP) Banyuwangi. The seawater, with a salinity of 35ppt, had been settled and sterilized using chlorine to reduce pathogenic microbial loads. *Amphipoda* sp. were sourced from the Brackishwater Aquaculture Development Center (BPBAP) in Situbondo, East Java. The animals were stocked at a density of 3 individuals per liter, totaling 60 individuals per aquarium in 3 weeks (21 days) with acclimations. Stocking was carried out in the morning to minimize stress due to high temperatures. Prior to stocking, *Amphipoda* sp. were acclimated to the environment by floating plastic bags containing the organisms in the aquaria for three days. Salinity acclimatization was followed by gradually adding aquarium water into the bags over 15 minutes until the animals exited the bags voluntarily. Three salinity treatments were applied (25, 30, and 35ppt), with adjustments made gradually in 2– 5ppt increments by mixing seawater and freshwater to achieve the target levels. Digital probes were used to detect water quality parameters such as temperature, pH and DO dissolved oxygen (DO) every day. Red Sea test kits were used to track ammonia (NH_3), nitrite (NO_2^-), and nitrate (NO_3^-) were monitored using Red Sea test kits.

Species identification

DNA isolation was performed using the gSYNC™ DNA Extraction Kit (Geneaid, GS300) following the manufacturer's protocol, and the purified shipworm DNA was subsequently amplified using the primers 18S_E18F (5'-GATCCMGGTTGATYCTGCC-3') and 18S_E1772R (5'-CWDCBGCAGGTTCACCTAC-3') with a polymerase chain reaction (PCR) mixture consisting of 1µl of DNA template, 1µl of primer 18S_E18F, 1µl of primer 18S_E1772R,

9.5µl of double-distilled water (ddH2O), and 12.5µl of MyTaq Red Mix, 2x (Bioline, BIO-25048). The amplification process involved initial denaturation at 95ºC for 3 minutes, followed by 35 cycles of denaturation at 95ºC for 15 seconds, annealing at 55ºC for 30 seconds, and extension at 72ºC for 45 seconds, with a final extension at 72ºC for 35 cycles. The PCR products were then analyzed using 1% agarose gel electrophoresis, and if the target band was detected, the samples were sent to First Base Laboratories, Malaysia, for sequencing. The sequencing chromatogram data were edited and analyzed for similarity using the Basic Local Alignment Search Tool (BLAST), with the sequencing results interpreted using the Sequencer Scanner V1 (ABI). Sequence reads from both forward and reverse primers were assembled into contigs using MEGA 11 software, and the contig sequences were subjected to BLAST analysis by uploading the nucleotide sequences to the platforms at https://blast.ncbi.nlm.nih.gov/Blast.cgi and http://blast.ddbj.nig.ac.jp/ to assess the similarity of the samples against available databases. Several sequence data sets from the databases were selected as references for comparison with the sample sequence data, and the DNA sequences were formatted using a text editor, saved in FASTA format, and prepared for subsequent analyses.

LCMS/MS analysis

Sample preparation for amino acid analysis began with the preparation of 6N HCl and 4N NaOH. The 6N HCl was prepared by adding 37% HCl to a 50mL volumetric flask containing water for LC, then filling to the mark. The 4N NaOH solution was made by dissolving 8g of NaOH in water to a final volume of 50mL. A 0.5g sample was mixed with 10mL each of 6N HCl and 4N NaOH, followed by hydrolysis using a Microwave Digestion System at 195°C for 30 minutes. The hydrolysate was filtered, and the filtrate was neutralized to pH 7, then diluted (dilution factor = 50) using 0.1N HCl. After passing through a 0.22µm syringe filter, the diluted samples was prepared for analysis by LC-MS/MS. Amino acid standard preparation involved preparing a 2.5 µmol/mL AA mix, followed by a 100 nmol/mL stock solution. Serial dilutions were made at 0.05, 0.1, 1, 10, 50, and 100 nmol/mL. The LC-MS/MS method used a mobile phase of 0.3% formic acid in acetonitrile (A) and acetonitrile :100 mM ammonium format (20:80) (B), with an Intrada Amino Acid column. MS parameters included a 400°C interface temperature, 650°C desolvation temperature, and gas flows of 3–15L/ min. High-resolution LCMS/MS analysis was conducted and modified according to established protocols using the Amino Acid Analysis by Triple Quadrupole LCMS-8060RX (Shimadzu).

Reproduction rate

The calculation of the reproductive rate in female samples was conducted throughout the presence of eggs in the female sample ennclosure at the end of the maintenance period. The reproductive rate was determined by counting the number of surviving female *Amphipoda* sp. individuals and the total quantity of eggs at the end of

the maintenance period. The reproductive rate was calculated using the following formula:

$$R_0 = l_x \ x. \ m_x$$

Explanation:

R0 : Reproductive rate **(**eggs/day**)**

lx : Number of surviving female individuals **(**individuals**)**

mx : Number of eggs **(**units**)**

Data analysis

A completely randomized design (CRD) was used to analyze the survival rate, reproduction rate, and egg count data. Statistical tests were performed, such as a homogeneity test to evaluate whether the data showed uniform variance and a normality test to ascertain whether the data were normally distributed. Following that, SPSS version 26 was used to do an analysis of variance (ANOVA) on the generated data.

RESULTS

Species identification

The DNA extraction results yielded a concentration of 58.9ng/ µL with a purity ratio of A260/280 of 2.02. Subsequently, DNA barcoding identification was performed, revealing the species *Amphipoda* sp. (678 bp) (Fig. 1). The results were then subjected to a BLAST analysis in NCBI, with a percent identity of 84.16%.

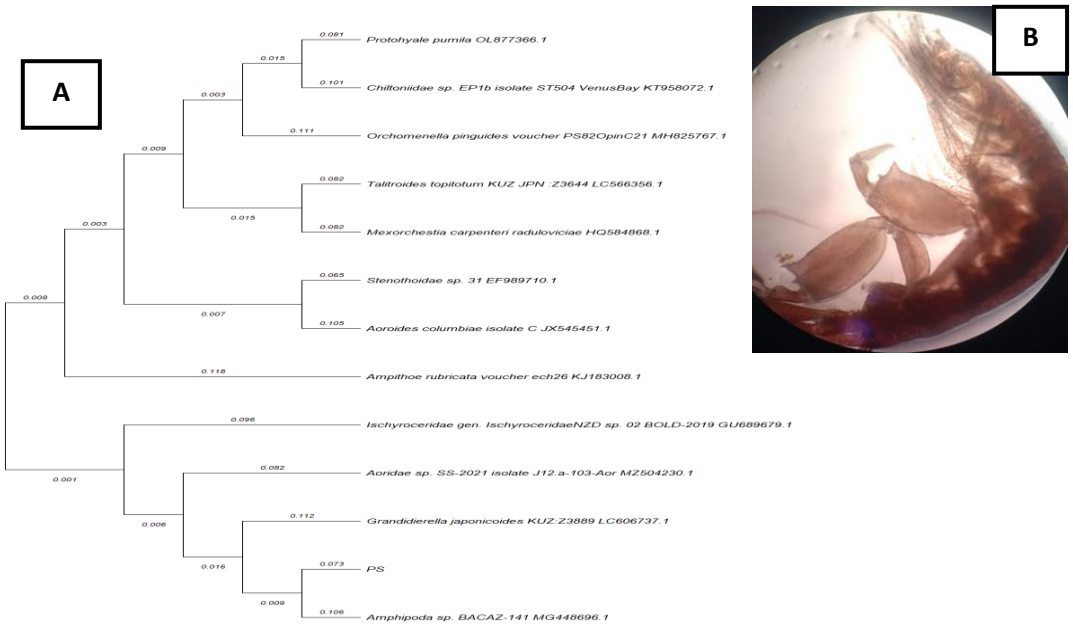

Fig. 1. Identification of *Amphipoda* sp. A**)** Phylogenetic Tree of *Amphipoda* sp. B**)** Mature Stage of *Amphipoda* sp.

Amino acid analyisis

The samples from BPPBAP Jepara and BPAP Situbondo were examined for amino acid profile. The highest variation in amino acids was found in the Situbondo sample, which contained L-alanine, L-glutamic acid, glycine, L-isoleucine, and L-leucine. In contrast, the Jepara sample only contained histidine, with other amino acid levels being below the detection threshold of the instrument, indicating that their content was very low (Table 1). The next step in the research is to culture the Situbondo sample based on the amino acid data.

Table 1. Amino acid profile of *Amphipoda* sp.

No	Amino Acid	Situbondo Sample (nmol/mL) (nmol/mL)	Jepara Sample (nmol/L)
1	L-Alanin	7,021	<0,05 **(**-10,108**)**
2	L-Arginine	N.D	<0,05 **(**-3,534**)**
3	L-Aspartic Acid	<0,05**(**-4,459**)**	N.D
4	L-Cystine	<0,05**(**-3,348**)**	<0,05 **(**-3,422**)**
5	L-Glutamic Acid	7,550	<0,05 **(**-4,955**)**
6	Glysine	5,464	N.D
7	L-Histidine	N.D	65,003
8	L-Isoleucine	1,488	N.D
9	L-Leucine	4,014	<0,05 **(**-3,245**)**
10	L-Lysine HCL	N.D	N.D
11	L-Methionine	<0,05 **(**-2,793**)**	N.D
12	L-Phenylalanine	<0,05 **(**1,533**)**	<0,05 **(**-3,131**)**
13	L-Proline	0,371	N.D
14	L-Serine	<0,05 **(**-2,501**)**	<0,05 **(**-4,480**)**
15	L-Threonine	<0,05 **(**-1,249**)**	<0,05 **(**3,453**)**
16	L-Valine	2,016	<0,05 **(**-4,705**)**
17	L-Tyrosine	<0,05 **(**3,655**)**	<0,05 **(**-3,956**)**
18	Tryptophan	N.D	N.D

Note: N.D (Not Detected).

Reproductive rate

The reproductive rate refers to the speed at which an organism produces offspring within a specific time period. *Amphipoda* sp. exhibits separate sexes, with individuals producing either male or female gametes. The highest reproductive rate was observed in treatment B with a salinity of 30ppt, reaching 510 eggs/day, followed by treatment C at 420 eggs/day, and treatment A 284 eggs/day (Fig. 2). The calculated F-value of 13.172 is greater than the 5% F-table value of 5.143 and also exceeds the 1% F-table value of 10.924, indicating a highly significant effect of different salinity levels on the reproductive rate of *Amphipoda* sp. Treatment B is significance than others. Salinity plays a crucial role in influencing various physiological aspects of female amphipods, including the organs and hormones involved in reproduction. Changes in salinity can affect reproductive organ function, such as the ovaries in female amphipods, and non-optimal

salinity levels may disrupt oogenesis (egg maturation), leading to reduced egg production and lower egg quality. This effect is likely due to osmotic stress, which influences energy metabolism and ion balance, both of which are essential for ovarian function. Additionally, the endocrine glands that regulate reproductive hormones, such as vitellogenin (a protein critical for yolk formation), may be disrupted by fluctuations in salinity. Since vitellogenin synthesis is influenced by environmental conditions, extreme salinity levels may suppress its production. Amphipods rely on specialized organs such as gills and antennal glands to maintain ion and water balance, and when environmental salinity is too high or too low, osmoregulation becomes impaired, causing energy to be diverted from reproduction to maintaining osmotic balance, ultimately reducing reproductive output.

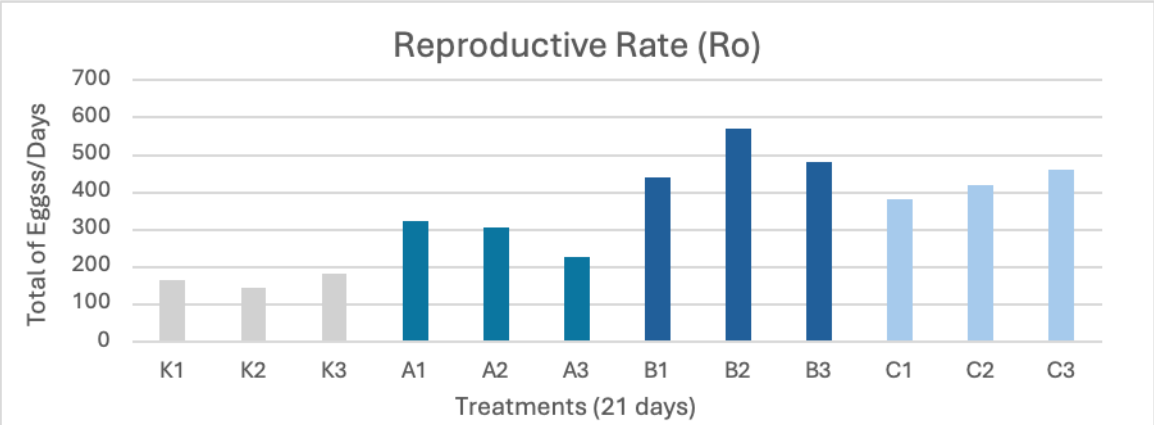

Fig. 2. Reproductive rate: The ideal salinity level for *Amphipoda* sp. during a 21-day period is 30 salinity that is utilized to gauge the success level of reproduction

Life cycle

In the juvenile phase, *Amphipoda* sp. undergoes size differentiation, indicating the transition from the juvenile to the adult stage (Fig. 3). One of the distinguishing features of this phase is the appearance of a whitish-black coloration on the dorsal side of the body. In the adult phase, *Amphipoda* sp. is significantly larger than in other stages, with a brownish-black coloration. This phase is characterized by the presence of well-developed gnathopods (claw-like appendages) in males, while in females, dark green to black granular egg-like structures become visible. Sexual dimorphism is evident in adult *Amphipoda* sp., where males are larger and possess more pronounced gnathopods compared to females. The dorsal coloration of adult *Amphipoda* sp. appears black.

Female *Amphipoda* sp. has a brood pouch located in the abdominal region that contains eggs, which are fertilized by the male. The inner middle surface of the coxa (segments 2–5) in adult females contains a brood plate structure known as the *marsupium* (oostegite), where the eggs are incubated. In young females, the brood plate appears as a simple protrusion or bud, which gradually elongates, enlarges, and develops coarse bristles as growth progresses. These bristles form a cradle-like structure that secures and

protects the fertilized eggs inside the brood pouch, where they continue to develop until hatching. This process is known as *demarsupiation*, wherein the female transfers the eggs into the marsupium and later releases the juveniles upon hatching. The presence of hatched juveniles inside the brood pouch is indicated by its reddish coloration, signifying that the female will soon release the juveniles.

The head, thorax, and abdomen are the three main body segments of the *Amphipod* sp. A pair of antennae oh the head act as sensory organs for identifying the surroundings and locating prey. Male amphipods, with their large eyes and strong swimming adaptations, are often attracted to dense swarms near nighttime light sources. The thorax comprises seven segments called *pereonites* and is supported by eight pairs of appendages. A distinctive feature of *Amphipoda* sp. is the presence of large gnathopods on the first thoracic segment, which are used for cutting and constructing their characteristic "barrel." Additionally, male *Amphipoda* sp. possesses a small penile funnel in the thoracic region, which appears too small for copulation but functions in the release of sperm or spermatophores for fertilization. A sexually mature male *Amphipoda* sp. is characterized by its larger size and well-developed, enlarged gnathopods.

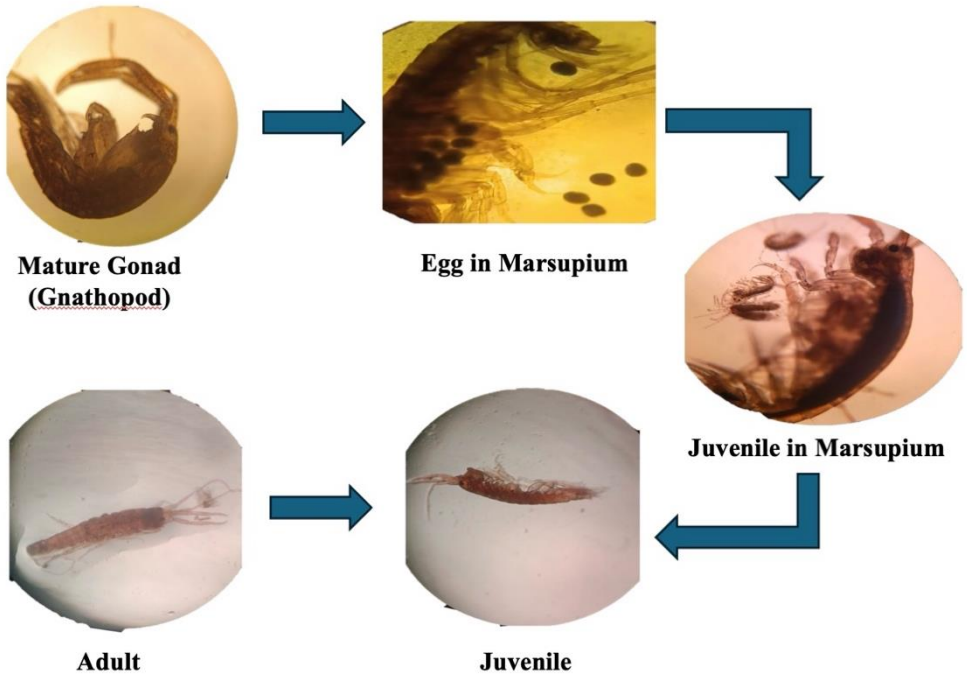

Fig. 3. Life cycle of *Amphipoda* sp.

Survival rate

The highest survival rate was observed in treatment B, with a value of 73% (Fig. 4). The phases in the research on the maintenance of *Amphipoda* sp. at different salinities consist of three phases: adaptation, exponential, and mortality. The adaptation phase is the process in which an organism responds to environmental changes that affect its physiological processes. During this phase, *Amphipoda* sp. adapts to the salinity

treatments added to each research container. The exponential phase occurs after the adaptation phase and is characterized by steady growth. The mortality phase is the final stage in the growth pattern, where the death rate exceeds the reproduction rate.

The survival rate of female *Amphipoda* sp. varied significantly across treatments, as indicated by a *P*-value of <0.05, with a significant value of 0.012. This demonstrates that the differences in feed treatments had a significant effect on the survival rate. The highest survival rate, at 73%, was observed in treatment B, followed by treatment C at 70%, and treatment A at 55%. The survival rate of female *Amphipoda* sp. showed values above 70%, indicating that the survival rate during maintenance was relatively good. This finding is consistent with the statement of **Renitasari *et al.* (2021)**, who noted that the optimal survival rate for crustacean or amphipod cultivation generally ranges from 70 to 90%. This survival rate is considered good for achieving maximum profit in cultivation activities.

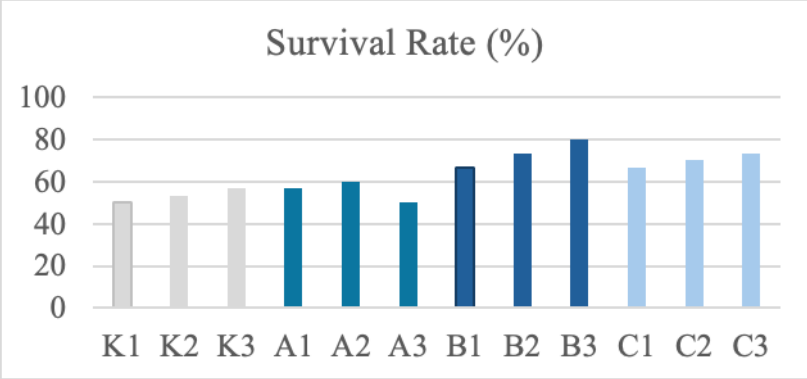

Fig. 4. Survival rate of *Amphipoda* sp.

Water quality analysis

During the maintenance of *Amphipoda* sp., the water quality parameters were controlled and maintained within normal limits, ensuring that they did not affect the growth and reproduction rates (Table 2).

Table 2. Water quality analysis

No	Parameter	Results	Range	Reference
1	Temperature	24,2 – 29,9ºC	15-25 ºC	**Lam-Gordillo *et al.*, (2023)**
2	pH	7,58 – 8,98	8,0 – 8,84	**Nailulmuna *et al.*, (2020)**
3	Dissolved Oxygen **(**DO**)**	5,01 – 5,56 mg/L	4,7 – 5,6 mg/L	**Fattah and Asbar (2015)**
4	Salinity	30 ppt	30 - 35 ppt	**Fattah and Asbar (2015)**
5	Ammonia NH3	0,2 – 0,8 mg/L	0,0978 – 1,705 mg/L	**Lam-Gordillo *et al.*, (2023)**
6	Nitrit **(**NO_{2-}**)**	0,05 – 0,2 mg/L	0,1 mg/L	**Pangestika *et al.*, 2020**
7	Nitrat **(**NO_{3-}**)**	0,05 – 0,2 mg/L	0,008 – 0,44 mg/L	**Pangestika *et al.*, 2020**

DISCUSSION

Molecular identification through DNA barcoding of *Amphipoda* sp. specimens revealed a DNA concentration of 58.9ng/ µL with an A260/280 purity ratio of 2.02 and a similarity level of 84.16% based on BLAST analysis that the specimen belongs to the *Amphipoda* taxonomic group. Morphological distinctions between juvenile and adult stages, including the development of gnathopods in males and the marsupium in females, support the evidence of sexual dimorphism as a marker of gonadal maturity. Amino acid profile analysis revealed that the sample from BPAP Situbondo exhibited a higher diversity of amino acids—such as L-alanine, L-glutamic acid, glycine, L-isoleucine, and L-leucine—compared to the Jepara sample, which was dominated by L-histidine with other amino acids falling below the instrument's detection threshold. This suggests an environmental influence on the nutritional quality of Amphipoda, which is critical in the context of live feed **(Vargas-Abúndez *et al.*, 2021; Hasnidar & Tamsil, 2023)**. These differences reinforce the importance of developing region-specific Amphipoda cultures, considering local environmental conditions.

Salinity has been shown to be a critical factor regulating the reproductive physiology of *Amphipoda* sp., particularly in vitellogenin production and oogenesis. The highest reproductive rate was observed at 30ppt salinity, reaching 510 eggs/day, indicating that this range provides an optimal osmotic condition for gonadal development, metabolic efficiency, and hormonal stability **(Rodríguez *et al.*, 2021; Silveyra *et al.*, 2022; Wang *et al.*, 2022)**. Both hypo- and hypersalinity conditions induce osmotic stress, which diverts energy away from reproduction toward homeostatic functions such as osmoregulation, and suppresses vitellogenin synthesis—an essential yolk precursor protein that affects egg quality and viability **(Su *et al.*, 2019; Silveyra *et al.*, 2022)**. Additionally, salinity fluctuations may interfere with the secretion of hormones such as ecdysteroids and juvenile hormones, thereby delaying oocyte maturation and causing histological abnormalities in the ovaries.

Synchronization of the *Amphipoda* sp. life cycle and reproductive timing is strongly influenced by salinity stability, which directly impacts breeding efficiency and the availability of high-quality live feed in aquaculture systems. In this context, salinity management plays a strategic role in enhancing reproductive performance while ensuring a consistent supply of live feed enriched with essential amino acids and long-chain polyunsaturated fatty acids like EPA and DHA **(Turcihan *et al.*, 2022; Suhaimi *et al.*, 2024)**. These findings suggest that *Amphipoda* sp. from stable salinity habitats and with superior amino acid composition hold significant potential as alternative live feed candidates in sustainable multitrophic aquaculture systems, especially when combined with co-culturing strategies involving phytoplankton **(Quirós-Pozo *et al.*, 2023)**. This research presents a promising approach to optimizing reproduction through salinity regulation. Additionally, the valuable insights from amino acid data highlight the superior potential of *Amphipoda* sp. as a high-quality and sustainable live feed. These findings

pave the way for developing proper and standardized cultivation protocols for *Amphipoda* sp.

CONCLUSION

This study demonstrates that salinity optimization, particularly at 30ppt, effectively enhances reproductive performance and synchronizes the life cycle of *Amphipoda* sp., while also its amino acid composition, an essential trait for high-quality and sustainable live feed. The integration of DNA-based species identification, reproductive evaluation, and biochemical analysis confirms the potential of *Amphipoda* sp. as a reliable candidate for live feed in aquaculture, and highlights the need for standardized cultivation protocols based on environmental parameter control. *Amphipoda* sp. has a nutritional value that supports its potential use in aquatic farming as a premium live feed. Future research and industrial application may focus on harnessing the exceptional nutritional profile of *Amphipoda* sp. to develop efficient, scalable, and eco-friendly live feed systems for aquaculture.

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