MARIA CRISTINA MIGLIO TOLEDO

EVALUACIÓN DE LA SOBREVIVENCIA Y CALIDAD LARVAL DE *Macrobrachium rosenbergii* (DE MAN, 1879) EN UN SISTEMA DE RECIRCULACIÓN CERRADO

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PRÓ-REITORIA DE PÓS-GRADUAÇÃO

PROGRAMA DE PÓS-GRADUAÇÃO EM RECURSOS PESQUEIROS E AQUICULTURA

Evaluación de la sobrevivencia y calidad larval de *Macrobrachium rosenbergii* (De Man, 1879) en un sistema de recirculación cerrado.

Maria Cristina Miglio Toledo

Trabalho de tese a ser julgado pelo Programa de Pós-graduação em Recursos Pesqueiros e Aquicultura da Universidade Federal Rural de Pernambuco como exigência para obtenção do título de Doutor.

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Trabalho de tese apresentado como exigência para obtenção do título de doutor, realizado em 27/02/2023 pela seguinte Banca Examinadora:

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Indice

Resumen

La producción del camarón gigante de agua dulce *Macrobrachium rosenbergii* en el Perú se realiza en zonas de selva tropical, con temperaturas altas y sin acceso al agua de mar, por ello los productores de camarón han optado por establecer sus hatcheries comerciales en estas zonas. Para lo cual utilizan sistemas de recirculación (SRA), el agua salobre para la producción larval, se consigue mezclando agua dulce con sales marinas comerciales o agua de mar, aprovechando el agua preparada para varios ciclos de producción. Con el uso continuo del agua salobre disminuyen las concentraciones de algunos elementos necesarios en los procesos osmoregulatorios y de crecimiento de las larvas generando un efecto negativo en la producción de PL y su supervivencia. Por ello el objetivo general del presente trabajo de investigación ha sido evaluar la supervivencia larval de camarón *M. rosenbergii* producido comercialmente en una hatchery en la Región San Martin en el Perú, a través de la evaluación de la calidad iónica del agua salobre en un SRA, utilizada sucesivamente en ciclos de producción larval. Para lo cual se desarrollaron dos experimentos, uno considerando agua de mar artificial que había sido utilizada en el SRA durante 240 días (E1) y en otra etapa agua de mar artificial recién preparada (E2). En ambos experimentos 100 zoea I/ L fueron acondicionadas en 4 tanques de 1000 L y alimentadas con Artemia y flan de huevo, siguiendo los protocolos de rutina aplicados en el criadero. Se estimó la supervivencia y el índice de estadio larvario (ISL) cada 5 días. La T °C, S ‰ y el pH se registraron diariamente, mientras que los iones y compuestos de nitrógeno cada 5 días. Se realizaron correlaciones de Spearman y Pearson entre la supervivencia de las larvas y la concentración de iones y la relación Mg/Ca. En el experimento E1 se obtuvo una supervivencia final de 32% ± 7.5% y el ciclo duro 23 días y en E2 se obtuvo 50% ± 7.5% de supervivencia con 21 días de ciclo larval. El índice de estadio larval no difirió significativamente entre los experimentos. Los parámetros de calidad del agua fueron apropiados para la especie. Aunque las concentraciones de nitrato (NO_3) se mantuvieron en 500 mg/L en E1, así mismo mostró una disminución de ion magnesio, aproximadamente la mitad en comparación con el agua de E2. Las concentraciones de magnesio y potasio disminuyeron en ambos experimentos. Se encontró una correlación positiva entre la supervivencia larvaria y el magnesio ($r = 0.54$) y potasio ($r = 0.78$) en E1, pero no se observó lo mismo en E2. La relación Mg/Ca presento un promedio de 1.2, en E1 mientras que la relación promedio fue de 2.1 en E2. Concluyendo que la disminución de los iones magnesio y potasio, por el uso continuo del agua en RAS, podría afectar la supervivencia de las larvas. Un segundo capítulo del trabajo de investigación fue evaluar la calidad de las postlarvas (PL) obtenidas en dos hatcheries comerciales con la aplicación de pruebas de estrés a la formalina (TF) y amonio (TA). Las PL evaluadas fueron de la hatchery comercial localizada en la Región San Martin y fueron producidas con agua recién preparada en el ensayo E1. Posteriormente, se evaluaron PL producidas, con agua usada en varios ciclos de producción, a la cual se adiciono magnesio para compensar la pérdida de este ion (ensayo E3). Las pruebas de estrés también se practicaron en PL de una hatchery artesanal localizada en Lima que utiliza un sistema estático con agua de mar natural. Esta hatchery recibe larvas zoea I, vía aérea, de la Región San Martin. Inicialmente se calculó la Concentración letal media (CL_{50}) para cada uno de los compuestos, la concentración de la CL_{50} calculada, fue usado para las pruebas finales de estrés. Es así que al final de cada etapa de producción, 120 PL fueron expuestas a formalina (600 mg/L por 1 hora) y amonio (30 mg amonio total/L por 24 horas). El criterio para definir una buena calidad de PL, consideró una supervivencia del 60% o más como un valor aceptable. Las PL de los ensayos E1 y E2, se consideraron de buena calidad. Sin embargo, hubo diferencias significativas entre las pruebas TA (75 %) y TF (48 a 57 %) en el ensayo E3, las PL no fueron consideradas de buena calidad. En todas las pruebas de estrés, el grupo control presentó 100% de supervivencia. Las PL del ensayo E3 fueron sometidas a un análisis histológico de las branquias el cual mostró daño leve en la prueba de estrés TA y efectos moderados a severos en la prueba de estrés TF. Las pruebas de estrés con amonio y formalina demostraron ser una herramienta eficaz para detectar PL débiles o estresadas de *M. rosenbergii*, en función al origen larval o manejo en la producción, por lo que se recomienda su implementación en condiciones de criadero.

Palabras clave: agua de mar artificial, *Macrobrachium rosenbergii*, larva, post larva, pruebas de estrés.

Abstract

The production of the giant freshwater prawn *Macrobrachium rosenbergii* in Peru is carried out in tropical jungle areas, with high temperatures and no access to seawater, which is why prawn producers have chosen to establish their commercial hatcheries in these areas. For which they use recirculation aquaculture systems (RAS), the brackish water for larval production is obtained by mixing fresh water with commercial sea salts or sea water, the water prepared it is used for several production cycles. The continuous use of brackish water, the concentrations of some elements necessary in the osmoregulatory and growth processes of the larvae decrease, generating a negative effect on the production of PL and their survival. Therefore, the general objective of this research has been to evaluate the larval survival of commercially produced *M. rosenbergii* in a hatchery in the San Martin Region in Peru, through the evaluation of the ionic quality of reconstituted brackish water in a RAS, used successively in larval production cycles. Larval production of giant river prawn *M. rosenbergii* and water quality in a commercial hatchery using a closed recirculation system (RAS) with artificial seawater, considering water used during 240 days (E1) and freshly prepared water (E2), was evaluated. An average of 100 larvae/L (zoea I) was stocked in four tanks of 1000 L; in both treatments, they were fed Artemia and egg custard. Survival and larval stage index (LSI) were estimated, every 5 days. Temperature, salinity and pH were monitored daily, while nitrogen compounds and ions were monitored every 5 days. Spearman and Pearson correlations were performed between larval survival and ion concentration and Mg/Ca ratio. Final larval survival differed significantly between treatments E1 (32% ± 7.5%) and E2 (50% ± 7.5%). Larval cycle lasted 23 and 21 days for E1 and E2 respectively. LSI did not differ significantly between the treatments. Water quality parameters were appropriate for the specie, although nitrate (NO_3) increased to 500 mg/ L in E1 treatment. Reused water showed a depletion of magnesium (~half of it) when compared to freshly prepared water. Magnesium and potassium concentrations decreased in both treatments during E1 cycle. A positive correlation was found between larval survival and magnesium ($r = 0.54$) and potassium ($r = 0.78$) in E1, but the same was not observed in E2. Mg/Ca ratio in E1 showed an average of 1.2, whereas, in E2, the average ratio was 2.1. The depletion of magnesium and potassium ions, through the continuous use of the water in RAS, could affect the survival of larvae. A second chapter of the research was to evaluate the quality of the post larvae (PL) obtained in two commercial hatcheries with the application of stress tests. The tests used were formalin (TF) and ammonium (TA). The PL used in the tests came from the hatchery located in the San Martin Region and were produced with freshly prepared water in trial E1 and in water used in several production cycles, to which magnesium was added to compensate the loss of this ion (trial E3). Stress tests were also performed on PL from an artisanal hatchery located in Lima that uses a static system with natural seawater. This hatchery receives zoea I larvae, from the San Martin Region. Initially, the Median Lethal Concentration (LC_{50}) was calculated for each of the compounds, the calculated LC_{50} concentration was used for the final stress tests. Thus, at the end of each production stage, 120 PL were exposed to formalin (600 mg/L for 1 hour) and ammonia (30 mg TAN/L for 24 hours). A survival of 60% or more was the criteria to define good PL quality. The PL from trials E1 and E2 were considered to be of good quality. However, there were significant differences between the TA (75%) and TF (48 to 57%) tests in the E3 trial, the PL were not considered of good quality. In all stress tests, the control group presented 100% survival. The PL from assay E3 underwent histological analysis of the gills, showed slight damage in the TA stress test and moderate to severe effects in the TF stress test. The stress tests applied proved to be an effective tool to detect weak or stressed PL of *M. rosenbergii*, depending on the larval origin or production management, its implementation and application feasibility should be evaluated and will depend on the production center.

Key words: artificial sea water, *Macrobrachium rosenbergii*, larvae, post larvae, stress tests.

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1. Introducción

La producción de camarón gigante de Malasia

El camarón gigante de agua dulce *Macrobrachium rosenbergii* (De Man, 1879), perteneciente a la familia *Palaemonidae*, es originario de la región Indo- Pacífico (NEW 2002, NEW *et al*., 2010). Es una especie que habita aguas dulces interiores pero sus estadios larvales requieren de aguas salobres para su desarrollo. Se produce principalmente en países asiáticos como China, Bangladesh, Tailandia, Vietnam, Myanmar, Taiwán y Malasia (FAO,2023).

En el reporte de FAO (2020) se menciona un incremento de la producción global de camarón gigante de agua dulce en un 7 % del 2010 al 2018, alcanzando el año 2020 las 294,018.60 t con un valor total de USD 2´383,882.198 (FAO, 2023). Representando la producción de esta especie el 2.5% de la producción total, entre seis crustáceos de importancia comercial producidos a nivel mundial (FAO, 2020). En la figura 1 se aprecia la evolución de la producción y el valor comercial mundial del camarón *M. rosenbergii*.

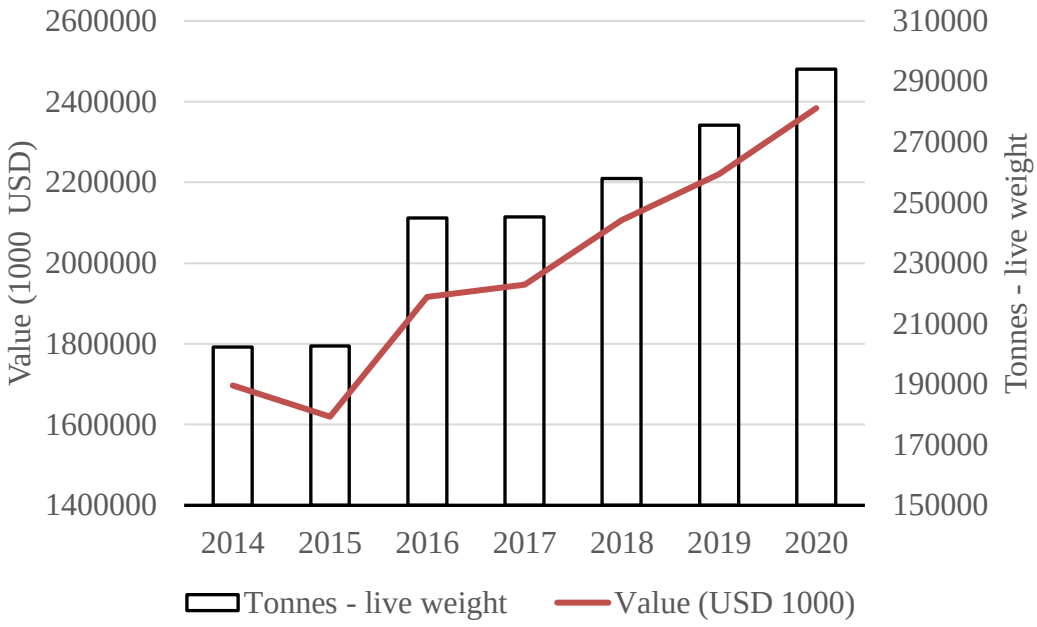

Figura 1 Producción y valor comercial mundial de camarón *M. rosenbergii*.
Fuente: Food and Agriculture Organization of the United Nations - FAO Fisheries and Aquaculture Information and Statistics Branch online (2023) disponible en https://www.fao.org/fishery/statistics-query/en/aquaculture

En el Perú, la producción de *M. rosenbergii* es relativamente baja, para el periodo 2012 al 2021 la producción promedio fue de 29 ± 18 toneladas métricas (figura 2) y la totalidad de ella se produce en la Región San Martin (PRODUCE, 2021). La acuicultura en San Martín reporta un promedio de 2140 ± 576 toneladas métricas para el período 2012 al 2021 y está representada por la producción de tilapia (65 ± 12 %) *Oreochromis niloticus* y las especies nativas gamitana (15 ± 5 %) *Colossoma macropomum*, paco (17 ± 13 %) *Piaractus brachypomus* y camarón de Malasia (1.3 ± 0.7 %) *M. rosenbergii* (PRODUCE, 2021).

Con respecto a las concesiones acuícolas en San Martín, el cultivo de tilapia representa el 92,08% del total de la superficie otorgada como derecho acuícola en la región, que se produce en sistemas de monocultivo y policultivo A (tilapia + camarón) y otros. El 31,18% del total de la superficie otorgada como derecho acuícola (161,24 hectáreas) corresponde al cultivo de camarón gigante, incluido el monocultivo y el policultivo con tilapia previamente mencionado (PNIPA, 2018).

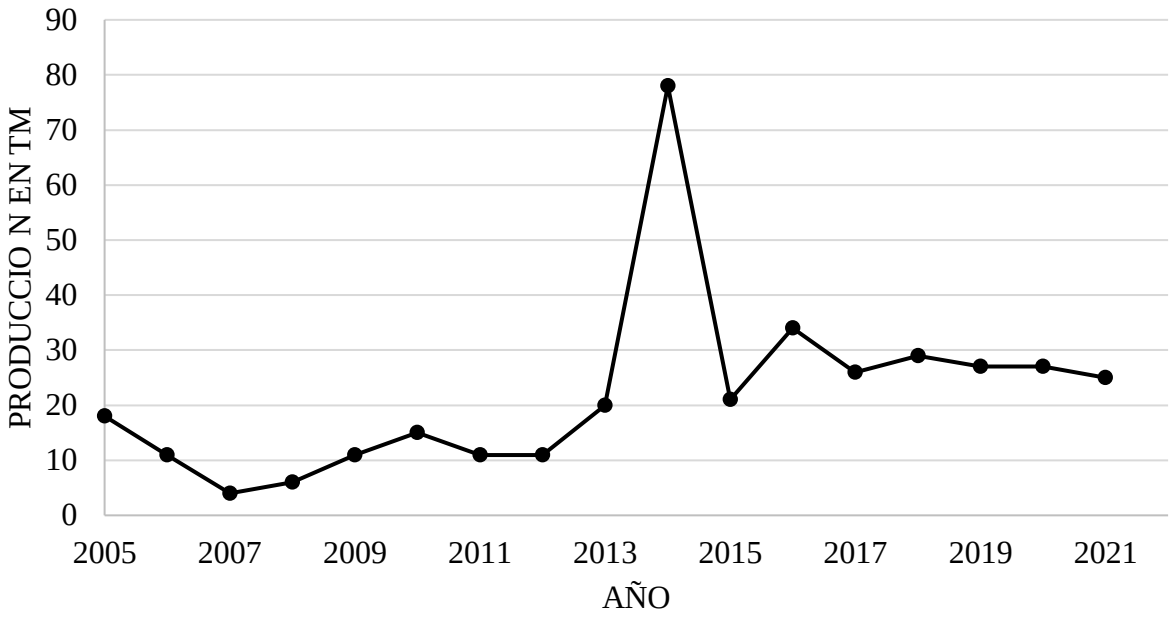

Figura 2 Producción de Camarón *M. rosenbergii* –Perú.
Fuente: PRODUCE (2021).

El Plan Regional de Acuicultura del Gobierno regional de San Martín del 2014 al 2023, (GORE SAN MARTÍN, 2013) plantea como estrategia consolidar la producción de camarón de Malasia en la región, queriendo posicionar la marca "camarón tropical" e incluso darle un valor agregado, con la elaboración de diversos productos. Los problemas identificados en el diagnóstico del Plan Regional de Acuicultura, así como en el taller Macrorregional sobre la innovación y futuro de la acuicultura y pesca en el macrorregión nororiental realizado por el proyecto PNIPA en el año

2017, han sido entre otros el insuficiente abastecimiento de semilla de calidad tanto para peces como camarón.

La Granja Acuícola Camaronera Las Palmas SAC, es un centro privado de producción de semilla de *M. rosenbergii* en la zona nororiental y es donde se desarrolló la presente investigación. La granja se localiza cerca de la ciudad de Tarapoto (400 Km de Chiclayo, ciudad costera más cercana) y está facultada a producir larvas de *M. rosenbergii* en una hatchery con una capacidad instalada de 400,000 PL a 600,000 PL mensualmente. Su infraestructura consta de una sala de microscopía, sala de preparación de alimento, sala de cultivo larval. El sistema de producción adoptado es un sistema de aguas claras con recirculación cerrado con agua salobre reconstituida con sales marinas.

GASTELU (2012), con respecto a la hatchery Las Palmas, describió el sistema de recirculación indicando que se utilizan filtros biológicos y tratamiento de agua, el cultivo de larvas se desarrolla a salinidades entre 13 y 16 ‰. Adicionalmente mencionó que el agua de mar era transportada desde el litoral en un viaje de 28 horas, y era combinada con agua dulce permitiendo un manejo productivo por más de 2 años, luego de ese tiempo se incorporaba un 50% de agua de mar al sistema para continuar la producción. El cultivo larval se realiza en un periodo de 24 días obteniéndose supervivencias promedio de 68%, luego las PL son aclimatadas a agua dulce.

En el año 2011 se mejoraron las instalaciones y se cambió el uso del agua de mar natural por agua de mar preparada con sales artificiales de la marca Marine Enterprise International Inc. (13.6 kg de la mezcla de sales permitía preparar 1000 litros de agua a 14 ‰). La eficiencia, medida en supervivencia larval, de esta agua de mezcla fue similar a la del agua de mar (supervivencia promedio de 65%, tiempo de cultivo larval de 26 días), sin embargo, según los autores era necesario renovar el 50% del volumen cada 10 meses, al observar una disminución en la supervivencia, estableciendo que los iones eran captados por las larvas y el empobrecimiento del agua era inevitable en el tiempo (GASTELU y SHEEN, 2016).

Debido a la problemática encontrada, observaciones prácticas indicaban que el empobrecimiento del agua de cultivo se daba entre el sexto y octavo mes de uso continuo, pues la supervivencia larval declinaba en un rango entre 60 % a 45 % con la consecuente pérdida económica para la granja.

Sistemas de producción de larvas y post larvas de camarón gigante

Las fases particulares en el ciclo de *M. rosenbergii* incluyen: huevos, larvas, post larvas (PL), juveniles y adultos. DANIELS *et al*. (2010), mencionan que las hembras de camarón que llevan los huevos adheridos al abdomen son denominadas hembras ovígeras o grávidas, la fuente más común de reproductores para los criaderos comerciales son los camarones adultos obtenidos de los estanques de engorde. Siendo también frecuente que los productores obtengan reproductores silvestres del ambiente natural.

HABASHY (2013) evaluó el comportamiento reproductivo *M. rosenbergii* en condiciones de laboratorio, indicando que las hembras presentaron ovas de color naranja brillante que cambiaron gradualmente a un color más oscuro a medida que avanzó el proceso de maduración del embrión. El período de incubación en su investigación varió de 18 a 24 días para diferentes tamaños de hembras y la tasa de eclosión varió del 65% al 91%. El investigador concluye que el número de huevos y larvas eclosionadas fue directamente proporcional al tamaño de los reproductores presentándose una relación lineal entre la fecundidad y el peso y la longitud de las hembras. WILDER *et al.,* (2004) refieren que, en la producción de *M. rosenbergii* en Vietnam, existe una baja tasa de maduración de la hembra en cautiverio, comienzan a portar huevos muy temprano, incluso a los 7-10 g, lo que conduce a huevos y larvas de mala calidad, y en sucesivas generaciones, las hembras maduran aún más precozmente. Refieren que, en la naturaleza, los camarones hembra no suelen quedar grávidas hasta los 20-40 g y los huevos obtenidos de estas hembras son de buena calidad y proporcionan altas tasas de supervivencia después de la eclosión.

Las hembras grávidas migran hacia los estuarios para poder desovar y cargan los huevos hasta por tres semanas, durante las cuales, los huevos, gradualmente van cambiando a una coloración naranja-marrón hasta que finalmente adquieren un color gris. Luego de dos o tres días, las larvas eclosionan como zoea en ambientes estuarinos y atraviesan por once estadios larvales (UNO y KWON, 1969) antes de metamorfosear a PL, luego migran hacia cuerpos de agua dulce. Cuando las larvas son liberadas después de la eclosión, nadan cabeza abajo y con la cola hacia adelante (NEW, 2002) y poseen una coloración traslúcida, revelando todos sus órganos (BROWN *et al.*, 2010).

Al llegar a etapa de PL, éstas se asemejan a los adultos, cambiando de una condición de nado libre y pelágica a bentónica, convirtiéndose en reotácticas positivas por lo que pueden migrar aguas arriba (NEW, 2002).

En acuicultura, la larvicultura de esta especie se realiza mediante la obtención y el desarrollo de las larvas en agua salobre (12 - 16 partes ‰) hasta completar la metamorfosis en PL, las cuales ya pueden ser cultivadas en agua dulce. Las PL pueden ser sembradas directamente en los estanques de

engorde o puede optarse por una fase intermediaria llamada pre-cría, en donde son cultivadas hasta llegar a estadio juvenil de 2 - 3 g (COYLE *et al*., 2010).

El crecimiento en esta especie ocurre a través de las mudas o ecdisis cuando los individuos periódicamente cambian su exoesqueleto, haciendo crecer el tejido somático (ISMAEL Y NEW, 2000; BROWN et al., 2010), el periodo entre las mudas se denomina intermuda y es dependiente del tamaño del camarón y de la dureza del agua (ADHIKARI *et al*., 2007). La frecuencia de la muda varia naturalmente dependiendo de la talla, edad, temperatura, salinidad y en la disponibilidad de alimento (ISMAEL Y NEW, 2000), siendo que durante el proceso de muda la vieja cutícula es reemplazada por una nueva.

DAVID *et al.* (2016) mencionan que, a pesar de la alta producción de *M. rosenbergii* en el mundo, la producción de PL es un cuello de botella porque carece de información basada en investigación científica. La larvicultura de esta especie se lleva a cabo en tanques de circulación abierta o en sistemas de recirculación (SRA) en ambientes cubiertos, siendo los SRA más eficientes en conservar el agua y el calor y generar menos efluentes, haciendo de estos sistemas más efectivos y sostenibles. Adicionalmente a que en la actualidad muchas hatcheries se localizan en zonas lejanas al litoral y es necesario mantener las condiciones del medio de cultivo.

VALENTI *et al.* (2010) mencionan que en sistemas de cultivo de aguas claras y de recirculación las larvas de *M. rosenbergii* sobreviven a densidades que varían en 60 a 100 larvas /l. Por su parte, DAVID *et al.* (2016) mencionan que en una experimentación realizada probando densidades de siembra en sistema de agua clara, la densidad de 100 larvas/l presentó una mayor eficiencia en la tasa de metamorfosis, pues aproximadamente el 50 % de las larvas se volvieron PL, en densidades mayores (140 larvas/l) la sobrevivencia fue de 35 %. SUDHAKAR *et al*. (2013) señalan una sobrevivencia de 73 % en larvas criadas de hembras colectadas del medio natural, indicando que la tasa de sobrevivencia depende de varios factores, entre ellos la densidad de siembra, el pH del agua y la alimentación.

Importancia de los componentes iónicos en la larvicultura de *M. rosenbergii*

En la larvicultura de *M. rosenbergii* no solo la salinidad es importante, sino también los componentes iónicos tales como el sodio, potasio, calcio y magnesio, macro elementos, que cumplen un rol significativo en la osmorregulación y crecimiento de los crustáceos (CHARMANTIER *et al*., 2009) (tabla 1). El calcio y el magnesio son dos macro elementos muy importantes que impactan la dureza del exoesqueleto larval, la frecuencia de la muda, la osmolalidad de la hemolinfa y la supervivencia larval (ADHIKARI *et al*. 2007; RAFIEE *et al*. 2015; TAVABE *et al*. 2013). RAIZADA *et al.* (2015), indican que la ausencia de magnesio en crustáceos no permite la hidrólisis de ATP por la bomba sodio potasio. El magnesio es esencial para formar el complejo ATP- Mg^{2+},

que es importante en la aclimatación a medios de baja salinidad. Las concentraciones de estos elementos en la etapa larval de *M. rosenbergii* sugeridos por diversos autores se muestran en la tabla 2.

Tabla 1: Rango de componentes iónicos en distintas aguas utilizadas en hatcheries de camarón *M. rosenbergii*

Variables	Agua dulce (mg/L)	Agua marina (mg/L)	Agua salobre (mg/L)
Cloruros (Cl)	40 - 225	19000 - 19657	6600 – 7900
Sodio (Na)	28 - 100	5950 - 10500	3500 – 4000
Potasio (K)	2 - 42	400 - 525	175 – 220
Calcio (Ca)	12 -24	390 - 450	175 - 195
Magnesio (Mg)	10 -27	1250 - 1345	460 – 540
Silicatos (SiO_2)	41- 53	3 -14	5- 30
Plomo (Pb)	< 0.02	< 0.03	< 0.3
Cobre (Cu)	< 0.02	< 0.03	<0.06
Zinc (Zn)	0.2 – 4.0	0.03 – 4.6	< 0.03
Manganeso (Mn)	< 0.02	< 0.4	< 0.03
Fierro (Fe)	< 0.02	0.05 – 0.15	0.3
Cromo (Cr)	<0.01	<0.005	<0.01
Oxígeno disuelto (DO_2)	>4	> 5	> 5
Dureza total (como $CaCO_3$)	< 120	-	2325 - 2715
Solidos disueltos totales (SDT)	217	-	-
Amonio (NH_3- N)	-	-	< 0.1
Nitrito (NO_2- N)	-	-	< 0.1
Nitrato (NO_3-N)	-	-	< 20
pH	6.5 – 8.5	7.0 – 8.5	7.0 – 8.5
Temperatura (°C)	-	-	28 - 31

Fuente: NEW (2002)

Tabla 2: Concentraciones sugeridas de macro elementos en el cultivo larval de *M. rosenbergii*

Elemento	**Símbolo**	**Sugerido (mg/L^{-})**	**Autor**
Calcio	Ca	240 (ratio Mg/Ca 1.25)	TAVABE *et al*. (2013)
Potasio	K	150	TAVABE *et al*. (2015)
Magnesio	Mg	300 (ratio Mg/Ca 1.25)	TAVABE *et al*. (2013)
Sodio	Na	4000	TAVABE *et al*. (2015)
Na/K	RATIO	K 80% ~ de agua de mar	RAIZADA *et al*. (2015)
Mg/Ca	RATIO	1.8 – 2	NEW *et al.* (2010)

FUNGE-SMITH *et al.* (1995) señalan que *M. rosenbergii* es una especie que puede controlar el gradiente de presión osmótica y la concentración de su hemolinfa cuando está expuesta a un amplio rango de salinidades ambientales, desde agua dulce hasta su punto isosmótico (Tabla 3).

STERN *et al*. (1987) determinaron la regulación osmótica e iónica de ciertos iones clave (Na^+, Cl^-, K^+, $Ca^{2+,}$ Mg^{2+}) en *M. rosenbergii* mantenido durante largos períodos en agua dulce, agua de mar diluida al 15‰ y al 24‰ y en aguas de composición iónica variable. Concluyendo que *M. rosenbergii* tiene la capacidad de regular y mantener su osmolaridad hemolinfática y composición iónica (Na^+, Cl^-, K^+, Ca^{2+} y Mg^{2+}) en medios que varían desde agua dulce hasta concentraciones cercanas a la iso-osmoticidad.

Tabla 3 Punto isoosmótico para diferentes estadios de *M. rosenbergii*

Punto isoosmótico (partes por mil)	**Referencia**
17 – 18	SANDIFER *et al.* (1975) – PL
14.5 – 15.6	CHENG *et al.* (2003) – Femenino, masculino

STERN *et al*. (1987) refieren que las concentraciones osmóticas en sangre se regularon hiperosmóticamente en un rango de 450–500 mOsm/l con la excepción de agua de mar al 24‰ donde la concentración osmótica en sangre aumentó a 636 mOsm/l. Las concentraciones sanguíneas de Na^+ y Cl^- se mantuvieron hiperiónicas en todos los medios excepto en el agua de mar al 24‰ en la que ambos iones eran hipoiónicos. Las concentraciones de K^+ y Ca^{2+} en sangre fueron hiperiónicas en todos los tratamientos mientras que el Mg^{2+} se mantuvo como regla hipoiónico.

FUNGE-SMITH *et al. (*1995) señalan que las concentraciones de sodio y cloro en la hemolinfa son hiper reguladas en salinidades por debajo del punto iso iónico (0 – 15 ‰) y en salinidades por

encima del punto iso iónico la concentración de ambos iones es como la del medio acuático. Los iones calcio y potasio son hiper regulados en todas las salinidades, mientras que el magnesio siempre se mantiene a más bajas concentraciones que el medio. Los mismos autores mencionan que la concentración de bromo fue hiper regulada desde el agua dulce hasta su punto iso iónico, mientras que el estroncio fue hiper regulado hasta los 18 ‰, sugiriendo que ambos iones son importantes en la fisiología de la especie.

La regulación iónica y osmótica en los crustáceos se lleva a cabo en los tejidos branquiales multifuncionales, pared del cuerpo, el intestino, conjuntamente con los órganos excretores (HENRY *et al*. 2012, BOUDOUR *et. al.* 2016). Las glándulas antenales representan los órganos excretores urinarios de los crustáceos decápodos, ellas intervienen en la regulación de la composición y el volumen de la orina y de los fluidos extracelulares. Asimismo, son capaces de regular la concentración de solutos orgánicos e inorgánicos y particularmente de los iones divalentes tales como magnesio, calcio y sulfatos (BOUDOUR *et al.,* 2016). Al nivel de la cavidad branquial, adicionalmente de la actividad respiratoria, las branquias son el principal sitio de intercambio iónico trans epitelial, el transporte iónico, una actividad de singular importancia de funciones esenciales que incluyen la osmorregulación, homeostasis del calcio, excreción de amonio y regulación del pH extracelular (HENRY *et al.* 2012).

Las larvas de *M. rosenbergii* no poseen las mismas capacidades osmoregulatorias que los adultos (BROWN *et al.* 2010). El sistema de osmorregulación en estos primeros estadios no está completamente desarrollado y durante los estadios larvales este proceso se atribuye principalmente a la actividad de la enzima Na/K-ATPasa (HUONG *et al.*, 2004), y se infiere que las células relacionadas con el transporte iónico se encuentren localizadas en los branquioestegitos (ITUARTE *et al.*, 2008).

Pruebas de estrés para evaluar la calidad larval y de PL

Las tasas de supervivencia de las larvas son la forma más común de medir la eficiencia de un criadero, mientras que la calidad de las larvas se puede asegurar manteniendo un estado genético, nutricional y de salud adecuado de los reproductores y proporcionando una nutrición larvaria correcta. Se han llevado a cabo programas de mejora genética con *M. rosenbergii* en Vietnam, China, India, Tailandia e Indonesia, siendo la principal característica de selección la tasa de crecimiento (PILLAI *et al.*, 2022). El termino calidad larval es ampliamente usado para referir la condición fisiológica, performance durante el cultivo (supervivencia y crecimiento) y resistencia a las pruebas de estrés (manipulación, cambios en las condiciones ambientales, resistencia a patógenos). La búsqueda y establecimiento de un criterio universal para evaluar la calidad larval, es una preocupación a nivel de investigación y de producción. La calidad de los huevos y nauplios depende principalmente de las

condiciones fisiológicas de los reproductores, pero también de las condiciones ambientales que prevalecen en el desove y los tanques de eclosión (RACOTTA *et al.* 2003). Algunos criterios utilizados para evaluar la calidad larvaria de crustáceos se concentran en cinco categorías: bioquímica, morfología, comportamiento, rendimiento productivo y supervivencia a pruebas de estrés (RACOTTA *et al*., 2004).

También se ha evaluado la calidad de las larvas de *M. rosenbergii* en relación con la duración del ciclo larvario, aparición del primer estadio de PL, desarrollo del proceso de metamorfosis, observación del proceso de muda, entre otros. El índice de calidad larvaria (ICL) presentado por TAYAMEN y BROWN (1998) ha sido capaz de monitorear el estado de la operación del criadero y la salud de la producción larvaria en diferentes etapas de crianza larvaria, al calificar diferentes criterios de calidad observados en el microscopio basados en criterios morfológicos y de comportamiento.

Por lo general, después de que se completa la metamorfosis larvaria a PL (90 % de PL), los individuos deben aclimatarse al agua dulce. Este procedimiento se realiza en tanques, donde el agua se cambia progresivamente en 24 -48 horas de 14 – 12 ‰ a 0 ‰, sin embargo, es un proceso que no es detallado claramente, por ejemplo, en Tailandia se realiza en 2 a 3 horas y en China en 6 a 8 horas (VALENTI *et al*., 2010). Las PL se alimentan con alimentos formulados y los tanques se limpian y el 50 % del agua se reemplaza diariamente (VALENTI *et al*., 2010). El agua utilizada para reducir la salinidad es la misma que se utiliza durante la fase de crecimiento, siendo continuamente aireada, manteniendo bajos los niveles de amonio, además de controlar el pH.

Las PL pueden venderse después de algunos días, las características de calidad de las PL que se deben observar y considerar como protocolo de calidad son: homogeneidad de tamaño, nado sincronizado y contra corriente, ojos morados, no estar en muda. Y bajo el microscopio o estereoscopio observar hepatopáncreas lleno y vacuolado, movimiento peristáltico del intestino, primera porción de intestino llena, ausencia de necrosis y manchas blancas en los músculos abdominales, ausencia de ectoparásitos y hongos y ausencia de mortalidad visible (GASTELU, com. pers. 2022)

La calidad de las larvas producidas en los criaderos de camarón (*L. vannamei*) se evalúa con base en indicadores estándar que incluyen: tasa de crecimiento y tamaño (homogeneidad de tamaño), estado nutricional, salud y estado general, y composición bioquímica del cuerpo, resistencia al estrés (prueba de estrés) y los síntomas de la enfermedad, incluido el uso de métodos moleculares para identificar la contaminación viral y bacteriana (MIRZAEI *et al*. 2021).

Las pruebas de estrés son aplicadas en estadios iniciales, así como en estadios post larvales y se centran principalmente en exponer a los organismos a condiciones ambientales adversas modificando la temperatura, la salinidad, la concentración de oxígeno disuelto o exponiendo a los

organismos a un químico estresor (metales pesados, pesticidas, amonio, por ejemplo) o a un patógeno (reto). HERNANDEZ – HERRERA (2001) indica que la prueba de estrés a la formalina consiste en exponer PL de *L. vannamei* por un periodo determinado en una solución de formalina, cuya concentración varía de acuerdo a la edad de la PL, registrando la supervivencia. KHASANI *et al*. (2018) probaron concentraciones de 500 y 750 mg/L de formalina en PL de *M. rosenbergii* durante una hora. BART y YEN (2003) realizaron diversos desafíos (oxígeno, salinidad y formalina) para comparar el desempeño de PL de 27 días de *M. rosenbergii* de Tailandia y Vietnam. Entre ellos, aplicaron una prueba predictiva de formalina al 2 % (200 mg/L), observando que el tiempo necesario para alcanzar el 90 % de mortalidad fue entre 74 y 95 h, mientras que el 50 % de mortalidad se alcanzó entre 46 y 48 h.

KHASANI *et al*. (2022), usando métodos sugeridos por un Estándar Nacional de Indonesia para semillas de *M. rosenbergii*, aplicaron pruebas de tolerancia a condiciones ambientales subóptimas a tres poblaciones de camarón gigante de agua dulce provenientes de programas de mejoramiento genético a varios factores ambientales estresantes (pH, temperatura, salinidad y formalina). Las pruebas se realizaron sometiendo a las PL a cambios bruscos de salinidad y temperatura, y exponiéndolas a pH bajo y formalina. La prueba de tolerancia a formalina se realizó transfiriendo PL directamente a medios con 500 mg/L de formaldehído. La mortalidad de las PL se observó a los 15, 30, 45, 60 y 120 minutos después de la prueba para calcular la tasa de supervivencia. Los autores consideraron que la mortalidad de las PL < 20 % indicaba que las PL se clasifican como tolerantes (80 % de supervivencia) a estresores ambientales o condiciones ambientales subóptimas.

Asimismo, SAMOCHA y PRAGNELL (2019), realizando pruebas de estrés con formalina en PL de *L. vannamei*, indicaron que la supervivencia esperada varió de 40 a 50 % para PL 1 y PL 7 días, respectivamente, estos autores indican además que la tolerancia a la formalina, aumenta con la edad de la PL, de 300 mg/L en PL1 a 600 mg/L en PL7. VILLALON (1991), realizando pruebas de estrés por salinidad para PL de *L. vannamei*, ha sugerido que cuando el valor de supervivencia en esta prueba es menor al 60 %, se debe rechazar el lote de PL.

CAVALLI *et al*. (2000) realizaron pruebas de estrés sometiendo a los organismos a concentraciones de amonio, para *M. rosenbergii* en relación a la calidad de los reproductores y nutrición larval, concluyendo que esta prueba era adecuada para la determinación de la calidad larval de esta especie. LIU *et al.* (2022) utilizaron una prueba de estrés de amonio para evaluar los efectos de 8 semanas de alimentación con aceite de árbol de té en el crecimiento, respuesta fisiológica y de inmunidad no específica de camarón gigante de agua dulce. Se utilizó una prueba de estrés de 20 mg/L durante 24 horas. Estos autores registraron la tasa de supervivencia cada 6 horas, que mejoró significativamente con el aceite de árbol de té dietético, siendo la mortalidad más alta (alrededor del 60%) en el grupo control. DUTRA (2017) ha demostrado que la concentración de amonio total de 20

mg/L presentó una mortalidad de 48± 10 % después de 96 horas en juveniles de *M. amazonicum*, en concentraciones de 40 mg/L la mortalidad alcanzó el 100% en 72 horas y en la concentración de 80 mg/L la mortalidad alcanzó el 100% después de 48 h de exposición.

Como mencionan LIN *et al*. (2022), los efectos tóxicos del amonio en los crustáceos acuáticos pueden afectar la osmorregulación, el estrés excesivo por amonio podría eventualmente inducir una alta mortalidad. Ellos enunciaron que la concentración letal media (CL_{50}) es el indicador más intuitivo del efecto tóxico agudo del nitrógeno amoniacal en los crustáceos. ROMANO y ZENG (2013) señalaron que el estrés por amonio en los crustáceos acuáticos podría causar varios cambios morfológicos y fisiológicos de las branquias, incluido el engrosamiento/desprendimiento del epitelio, constricción/colapso de las laminillas, alteración/destrucción de las células pilares, necrosis e infiltraciones de hemocitos.

2. Objetivos del Trabajo

Objetivo General

Evaluar la supervivencia larval de camarón *Macrobrachium rosenbergii* producido en la Región San Martin en el Perú, a través de la evaluación de la calidad iónica del agua salobre reconstituida en un sistema de recirculación cerrado, utilizada sucesivamente en ciclos de producción larval.

Objetivos específicos

1. Evaluar las deficiencias iónicas en agua salobre reconstituida para la producción de larvas de *M. rosenbergii* en ciclos sucesivos, con relación a la supervivencia (Artículo 1).
2. Evaluar la metamorfosis y calidad larval de *M. rosenbergii* producido en un sistema de recirculación cerrado, en ciclos sucesivos (Artículo 1).
3. Evaluar la calidad de PL de *M. rosenbergii* con la aplicación de pruebas de estrés (Artículo 2).

3. Capítulos de Tesis

3.1 Capítulo I: artículo publicado online en la revista científica Aquaculture Research en 17 July 2021. https://doi.org/10.1111/are.15468

Survival and metamorphosis of giant river prawn *Macrobrachium rosenbergii* larvae in a commercial recirculation system with artificial seawater.

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ORIGINAL ARTICLE

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Survival and metamorphosis of giant river prawn *Macrobrachium rosenbergii* larvae in a commercial recirculation system with artificial seawater

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Abstract

Larval production of giant river prawn *Macrobrachium rosenbergii* and water quality in a commercial hatchery using a closed recirculation system (RAS) with artificial seawater, considering water used during 240 days (E1) and freshly prepared water (E2), was evaluated. An average of 100 larvae L^{-1} (zoea I) was stocked in four tanks of 1000 L; in both treatments, they were fed Artemia and egg custard. Survival and larval stage index (LSI) were estimated, every 5 days. Temperature, salinity and pH were monitored daily, while nitrogen compounds and ions were monitored every 5 days. Spearman and Pearson correlations were performed between larval survival and ion concentration and Mg/Ca ratio. Final larval survival differed significantly between treatments E1 (32% ± 7.5%) and E2 (50% ± 7.5%). Larval cycle lasted 23 and 21 days for E1 and E2 respectively. LSI did not differ significantly between the treatments. Water quality parameters were appropriate for the specie, although nitrate (NO_3) increased to 500 mg L^{-1} in E1 treatment. Reused water showed a depletion of magnesium (~half of it) when compared to freshly prepared water. Magnesium and potassium concentrations decreased in both treatments during E1 cycle. A positive correlation was found between larval survival and magnesium ($r = 0.54$) and potassium ($r = 0.78$) in E1, but the same was not observed in E2. Mg/Ca ratio in E1 showed an average of 1.2, whereas, in E2, the average ratio was 2.1. The depletion of magnesium and potassium ions, through the continuous use of the water in RAS, could affect the survival of larvae.

KEYWORDS
artificial seawater, *Macrobrachium rosenbergii* larvae, metamorphosis, survival

1 | INTRODUCTION

The giant river prawn *Macrobrachium rosenbergii* was introduced in Perú from Israel and Panama by Agrarian National University La Molina in 1983 for the purpose of research, and a pilot experimental hatchery was established (Nava and Vicencio, 1987). Subsequently, in 1984, two commercial hatcheries were installed in San Martin region (Orbegoso, 2000) in order to benefit from the climatic conditions of the region and for the sake of proximity to the production fields. Thus, since then, more hatcheries were installed and closed in the region and also in Lima.

According to Catastro Acuícola Nacional (2019), there are currently six hatcheries licenced to produce post larvae (PL) of *M. rosenbergii*. One is situated in Lima and the rest are situated in San Martin;

however, only two hatcheries were dedicated to exclusively produce *M. rosenbergii*, as the others preferably produce fries from native fish and tilapia. The largest hatchery of Las Palmas in San Martin has adequate facilities to produce 400,000–600,000 PL monthly in a closed recirculation system (RAS). Las Palmas hatchery, formerly known as Calipuy laboratory, was established in 1989 to provide PL to their own fields. It is located 400 Km from the nearest seacoast city, and transportation of seawater could take up to 28 h. Initially, seawater was used in combination with freshwater to reach a salinity of 13‰–14‰, maintaining the prepared water for 2 years in RAS. The larval average survival, as at then, was 68 per cent in a 24-day production cycle. Since the cost to transport seawater to Las Palmas was expensive, by 2011, the facilities were improved and artificial seawater replaced natural seawater in order to prepare the mixture of 13‰–14 ‰. The larval survival percentage, using prepared salt (Marine Enterprise International Inc.), was 65 per cent in a 26-day production cycle. However, it was necessary to renovate 50% of RAS water volume every 10 months due to the diminished survival percentage (Gastelú and Sheen, 2016).

The larval development of *M. rosenbergii* has eleven stages, which occur in brackish water before metamorphosis to PL and migration to freshwater (Brown et al., 2010). The cost of transporting seawater to interior areas is not profitable, and hatcheries located further inland need to prepare artificial seawater using commercial salts or mineral supplements that are locally available. Commercial salt formulation does not always meet the requirements of aquatic organisms, especially in the composition of trace elements, since the mixtures are simplifications of seawater. Therefore, RAS with artificial seawater often presents inappropriate salt mixture, as well as a wide variation in the concentration of critical substances (Colt and Huguenin, 2002).

Mallasen and Valenti (1998a) indicated that ionic composition of artificial seawater for culture may be species-specific, and therefore, evaluative studies should be carried out before a particular formulation for commercial cultures is recommended. Besides, they suggest that delays in larval development or increases in mortality could occur in reused water due to the reduction in the concentration of any essential chemical element or the accumulation of metabolites, pheromones or toxic substances.

The optimal condition of salinity, ionic composition and osmoregulation of larvae, juvenile and adults of *M. rosenbergii* has been well-investigated (Funge-Smith et al., 1995; Mallasen & Valenti, 1998b ; Huong et al., 2004; Brown et al., 2010; Tavabe et al., 2017). Valenti et al. (2010) indicated that the best productive results of larval production were at salinities between 12 and 16 ‰, in addition to the salinity, sodium (Na^+), potassium (K^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}) concentrations, as well as, impact osmoregulation and growth in crustaceans (Charmantier et al., 2009). Mallasen and Valenti (1998b b) suggested that optimal *M. rosenbergii* larval development can be attained in artificial seawater with an adequate content of ions such as Na^+, K^+, Ca^{2+}, Mg^{2+}, chloride (Cl^{-1}), sulphate (SO_4^{-2}), bicarbonates (HCO_3^{-1}) and bromide (Br^{-1}). A subsequent study by Ismael et al. (2001) concluded that *M. rosenbergii* larvae produced in artificial seawater prepared with these eight ions can reach a similar biomass with those reared in seawater, but required a longer period of time (6–10 days) for the larvae to reach the same size as those reared in natural medium. In addition, more energy was spent to maintain the ionic equilibrium.

Calcium and magnesium are two macro-elements that are important for larval exoskeleton hardness, ecdysis frequency, haemolymph osmolality and larval survival (Adhikari et al., 2007; Rafiee et al., 2015; Tavabe et al., 2013). New (2002) suggested that artificial brackish water should contain magnesium and calcium concentrations around 500 and 200 ppm respectively. Tavabe et al. (2013) reported that a balance of 240 ppm calcium and 300 ppm magnesium, with Mg/Ca ratio of about 1.25, is optimal for larviculture and that this balance, at 30 days post hatch, showed the highest larval survival (40% ± 2.6%). Raizada et al. (2015) indicated that the absence of magnesium in crustaceans prevented ATP hydrolysis by the Na^+/K^+ pump, since magnesium is essential for the formation of ATP-Mg^{2+} complex and thus important in low salinity media acclimatization. Tavabe et al. (2017) investigated the Na/K-ATPase activity in *M. rosenbergii* larvae, using different sodium vs potassium and calcium vs magnesium concentrations in specific development stages. The authors observed that Na/K-ATPase activity is widely variable during larval development and is affected by the interaction of the four macro-elements.

Las Palmas hatchery has lately experienced a further decrease in the survival of *M. rosenbergii* larvae. Therefore, this study aimed to evaluate the ionic deficiencies in reused water (RW) in RAS, as well as larval survival and metamorphosis of *M. rosenbergii* at this hatchery, in comparison with freshly prepared water.

2 | MATERIALS AND METHODS

The study was conducted at Las Palmas hatchery, which is located at 6°31'14.9"S and 76°19'59.5"W, San Martin region. This hatchery uses a closed RAS for *M. rosenbergii* larviculture. It has three 2.5 m^3 reservoirs that are located outside of the hatchery facilities, as well as three 10 m^3 reservoirs inside the facilities, in which the treated freshwater is received for use in cultivation. The RAS is equipped with nine tanks of 1000 L capacity for larval production, connected to each other by a distribution tank that recirculates the water from the 10 m^3 reservoir. Each production tank is connected to a 200-L cylinder that is implemented with a mechanical filtration system (perlon synthetic fibre filter) and biological filtration system using ceramic rings, as a substrate for bacterial attachment. The recirculation system is activated when the larvae reach the third stage of metamorphosis, the effluent from the production tank goes through an initial filtration that prevents the passage of larvae, initially with 250-micron-mesh, until it is replaced by a 450-micron-mesh when larvae reach the seventh stage. The water recirculation is provided by submersible hydraulic pumps (2000 litres $hour^{-1}$) (Venusaqua, China) that drive the water from the distribution tank to the production tanks, and water renovation ratio is approximately 50 per cent per hour.

In addition, an ultraviolet (UV) system (Odyssea, China) and an artisanal skimmer are installed in the distribution tank. Two blowers (Sweetwater, USA) of 1HP each were used to ensure an oxygen saturation around 70 per cent in every tank and treatment system.

2.1 | Experimental design

In order to evaluate the ion deficiencies in RW, two treatments were established with artificial seawater that was used during 240 days (E1) and freshly prepared artificial seawater (E2) using four tanks of RAS system for each treatment. The treatments lasted 23 and 21 days for E1 and E2 respectively.

2.2 | Ovigerous females and larval stocking

Ovigerous females from rearing ponds with an average weight of 25 (±2 g) were used for larval production in both treatments (n = 40 to 60), with a mean larval production of 13,000 per female. The selected egg-bearing females with brownish egg masses were disinfected with 2 ml of formalin in 30 L of water per 30 min and permanent aeration. These females were transferred to the spawning tanks (1200 L) provided with shelters, previously the chelas of the second pair of periopods were removed to prevent the ovigerous mass from being injured. The water was kept with salinity of 10 ‰ and temperature of 29°C until larval hatching. Larvae were kept at the same water quality (pH, salinity) of the production tank.

After hatching, larvae were collected by positive phototropism and their number was estimated by taking the mean number of 3 samples of 100 ml and multiplying this number by volume of the container. Afterwards, the larvae were transferred to the larval rearing tanks of the RAS system. *M. rosenbergii* larvae were stocking in these production tanks at different dates, due to the asynchronous hatching of the larvae. The average stocking density was 100 larvae per litre, which corresponded to a total of 440,000 and 406,000 zoea I, which was stocked at treatments E1 and E2 respectively.

2.3 | Feeding

The larvae were fed Artemia nauplii (Brine shrimp direct Grade A, USA) twice until the presence of the first PL, considering 30 nauplii per larva per day. The Artemia cysts were decapsulated using sodium hypochlorite (5.5 per cent), after hydration in freshwater for 1 h with constant aeration. After a colour change from brown to bright orange was observed, the chlorine concentration was diluted by adding water and the decapsulated cysts were immediately collected on a 105-micron-mesh and washed with plenty of water at low pressure. Then, the cysts were placed in 'carboys' type containers, with water at 25 ‰ of salinity, prepared with commercial coarse salt without the addition of iodine. The hatching temperature was kept at 28°C. Nauplii, instar I stage was collected 20 h later, in a 105-micron-mesh and washed with abundant freshwater to remove the shells. They were then placed in a jar with culture water and supplied to each tank.

A semi-moist feed was offered from zoea III stage until PL harvest to satiety. This diet was composed of a combination of ingredients, including vegetable oil, fishmeal, prawn eggs, chicken eggs and soy protein, resulting in approximately 60% of crude protein and 22% of lipid content. Since appropriate particle size is required for different larval stages, 250-micro-filter was used from zoea II to III; 450-micro-filter was used from zoea III to VIII, and 600-micro-filter was used from zoea VIII to XI in order to micronize the diet throughout the larval cycle production.

2.4 | Artificial seawater preparation

The artificial seawater was prepared with commercial salt (Blue Treasure SPS Sea Salt™ China) using treated freshwater from the Ahuashiyacu Creek (pH = 8.2; EC = 1.15 dSm^{-1}; Ca^{2+} = 32.56 mg L^{-1}; Mg^{2+} = 15.62 mg L^{-1}; K^{+} = 8.99 mg L^{-1}; Na^{+} = 143.98 mg L^{-1}). Freshwater was transferred through an irrigation channel from the creek to three external reservoirs (2500 L per tank). Water was filtered of sand and disinfected with 4% sodium hypochlorite. Chloride was checked using a commercial kit (JBL-Germany) before addition of salt. The sea salt mixture water at the salinity of 35 ‰ contains: Na^{+} = 9300 mg L^{-1}, Cl^{+} = 17300 mg L^{-1}, Ca^{2+} = 430 mg L^{-1}, Mg^{2+} = 1400 mg L^{-1}, K^{+} = 360 mg L^{-1}, SO_4^{2-} = 2260 mg L^{-1} Sr^{2+} = 9.0 mg L^{-1}, Rb^{2+} = 0.11 mg L^{-1}, Fe = 0.06 mg L. $^{-1}$, Br = 20 mg L^{-1}, B = 4.0 mg L^{-1}, F = 0.8 mg L^{-1}, and trace elements.

The water used in E1 was recycled in RAS for 240 days using 8 tanks in average. The 1000-L production tanks were used 58 times, the renewal rate was 50 per cent per hour, and the water quality of the system remained uniform. At the beginning of each production cycle, the evaporated or eliminated (involuntary) water volume was added and the appropriate salinity level (14‰) was kept. It should be noted that during this procedure, common industrial salt (NaCl) and potassium, calcium and magnesium supplements could have been added, but no record of this supplemented quantity was found. The information collected from the hatchery production records indicated that the total number of larvae produced during this time was 5,930,000 larvae, obtained in 27.8 days of cycle in average, reaching a production of 2,865,715 post larvae after freshwater acclimatation. Average larval survival during this time was 48.3% (range from 30 to 69.7%), but survival started to decline from the fourth production cycle.

At the end of the E1 production cycle, each tank was washed and disinfected. Before starting the E2 treatment, the filters were disconnected from the production tanks and kept with water. Dirty mechanical filters were flushed with pressure water and disinfected with a 0.5 per cent sodium hypochlorite water solution. Artificial seawater was prepared for E2 at the salinity of 13‰ by mixing the appropriate commercial salt mentioned before, content in freshwater. Both treatments were managed without water reposition.

2.5 | Water quality monitoring

For both treatments, water quality evaluation was undertaken from July until September of 2018. Temperature (°C), salinity (‰), pH and dissolved oxygen (mg L^{-1} and saturation [%]) were monitored daily with a commercial digital thermometer (Hidance), refractometer (Ketotec), potentiometer (Aceche) and oximeter (Milwaukee) respectively. Nitrogen compounds were evaluated for total ammonia (NH_3: NH_4 detection limit 0.1–5.0 mg L^{-1}), nitrite (NO_2 detection limit 0.025–1.0 mg L^{-1}) and nitrate (NO_3 detection limit 1–240 mg L^{-1}) using a colorimetric kit (JBL). The water quality parameters and samples were taken early morning, daily from the distribution tank.

Ions were evaluated in water samples taken every five or seven days from each rearing tank of both treatments. Water samples were preserved in refrigeration and sent to the Soil Lab at Agrarian National University La Molina. Potassium, calcium, magnesium and sodium ions were analysed by high-resolution atomic absorption spectrophotometry (Jena Analytical). Chlorides were analysed by titration with silver nitrate and sulphates by Turbidimetry according to Bazán (2017). Results were expressed in meq. L^{-1} and transformed to mg L^{-1}.

2.6 | Survival estimation

In order to estimate survival in both treatments, the counting protocol was followed for the hatchery procedures and individuals were counted at the beginning (zoea I) of the production cycle and weekly until the final stage (zoea XI and PL). Total larval number from each tank was estimated by taking the mean number from three replicates of one litre samples and multiplying this number by volume of the tank. Survival percentage was estimated for the initial stocking number.

A polyvinyl chloride tube of 1-inch diameter and ½ inch height was used as a volumetric standard (Valenti et al., 2010) to calculate the final number of PL. The average of the triplicate counting was used as standard and multiplied by the number of units generated to obtain the quantity of PL harvest.

2.7 | Larval Stage Index (LSI) estimation and larval condition observations

Larval stage index (LSI) was estimated according to the formula proposed by Manzi et al. (1977). In order to recognize the stage, a microscope (Labor Tech) was used following the criteria suggested by Uno and Kwon (1969).

$$LSI = (\Sigma ni\,Ei)/n$$

where:

ni =number of larvae at stage Ei; n =number of larvae examined; E =larval stage; LSI =ranges varied from 1 to 12.

Time of the first PL stage appearance was assessed.

Larval condition was observed daily in a sample of 10 larvae per tank, considering morphology and behaviour according to the criteria mentioned by Tayamen and Brown (1999) using a microscope (Labor Tech-Italy). The characteristics observed were as follows: conditions of the hepatopancreas–gut lipid content, pigmentation state of the chromatophores, body coloration, melanization–presence of black spots, state of the rostrum, setae–disfigured or damaged, and presence of infesting organisms. In the case of swimming behaviour, sluggish–circular erratic or very active movement was observed. More also, photopositive response was also evaluated.

From the day 14 (E1) and day 15 (E2) to the end of the production cycle, the larval condition index (LCI) (adapted from Tayamen & Brown, 1999) was verified for *M. rosenbergii* larvae from zoea IX to zoea XI. Each item checked received a value from 0 to 2, where 0 =poor, 1 =good and 2 =excellent. The larval condition index (LCI) was determined according to the formula:

$$LCI = \Sigma P/n$$

where:

P = total number of points assigned to each larva examined; n = number of larvae analysed; LCI ranges varied from 0 to 2.

2.8 | Statistical analyses

Survival values, LSI, water quality and ionic composition data were organized and displayed graphically with Microsoft Excel package of Office 2010 for Windows and were evaluated via descriptive statistics and analysed using R studio version 3.6.3 (RStudio Team (2020). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/).

Anderson–Darling and Shapiro–Wilk (water quality) tests of normality were performed for data. Mann–Whitney test was applied in the absence of this characteristic at a significance level of 0.05 to verify the differences between treatments for larval survival percentage and LSI. Cation and anion concentrations (transformed to mg L^{-1}), as well as ionic ratio Mg/Ca, were evaluated with Mauchly´s test to assess the statistical assumption of sphericity when using repeated-measures ANOVA and were tested for weekly sampled in every treatment and among treatments at p-value < 0.05. The corresponded data were organized by culture day, in order to correlated with duration of each culture stage, LSI and survival percentage using Spearman or Pearson correlation at p-value < 0.05.

2.9 | Ethics statement

Ethical review and approval were not required for the animal study because animals were sampled as part of the daily aquaculture procedure.

3 | RESULTS

3.1 | Larval survival, larval stage index and larval condition

Reused water (E1) resulted in a final average larval survival of 32.27% ± 7.5%, with a final average of 35,500 PL (35 PL per litre). Nevertheless, the average larval survival in freshly prepared water (E2) was 50.15% ± 7.5%, with a final average of 50,750 PL (50 PL per litre). The weekly survival percentage was significantly higher for E2 when treatment compared to E1 (Table 1).

Larval stage index was not significantly different from day 11 in both cycles (Table 2). An average value of LSI for E1 and E2 tanks is presented in Figure 1. Close to stages VIII, IX and X (13–15 days post hatching), three stages were observed in the same tank in both treatments (E1 and E2). The first PL was observed between 18 and 20 days for E1 tanks and between 18 and 19 days for E2 tanks. The cycle duration for E1 and E2 was 23 days and 21 days respectively. Harvest was on average with 70% of PL. Assuming that the remained larvae were mainly zoea XI and some zoea X for both cycles, LSI would be in the range from 11.5 to 11.7.

Larval conditions observed did not show any irregularity. Details of IX, X and XI zoea stages are showed in Figures 2 and 3, biofouling and deformation in rostrum and black spots in some individuals of E1 (Figure 2) and some infesting organism and minor necrosis in some individuals of E2 (Figure 3). LCI for E1 was 1.24 ± 0.05 and 1.63 ± 0.05 for E2 (Table 3), and there was a significant difference between sample medians.

3.2 | Water quality

There was statistical evidence to ensure that the water quality parameters did not show a normal distribution ($p < 0.05$). The mean values of the water quality parameters for E1 and E2 are shown in Table 4.

The mean temperature of the water during E1 experimental period was 28.8 ± 0.4°C, with minimum and maximum temperatures of 28 and 29°C respectively (Table 4) during 23 days, meanwhile E2 experimental period last 21 days, with a mean temperature of 28.8 ± 0.5°C. The temperatures of both treatments were statistically similar ($p > 0.05$).

TABLE 1 Mean ± SD of *Macrobrachium rosenbergii* larval survival percentage (%) with reused (E1) and freshly prepared water (E2) during 23 and 21 days respectively ($n = 4$ tanks)

Time	E1	E2	*p*-Value
Week 1 (6–7 days)	79.54 ± 2.1(a)	93.21 ± 2.16 (b)	0.03
Week 2 (16. 14 days)	62.95 ± 7.1 (a)	84.93 ± 7.14 (b)	0.03
Harvest (23–21 days)	32.27 ± 7.5 (a)	50.15 ± 7.51 (b)	0.03

Note: Means with different superscript letters are significantly different between treatments ($p < 0.05$).

The water quality parameters such as pH, ammonium, nitrites and nitrates registered in each treatment showed evidence of being statistically different ($p < 0.05$) (Table 4). The pH value ranged between 7.6 and 8.0 in both treatments, with a mean value for E1 0f 7.9 ± 0.1 and E2 of 7.7 ± 0.1. Salinity was 14 ‰ in E1 and 13 ‰ in E2.

Ammonia concentration was maintained under 0.05 mg L^{-1} in E1 treatment, whereas in E2, the ammonia concentration increased at the third day of culture, reaching values between 0.4 and 0.6 mg L^{-1} and then from day 10 onwards remained at levels between 0.1 and <0.5 mg L^{-1}. Nitrite average concentration was 0.3 ± 0.0 mg L^{-1} in E1 and 0.36 ± 0.21 mg L^{-1} in E2 treatment. Nitrate was 500 ± 0.0 mg L^{-1} in E1 during 23 days of production; meanwhile, an average of 3. 6 ± 1.9 mg L^{-1} was registered in E2.

Temperature, pH and salinity remained within the standards required by *M. rosenbergii* larvae and did not differ significantly ($p \geq 0.05$) among the treatments.

3.3 | Water ionic composition

The ionic composition of water for E1 and E2 production cycles by week is presented in Table 5.

The values of cations and anions in the water samples did not present normality ($p < 0.05$), and with Mauchly's test ($\alpha > 0.05$), it was established that the assumption of homogeneity of variances (sphericity) was fulfilled. The concentrations of ions evaluated in treatment E1 were significantly different that in E2 (repeated-measures ANOVA $p < 0.05$), except for the calcium ion (Table 6).

RW showed a depletion of magnesium (~half of it) since the beginning of production cycle when compared with freshly prepared water, while potassium, sodium and chloride showed higher values in E1 due to salinity difference observed regarding E2. Magnesium ($p = 0.029$) and potassium ($p = 0.03$) concentrations showed significant differences in E1 from the initial concentration through the final concentration (Table 5). Similar results were obtained for E2 regarding magnesium ($p = 0.029$) and chloride ($p = 0.027$).

In E1 treatment, the Spearman correlation coefficients (r) of calcium with time (post-hatching day—phd), LSI and survival were moderate to weak ($r = 0.53$, 0.43 and −0.23 respectively), whereas, with respect to potassium, coefficients were negatively very strong to positively strong ($r = -0.87$, −0.85 and 0.78 respectively). Meanwhile, the simple (Pearson) correlation coefficients for chloride were very weak and moderate for sodium and magnesium (Table 7).

Treatment E2 did not show any correlation, except for a moderate positive (r = 0.56 and 0.57) for sodium between time and LSI, while the correlation coefficient for chloride and time was moderate ($r = 0.5$).

Mg/Ca ratio initiated with an average of 1.5 at the beginning of the rearing process finally decreased to an average of 1.0 in E1(Table 5), showing significant differences ($p = 0.03$), whereas, in freshly prepared water, the average ratio was 2.5 initially, but finished with 2.0 at end of the cycle, without significant differences.

The correlation coefficient for Mg/Ca ratio was negatively moderate regarding LSI ($r = -0.65$) and time ($r = -0.66$) in E1, whereas, in E2, no correlation was found (Table 7).

TABLE 2 Mean ± SD of *Macrobrachium rosenbergii* larval stage index (LSI) per week (post-hatching day) for reused (E1) and freshly prepared water (E2) ($n = 4$ tanks)

Post-hatching day	E1	E2	
	Mean ± SD	Mean ± SD	*p*-Value
6	4.2 ± 0.1 (a)	4.9 ± 0.3 (b)	0.02
11	7.4 ± 0.1 (a)	7.6 ± 0.5 (a)	0.30
16	9.5 ± 0.3 (a)	9.7 ± 0.5 (a)	0.60
21	11.1 ± 0.1 (a)	11.5 ± 0.2 (a)	0.10

Note: Means with different superscript letters are significantly different between treatments ($p < 0.05$).

4 | DISCUSSION

4.1 | Larval survival, larval stage index and larval condition index

Although little information is available regarding the survival of *M. rosenbergii* in commercial hatcheries, it has been suggested that larval survival may be affected by different production systems, stocking densities and broodstock origins. Valenti et al. (2010) mentioned that survival rate varies between 40% and 50% in flow-through systems and between 60% and 80% in RAS of experimental and commercial hatcheries, with culture cycles during 29 to 35 days. David et al. (2016) evaluated the effect of stocking density (in two experiments: 50, 70 and 90 larvae L^{-1} and 80, 100, 120 and 140 larvae L^{-1}) on the survival and productivity of *M. rosenbergii* larvae in RAS using natural seawater. These authors observed with a period of

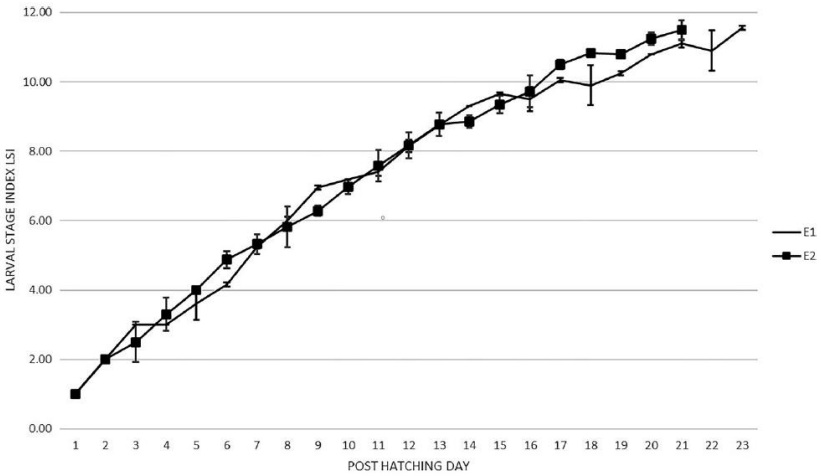

FIGURE 1 Average value ($n = 4$ tanks each) and standard deviation of *Macrobrachium rosenbergii* larval stage index (LSI) for E1 and E2 treatments during the culture cycle (post-hatching day)

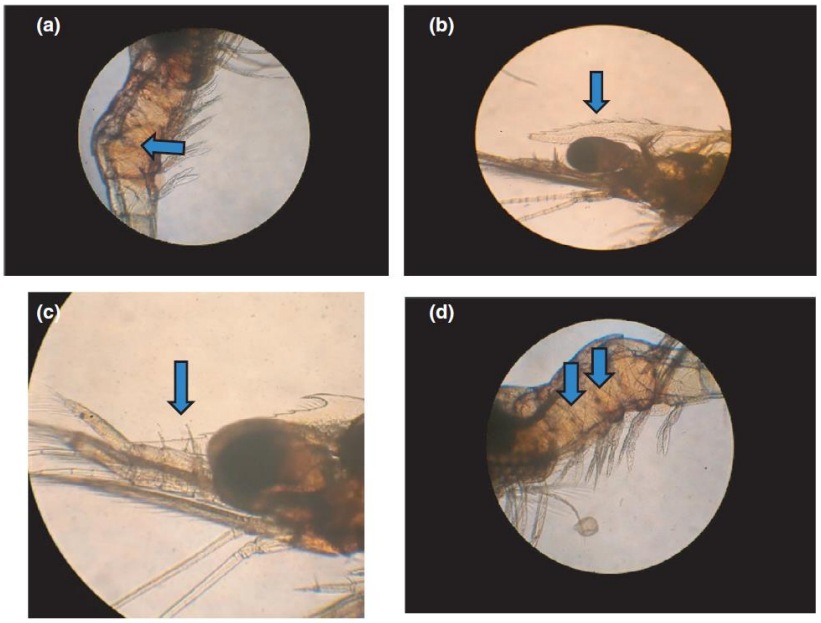

FIGURE 2 Larval characteristics quality observed for E1–larval stage from zoea IX to XI, according to Tayamen and Brown (1998) with modification. (a) body coloration, (b) state of the rostrum with deformation, (c) presence of infesting organisms and (d) melanization with black spots

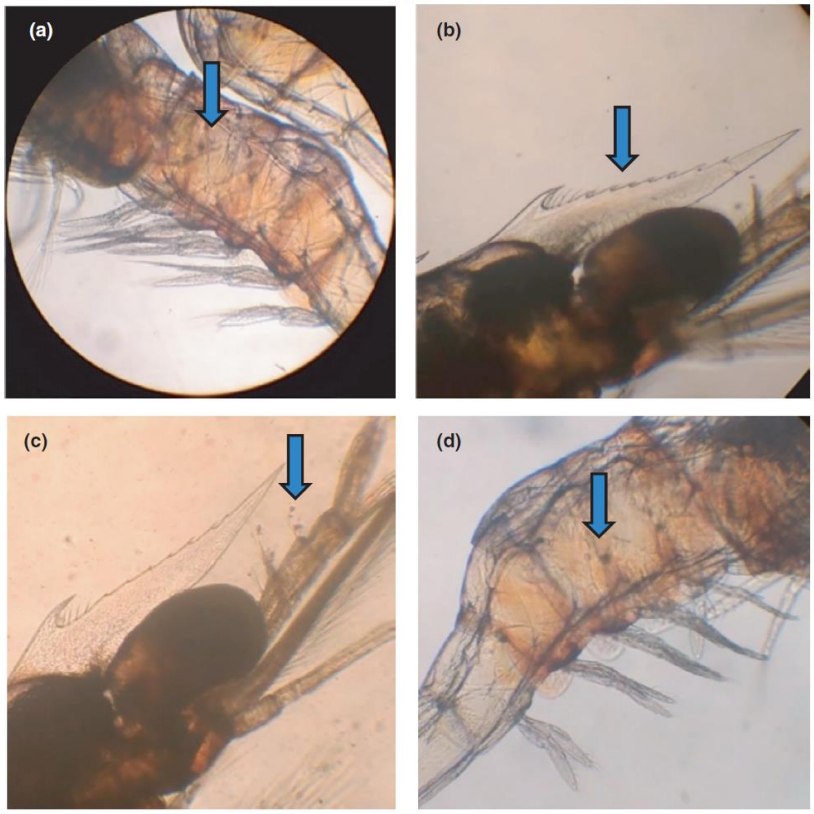

FIGURE 3 Larval characteristics quality observed for E2–larval stage from zoea IX to XI, according to Tayamen and Brown (1998) with modification. (a) Adequate corporal colour, (b) state of the rostrum and setae without deformation, (c) the presence of some infesting organisms and (d) melanization with some minor necrosis

TABLE 3 Mean ± SD of *Macrobrachium rosenbergii* larval condition index (LCI) per tank for reused (E1) and freshly prepared water (E2) (n = 4 tanks; n = 10 days (E1) and 8 days (E2); n = 10 larvae per tank)

	E1	E2
Tanks	Mean ± SD	Mean ± SD
T1	1.23 ± 0.08	1.63 ± 0.05
T2	1.24 ± 0.08	1.63 ± 0.04
T3	1.24 ± 0.07	1.62 ± 0.04
T4	1.24 ± 0.06	1.63 ± 0.05
Mean	1.24 ± 0.005 (a)	1.63 ± 0.005 (b)

Note: Means with different superscript letters are significantly different between treatments ($p < 0.05$).

culture ranged from 22 to 23 days that the intensification decreased survival, but increased productivity until densities of 100 larvae L^{-1}, with a survival rate of 55.0 ± 5.7%, these rates tended to decrease as the system was intensified.

Overall survival of *M. rosenbergii* in 15 hatcheries in the south-west region of Bangladesh was considered very poor (20%–35%) by Chattopadhyay (2016). This author argued that more than 95% of the hatcheries from the study were using wild broodstock and natural seawater, which was the probably causing the low survival due to viral and bacterial diseases. Therefore, survival rates observed in the present study (32%–50%) are comparable with those reported in other production systems.

Regarding the effect of the ionic composition of the water on the survival of *M. rosenbergii* larvae, Mallasen and Valenti (1998b b) compared the use of natural (NS) and artificial (AS) seawater for *M. rosenbergii* larviculture in RAS; their results reveal differences in the productivity (60 PL L^{-1} versus 44 PL L^{-1}, for NS and AS respectively), as well as in terms of the culture duration, being greater in AS (31 days) than NS (28 days) at 12‰. Besides, in reused water in two consecutive cycles, they did not find significant differences in culture duration, metamorphosis rate and productivity (Mallasen & Valenti, 1998b) although productivity of the natural water treatment was higher than that obtained with artificial seawater.

In our research, the weekly and final survival percentages obtained during E1 differ significantly from those of E2, and the survival percentage of *M. rosenbergii* larvae exposed to freshly prepared water was higher than that of animals cultured in RW. The E1 cycle lasted for two more days (23 days) than E2 (21 days). The previous week of harvesting (LSI ~9.5 to 11.5), the survival percentage showed a steep drop in both productive cycles, presumably due to the effect of cannibalism. According to Valenti et al. (2010), harvest should be performed when population inside the tank shows approximately 90% of PL, but the culture was finalized in Las Palmas when 70% of the larvae had reached PL. The remaining 30% was zoea XI, which showed cannibalism during the process of metamorphosis to PL, thus preying upon newly metamorphosed PL.

Parameters	Units	E1	E2
Temperature	°C	28.8 ± 0.4	28.8 ± 0.5
pH		7.9 ± 0.1 (a)	7.7 ± 0.1 (b)
Salinity	‰	14 ± 0	13 ± 0
Ammonium (NH_3 NH_4^+)	mg L^{-1}	0.05 ± 0 (a)	0.17 ± 0.18 (b)
Nitrite (NO_2)	mg L^{-1}	0.3 ± 0 (a)	0.35 ± 0.2 (b)
Nitrate (NO_3)	mg L^{-1}	500 ± 0 (a)	3.5 ± 1.9 (b)

Note: Means with different superscript letters are significantly different between treatments ($p < 0.05$).

TABLE 4 Water quality parameters data, during production cycles (E1) reused water and (E2) freshly prepared water, recorded in the distribution tank during 23 and 21 days respectively

TABLE 5 Mean ± SD ionic concentration (mg L^{-1}) in production tanks ($n = 4$) per week of the production cycle, with reused (E1) and freshly prepared water (E2)

Treatment	Ions	Dates of sampling (post-hatching day)				*p*-Value
E1		6	11	16	23	
	Calcium	196.4 ± 12.9	215 ± 37.4	253 ±55.7	236.2 ± 50.8	0.110
	Magnesium	285.6 ± 13.7 (a)	273.8 ± 27.2	261.8 ± 45.8	237.6 ± 26.3 (b)	0.029
	Potassium	158.3 ± 3.0 (a)	150.2 ± 11.7	117.7 ± 12.4	107.1 ± 9.6 (b)	0.030
	Sodium	5100.0 ± 316.2	5015.0 ± 375.3	5164.5 ± 157.4	5423.5 ± 113.8	0.191
	Chloride	9008.1 ± 443.7	8919.4 ± 637.9	8777.4 ± 78.7	9105.7 ± 93.9	0.561
	Mg/Ca	1.5 ± 0.2 (a)	1.3 ± 0.3	1.1 ± 0.2	1.0 ± 0.2 (b)	0.030
E2		6	11	15	21	
	Calcium	217.3 ± 43.9	244.0 ± 58.14	186.7 ± 13.1	207.0 ± 3.8	1.000
	Magnesium	526.0 ± 8.8 (a)	407.9 ± 37.7	401.6 ± 69.2	415.5 ± 46.2 (b)	0.029
	Potassium	155.4 ± 5.1	154.5 ± 14.7	151.7 ± 40.6	159.2 ± 33.0	0.309
	Sodium	3790.2 ± 49.1	3080.7 ± 480.4	3418.3 ± 233.1	3683.6 ± 130.8	0.309
	Chloride	7117.7 ± 20.4 (a)	6043.9 ± 639.9	6336.8 ± 507.5	6789.2 ± 67.9 (b)	0.027
	Mg/Ca	2.5 ± 0.5	1.7 ± 0.4	2.2 ± 0.5	2.02 ± 0.2	0.112

Note: Means with different superscript letters are significantly different from initial to final ionic concentration by treatment ($p < 0.05$).

TABLE 6 Ionic concentration (mg L^{-1}) (median) in production tanks ($n = 4$) of the production cycle, with reused (E1) and freshly prepared water (E2)

Treatment	Calcium	Magnesium	Potassium	Sodium	Chloride	Mg/Ca
E1	206.0	265.4	132.4	5210.1	8946.0	1.2
E2	204.5	437.1	159.1	3627.4	6762.8	2.1
p-value	0.3858	$1.679e^{-06}$	0.00786	$1.4e^{-06}$	$1.375e^{-06}$	$9.39e^{-06}$

Note: *p*-value < 0.05 are significantly different by treatment.

TABLE 7 Coefficient of correlations between ionic concentration (mg. L^{-1}) and Mg/Ca ratio and survival percentage, larval stage index (LSI) and post-hatching days (phd) in production tanks ($n = 4$) with reused (E1) and freshly prepared water (E2)

Treatment		Ca	Mg	K	Cl	Na	Mg/Ca
E1	Survival	−0.23	0.54[a]	0.78	−0.26[a]	−0.55[a]	0.44[a]
	LSI	0.43	−0.52[a]	−0.85	0.09[a]	0.46[a]	−0.65[a]
	phd	0.53	−0.55[a]	−0.87	−0.08[a]	0.48[a]	−0.66[a]
E2	Survival	−0.22	0.03	0	−0.15	−0.34	0.02
	LSI	0.12	−0.11	0.2	0.42	0.57	−0.06
	phd	0.19	−0.01	0.2	0.5	0.56	−0.01

Note: Coefficient of correlation with an asterisk.

[a]Indicates simple Pearson correlation significant values.

A similar LSI was found from post-hatching day 6 until the final day in both treatments. Nandlal and Pickering (2005) mentioned that, in captivity, all larvae developed at the same pace up to stage IV (synchronous larval development), from which the timing of moulting and appearance of the developmental stages differed between individuals until the PL stage. From 16 post-hatching days of both production cycles, the presence of three larval stages was observed in some tanks, which could also be related to decrease in the survival rate. Accordingly, Valenti et al. (2010) suggested that 3 or 6 stages occurring simultaneously in a production tank can generate problems in feeding, optimum parameters of production or emergence of illness.

The technique normally used to evaluate the performance (status and quality) of the larvae through daily observation was adequate and has allowed the optimization of the production management and operation protocols. From days 14 and 15 (for E1 and E2 respectively) until the final day of harvest, the larval condition index was recorded, which resulted in an average classified as good (1.24 ± 0.05 and 1.63 ± 0.05 for E1 and E2 respectively) in both treatments.

4.2 | Water quality

Over the duration of both production cycles, temperature, pH and salinity were within the acceptable range recommended for *M. rosenbergii* larval production (Valenti et al. 2010). The management procedures in the hatchery probably favoured these water quality conditions, including cleaning, filter change, tank siphoning, heathers with temperature control and tank covers.

The ammonia concentrations during the evaluations in E1 and E2 were within the safety level for *M. rosenbergii* larvae. Ammonia fluctuations in E2 were related to filter reactivation, since E2 treatment was performed with freshly prepared water as well as to biomass increment and prepared feed addition. Armstrong et al. (1978) suggested a LC_{50} (lethal concentration media) of 12.65 mg L^{-1} at 144 h, while Mallasen and Valenti (2005) found a LC_{50} of 8 mg L^{-1} at 96 h, with safety levels of total ammonia nitrogen at 1.2 and 0.8 mg L^{-1}, for *M. rosenbergii* larvae, reported, respectively, for each author.

Concerning nitrites, concentration in E1 was kept at 0.3 ± 0 mg L^{-1} during the evaluation period, probably due to the stability of the biological filters of each tank connected to the RAS. The maximum value of nitrite during E2 cycle was 0.6 mg L^{-1} (0.18 mg L^{-1} NO_2 – N). Accordingly, to Mallasen and Valenti (2006) *M. rosenbergii* larvae can tolerate chronic exposure to concentrations as high as 2 mg L^{-1} NO_2–N, without significant changes in survival, growth and general development, increasing ambient nitrite (NO_2-N) to 16 mg L^{-1}, delayed larval development, retarded larval growth and caused mortality.

Nitrate, meanwhile, does not represent a toxic element for *M. rosenbergii* larvae. According to Mallasen et al. (2004), survival rate, weight gain and larval development were evaluated for different concentrations of nitrate in two different phases: from zoea I through VIII and from stage VIII through PL metamorphosis. No effect was observed for concentrations up to 180 mg L^{-1} NO_3-N, and nitrate levels as much as 1,000 mg L^{-1} NO_3-N did not affect survival for zoea I through VIII. From stage VIII through PL metamorphosis, according to a linear model, the rate of survival and metamorphosis decreased as nitrate concentration increased, Mallasen et al. (2004). The nitrate concentrations throughout E1 initiated and consistently maintained a concentration of 500 mg L^{-1} (112 mg L^{-1} NO_3 - N) because the RAS did not consider a further nitrate reduction treatment and because no makeup water was added during the process. However, in the freshly prepared mixture (E2), nitrate concentration increased until 5 mg L^{-1}.

Therefore, the results indicate that water quality parameters in both production cycles did not interfere with the production results obtained in this work. Consequently, it can be ensured that the filtration systems and the daily maintenance and cleaning operations allowed the preservation of the water quality in the production tanks; however, it is necessary to mention that the accumulation of nitrates in the RAS, despite not having generated mortality or delayed larval development during the E1 cycle, should be controlled to avoid a future problem.

4.3 | Water ionic composition

As mentioned previously, the ionic composition of the culture water is important, so that the *M. rosenbergii* larvae can carry out their metabolic and osmoregulation processes, without adding a greater expenditure of energy. Experimental studies performed by Mallasen and Valenti (1998b) and Ismael et al. (2001) were carried out to determine the optimal ionic composition (macro and microelements) for M. *rosenbergii* larviculture, evaluating production parameters and larval metamorphosis.

Later studies, from Tavabe et al. (2013), Rafiee et al. (2015), Raizada et al. (2015) and Tavabe et al. (2017), defined through experiments with individual concentrations of main ionic elements (calcium, magnesium, potassium and sodium) and their interactions, the consequences of their deficits in terms of the processes of survival, metamorphosis and osmoregulation with the evaluation of Na/K-ATPase activity.

The objective of this work was to evaluate at a commercial production level, how the productive parameters would be affected with the continuous reuse of artificial seawater in a RAS, in that sense it can be seen that RW (E1) and freshly prepared water (E2) did not present ion deficiencies for *M. rosenbergii* larval production in terms of the values suggested by Tavabe et al. (2013), Tavabe et al. (2017) for Ca (240 mg L^{-1}); Mg (300 mg L^{-1}); K (150 mg L^{-1}) and Na (4000 mg L^{-1}).

Comparatively, the culture initiated with approximately half the concentration of magnesium (285.6 ± 13.7 mg L^{-1}) and constant decrease in E1 with respect to the E2 cycle showed a moderately positive correlation with survival and a negative correlation with LSI. Accordingly, Hangsapreurke (2008) stated that *M. rosenbergii* larvae require a magnesium concentration around 574 mg L^{-1} in environmental water, while low survival occurred during the final larval stage related to magnesium depletion in the culture water. Tavabe et al. (2013) quoting Weiling *et al.* (1995) mentioned that they compared brackish water prepared with a mixture of chemicals

in freshwater and deep well water, for *M. rosenbergii* larviculture. They found suitable contents of Mg^{2+} (300.0–440.0 mg L^{-1}) and Ca^{2+} (170.0–244.0 mg L^{-1}) in the mixed water, and the Mg^{2+}/Ca^{2+} ratios ranged from 1.8 to 2.2. Nevertheless, the authors did not investigate the interaction of several combined concentrations of the ions for optimal giant river prawn larviculture. During the production process, it was possible to observe a decrease in magnesium ion towards the end of the cycle ($p = 0.029$) in both production cycles, which indicate the use of this element by the larval biomass during the moulting process.

Calcium ion concentration did not show significant differences in E1 and E2, although, as mentioned by Tavabe et al. (2013) quoting Wilder *et al.* (2009) that calcium and magnesium levels in hatchery water greatly affect moulting frequency, a moderate correlation was found for calcium ion in E1 with metamorphosis, since the magnesium–calcium relationship showed a decrease from 1.5 to 1 during the production cycle, with a moderate negative correlation with the metamorphosis process. Tavabe et al., (2013) mentioned that *M. rosenbergii* growth, survival, development and overall larval quality were strongly affected by different concentrations of calcium and magnesium both separately and in combination. They indicated that a balance of calcium and magnesium in larviculture is very important after the 6th stage (about 10 days post hatching) of larval development and that the Mg^{+2}/Ca^{+2} ratio of the larval culture water should be maintained at about 1.25. The median value of Mg^{+2}/Ca^{+2} ratio in E2 was 2.1, which is superior to the value suggested. Meanwhile in E1, the average ratio was close to 1.0 ± 0.2, during the last week of culture period when the highest mortalities were observed.

Tavabe et al. (2015) found that the interaction of 4000 mg L^{-1} sodium and 150 mg L^{-1} potassium resulted in the best performance for *M. rosenbergii* larviculture, providing the highest final survival to PL metamorphosis ($40.6\% \pm 2.5\%$) in comparison of treatments with less concentrations.

The larvae of *M. rosenbergii* do not possess the same osmoregulatory capacities as adults (Brown et al., 2010). The osmoregulation system in these primary stages is not completely developed. During the larval stages, these processes were associated with the activity of Na/K-ATPase enzyme (Huong et al., 2004). Therefore, the potassium concentration during the production cycles is important for the activation of this enzyme (Raizada et al., 2015). This is probably the reason why our results showed a very strong correlation with survival and metamorphosis, due to the depletion of potassium during E1 treatment.

5 | CONCLUSIONS

The evaluation of both reused and freshly prepared water allows us to conclude that the RAS in the commercial hatchery can maintain the water quality in adequate conditions for the production of *M. rosembergii* larvae. However, the continuous use of water in the system presents the drawback of accumulation of nitrates and deficiency of magnesium and potassium ions, as well as accumulation of sodium and chlorides for up to 240 days of water reuse in seven continuous production cycles, under the protocols defined by hatchery operation.

The concentration of potassium and magnesium ions through the use of the water in the RAS could affect larval survival and metamorphosis. Therefore, the strategy to keep the water prepared in the recirculation system for several production cycles would consist of a continuous evaluation of the concentrations of those ions, supplying them when necessary, with locally available products. The magnesium/calcium ratio also has to be raised to appropriate levels, given its potential effect in metamorphosis.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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AUTHOR CONTRIBUTIONS

Maria Cristina Miglio: conception and design, acquisition of data, analysis and interpretation of data, writing the manuscript; Braulio Zaga: acquisition and preliminar analysis of data; Jose Carlos Gastelu: contributions to conception and design; William Severi: critical review of the content of the manuscript; Silvio Peixoto: critical review of the content of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, [MM], upon request from the reviewers.

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3.2 Capítulo II: articulo a ser presentado para su publicación en la Revista Aquaculture International

Quality assessment of *Macrobrachium rosenbergii* post larvae produced in commercial hatcheries using formalin and ammonia stress test

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ABSTRACT

The quality of *Macrobrachium rosenbergii* post larvae (PL) produced in two commercial hatcheries, were evaluated using stress test of formalin (TF) and ammonia (TA). The PL were produced using a recirculation aquaculture system (RAS) with freshly prepared artificial seawater (trial E1) and a static system with natural seawater (trial E2). In a second phase, the ionic balance of the water in the RAS was adjusted with the addition of magnesium ion (trial E3). Initially, the Median Lethal Concentration (LC_{50}) was calculated for each of the compounds, the calculated LC_{50} concentration was used for the final stress tests. Thus, at the end of each trial, 120 PL were exposed to formalin (600 mg/L 1 hour) and ammonia (30 mg total ammonia/L 24 hours). A survival of 60% or more was the criteria to define good PL quality. The PL from trials E1 and E2 were considered to be of good quality. However, there were significant differences between the TA (75%) and TF (48 to 57%) tests in the E3 trial, the PL were not considered of good quality. In all stress tests, the control group presented 100% survival. Additionally, PL from trial E3 were fixed in Davidson solution and preserved in 70% ethanol for gills histological analysis. The histological analysis of the gills of PL (E3) showed slight damage in the TA stress test and moderate to severe effects in TF stress test. The stress tests using ammonia and formalin proved to be an effective tool to detect weak or stressed PL of *M. rosenbergii*, thus their implementation under the hatchery conditions is recommended.

Key words: formalin, ammonia, stress tests, post larvae quality.

1. INTRODUCTION

The giant freshwater prawn *Macrobrachium rosenbergii* is among the six commercially most important species of crustaceans, with a global production of 234 thousand tons, representing around 2.5% of the total crustacean production (FAO, 2020). It is mainly produced in Asian countries such as China, Bangladesh, Thailand, Vietnam, Myanmar, Taiwan and Malaysia (FAO, 2023).

Daniels *et al.* (2010), mention that the most common source of broodstock for commercial hatcheries are adult prawns obtained from production ponds, although it is also common for producers to obtain wild gravid females from the natural environment. Habashy (2013) evaluated the reproductive behavior of *M. rosenbergii* under laboratory conditions, he concludes that the number of hatched eggs and larvae was directly proportional to the size of the reproducers, presenting a linear relationship between fecundity and the weight and length of the females. Wilder et al., (2004) report that, in the production of *M. rosenbergii* in Vietnam, there is a low maturation rate of the female in captivity, they begin to carry eggs very early, even at 7-10 g, which it leads to poor quality eggs and larvae, and in successive generations, the females mature even earlier. They report that, in nature, female prawns do not usually become pregnant until 20-40 g and the eggs obtained from these females are of good quality and provide high survival rates after hatching. Gravid females migrate to estuaries to spawn and carry eggs for up to three weeks, during which time the eggs gradually change to an orange-brown coloration until finally turning gray. After two to three days, the larvae hatch as zoea in estuarine environments and go through eleven larval instars (Uno and Kwon, 1969) before metamorphosing to PL, then migrating to freshwater bodies. When the larvae are released after hatching, they swim upside down and tail forward (New, 2002) and have a translucent coloration, revealing all their organs (Brown *et al.*, 2010). Upon reaching the PL stage, they resemble adults, changing from a free-swimming and pelagic to a benthic condition, becoming positively reotactic so they can migrate upstream (New, 2002).

In aquaculture, the larviculture of this species is carried out by obtaining and developing the larvae in brackish water (12 - 16 parts ‰) until completing metamorphosis into PL, which can now be cultured in freshwater. The larvae survival rate is the most common way to measure the efficiency of PL produced in a hatchery, while larval quality can be ensured by maintaining an adequate genetic, nutritional and health status of the broodstock and providing correct larval nutrition. Genetic improvement programs with *M. rosenbergii* have been conducted in Vietnam, China, India, Thailand and Indonesia, with the major selection trait being growth rate (Pillai *et al*. 2022). The quality of *M. rosenbergii* larvae has also been evaluated in relation to the duration of larval cycle, appearance of the first PL stage, development of the metamorphosis process, observation of the moulting process, among others. The larval quality index (LQI) presented by Tayamen and Brown (1998) has been useful to monitoring the status of the hatchery operation and overall larvae health in different rearing

stages, by scoring several quality criteria observed in the microscope. Usually, after larval metamorphosis to PL is completed (90 % of PL), the individuals must be acclimatized to freshwater. This procedure is done in tanks, where water is progressively changed in 24 to 48 hours, from 14 – 12 ‰ to 0 ‰ or depending of the country 2 to 3 hours in Thailand and 6 to 8 hours in China (New et al., 2010). PL are feed with formulated food and tanks are cleaned and 50 % of water is replaced daily (New et al., 2010).

PL can be sold after some days, the quality characteristics of the PL that must observe and considered as a quality protocol are: size homogeneity, synchronized swimming and against the current, black eyes, not be in molting. And under the microscope or stereoscope observe full and vacuolated hepatopancreas, peristaltic movement of the intestine, first portion of intestine filled, absence of necrosis and white spots on the abdominal muscles, absence of ectoparasites and fungi and absence of visible mortality (Gastelu, pers.comm. 2022). The quality of the larvae produced in shrimp (*L. vannamei*) hatcheries is evaluated based on standard indicators which include: growth rate and size (size homogeneity), nutritional status, health and general condition, and biochemical composition of the body, resistance to stress (stress test) and symptoms of the disease including using molecular methods for identifying viral and bacterial contamination (Mirzaei et al. 2021). Cuellar-Anjel et al. (2010) suggested that the quality of the shrimp PL should be the main concern in hatcheries, since their performance and resistance during culture conditions depends on it. They published a manual of good practices for *L. vannamei* hatcheries using larval quality evaluation and different stress tests as main criteria, measuring the tolerance (i.e., survival) of the animals in relation to a known extreme parameter. Therefore, PL that reveal a good resistance to stress tests will also probably be the strongest under environmental stress conditions.

Formalin stress test consists of exposing *L. vannamei* PL for a certain period to a solution of formalin, whose concentration varies according to the age of the PL (Hernandez-Herrera 2001). Leal *et al.* (2018) mentioned that formalin is one of the most applied chemicals in intensive aquaculture as a prophylactic measure or with therapeutic purposes. Khasani et al. (2017) tested concentrations varying from 500 to 750 mg/L of formalin in PL of *M. rosenbergii* for an hour. Bart and Yen (2003) performed diverse challenges (oxygen, salinity and formalin) to compared the performance of PL of 27 days old *M. rosenbergii* from Thailand and Vietnam. Among them, they applied a predictive test of formalin of 2 % (200 mg/L), observing that the time required to reach 90% mortality was between 74 and 95 h, while 50 % mortality was attained within 46 to 48 h. Khasani et al. (2022), using methods suggested by a National Standard of Indonesia for *M. rosenbergii* seed, applied tolerance test to three populations of giant freshwater prawn from genetic improvement programs to several environmental stressors (pH, temperature, salinity, and formaldehyde). These tests were carried out by giving sudden changes in salinity and temperature, and exposure of the juvenile PL to low pH and formalin. The

tolerance test to formalin was carried out by transferring PL directly to media with 500 mg/L of formaldehyde. The mortality of the PL was observed at 15, 30, 45, 60, and 120 minutes post the test to calculate the survival rate.

The ammonia stress test was also recommended as an effective criterion of *M. rosenbergii* larval quality (Cavalli et al. 2000). Studies showed that the lethal concentration (LC_{50}) for *M. rosenbergii* larvae ranges from 0.43 to 3.41 mg NH_3/L for a period of 96 hours at pH of 9 (Mallasen and Valenti 2005), and between 0.54 to 2.02 mg/L of N-NH_3 at pH 9 to 9.5 (Straus et al. 1991). Liu et al. (2022) used a stress test of ammonia to assess the effects of 8 weeks feeding trail with tea tree oil (TTO) in the growth, physiological and no specific immunity response of GFP using 20 mg/L ammonia stress test for 24 hours. These authors recorded the survival rate every 6 hours, which was significantly improved by the dietary TTO, being the highest mortality (around 60%) in the control group. As mentioned by Lin et al. (2022), toxic effects of ammonia on aquatic crustaceans can affect osmoregulation and excessive ammonia concentrations could eventually induce high mortality, thus they argued that semi-lethal concentration (LC_{50}) is the most intuitive indicator of the acute toxic effect of ammonia nitrogen on crustaceans. Romano and Zeng (2013) pointed out that ammonia stress on aquatic crustaceans could cause various gill morphological and physiological changes, including epithelial thickening/sloughing, lamellae constriction/collapse, disruption/destruction of pillar cells, necrosis, and hemocyte infiltrations. Dutra et al. (2017) evaluated the damages caused to the gill structure of *Macrobrachium amazonicum* juveniles subjected to different ammonia and nitrite concentration by histological analysis.

In Peru the production of *M. rosenbergii* is relatively low, for the period 2012 to 2021 the average production was 29 ± 18 metric tons and all of it is produced in the San Martín Region (PRODUCE, 2021), 31 % of the concession areas are used for monoculture or polyculture with tilapia. The Regional Aquaculture Plan of the regional Government of San Martín from 2014 to 2023, (GORE SAN MARTÍN, 2013) proposes as a strategy to consolidate *M. rosenbergii* production in the region, although one of the problems identified in the diagnosis of Regional Aquaculture Plan, as well as in the Macroregional workshop on innovation and future of aquaculture and fishing in the northeastern macroregion carried out by the PNIPA project in 2017, have been, the insufficient supply of quality seed for both fish and prawn.

Therefore, considering the above, the main goal of this study was to evaluate the quality of *M. rosenbergii* post larvae produced with different management systems in two commercial hatcheries using stress tests of formalin and ammonia.

2. MATERIALS AND METHODS

2.1 Post larvae origin and hatchery procedures

Post larvae of *M. rosenbergii* were produced at the Las Palmas hatchery, Tarapoto, San Martin, Peru using a recirculation aquaculture system (RAS). Two trials were performed with freshly prepared artificial seawater (E1), (Temperature 28 – 30 °C; pH 7.6 – 7.8; Salinity 13 ‰; Total ammonia 0.2 - 0.6 mg/L; NO_2 0 - 0.6 mg/L; NO_3 1.0 - 5.0 mg/L; Ca 204.5 mg/L; Mg 437.1 mg/L; K 159.1 mg/L; Mg/Ca 2.1) and with reused artificial seawater adjusted with magnesium ion replacement (E3), (Temperature 28 –28.7 °C; pH 7.7; Salinity 13 ‰; Total ammonia < 0.05 mg/L; NO_2 0. 1 mg/L; NO_3 5.0 mg/L; Mg 560 mg/L; Ca 160 mg/L; K 180 mg/L; Mg/Ca 3.5) using four production tanks of RAS for each trial.

Another trial was done with zoea I sent from Las Palmas hatchery to Aquaprawn hatchery, Chaclacayo, Lima, Peru, and were produced in a static system with natural seawater (E2) (Temperature 30 °C; pH 8.1 – 8.2; Salinity 16 ‰; Total ammonia 0.1 -1.0 mg/L; NO_2 0.1 – 6.00 mg/L; NO_3 120 -150 mg/L; Mg 401.6 mg/L; Ca 291.4 mg/L; K 137.2 mg/L; Mg/Ca 1.4). Aquaprawn uses 6 tanks of 350 L. Initially, a tank is stocked with 270 thousand larvae zoea I and after reaching zoea 3 (day 3) the tank is split in two tanks. Once zoea 5 stage is reached (day 6) the larvae content is split into 4 tanks, then after reaching zoea 8 stage (day 13) split into 5 tanks and finally in zoea 9 stage (day 15) into 6 tanks. Every two days, 50% of the water in the tanks is replaced with water treated in mechanical and biological filters.

The operational procedures to produce *M. rosenbergii* larvae in each of these trials are summarized in table 1.

The PL acclimatization to freshwater at Las Palmas hatchery (E1 and E3) began when larvae were metamorphosed to PL and individuals started to position themselves at the bottom of the culture tank and into the shelters. PL were collected, counted and transferred to rectangular concrete tanks of 1,300 L, where acclimatization to freshwater took place. In the first 24 hours, PL were transferred from 10 to 3 ‰ and in the next day from 3 to 0 ‰. The water used to reduce salinity is the same used during the growing phase, being continuously aerated, the values from temperature are between 26 – 28.5 °C, keeping ammonium levels low (total ammonia < 0.05 mg/L), as well controlling pH (7.7 to 8.0).

In the Aquaprawn hatchery (E2), the acclimatization process was carried out in the same culture tank, when approximately 90 % of the population had metamorphosed to PL. This process started by lowering the water level to half and completed with freshwater, allowing salinity to gradually decrease from 16 to 8 ‰, then to 4 ‰ and finally to 2 ‰ during approximately five to seven days. Before adding the freshwater, the temperature variation was not greater than 1°C. The PL are sold acclimatized at 2 ‰ and the process of acclimatization to freshwater (0 ‰) is only completed when they are stocked in the ponds designated by the buyer.

Table 1: Operational procedures used in the trials E1, E2 and E3 to produce *M. rosenbergii* larvae at Las Palmas and Aquaprawn hatcheries in Peru.

Operational procedures	Trial E1 (22/09/2018) Las Palmas hatchery	Trial E2 (03/12/2018) Aquaprawn hatchery	Trial E3 (14/12/2021) Las Palmas hatchery
System	Recirculation Aquaculture System (4 tanks of 1000 L).	Static system (6 tanks of 350 L), 50% water replacement every two days.	Recirculation Aquaculture System (4 tanks of 1000 L).
Water origin	Freshly prepared artificial seawater (Blue Treasure SPS Sea Salt, ™ China)	Natural seawater	Reused artificial seawater in the RAS, adjusted with magnesium ion replacement
Initial larval density	100 larvae/ L (Zoea I)	270 thousand larvae (Zoea I)	100 larvae /L (Zoea I)
Cycle duration	21 days	22 days	25 days
First PL	Day 19 th	Day 20 th	Day 22 th
Final survival % range	45 – 61 %	38 % (total)	31 – 40 %
Salinity during production (‰)	13	16	13
Female broodstock size	30 g	30 g	15 g
Larvae quality index (LQI)*	Good	Good	Good

*Tayamen and Brown (1998) modified

2.2 Experimental design

In order to estimate the quality of PL produced commercially in each system, they were exposed to stress tests at the end of each trial. These stress tests were performed by exposing PL to formalin concentration of 600 mg/L for a period of 1 hour (TF) and to ammonia with a concentration of 30 mg total ammonia/L for a period of 24 hours (TA). The concentrations used here were defined in preliminary tests, where the median lethal concentration (LC_{50}) for formalin

and ammonium was determined.

Stress tests for E1 and E3 were conducted at the Las Palmas hatchery, whereas stress test for E2 was performed at Agrarian University La Molina, Lima, Peru.

2.3 Preliminary test of formalin and ammonia

Both preliminary tests using formalin and ammonia were performed with PL conditioned to freshwater. PL were maintained at a density of 20 PL/L (Racotta 2004), temperature of 28°C ± 1°C, pH 8.0 ± 0.2 and in constantly aerated fresh water. Feeding was suspended for 24 hours before the tests.

For the ammonia preliminary test, a 1 L container was used to keep 10 PL. The design was completely randomized, with four increasing total ammonia concentrations (20, 30, 40, 50 mg/L), three replicates and a control. The desired ammonia concentrations were prepared from the addition of appropriate volumes of the NH_4Cl stock solution (1000 mg/L NH_4Cl stock solution; SPECTRUM). Ammonia is present in water in two forms, unionized (NH_3) and ionized (NH_4^+) (in this study, the term "ammonia" refers to the sum of NH_3 and NH_4^+).

For the preliminary test of formalin, five concentrations were used (40, 200, 400, 600, 800 mg CH_2O /L), with three replicates and a control. The formalin concentrations were prepared from a 40% commercial formaldehyde stock (Laboratorio ALKOFARMA EIRL).

The criterion used to determine the median lethal concentration (LC_{50}) was mortality observation after 24 hours for ammonia test and one hour for formalin test. PL with total absence of any vital signs and that did not react to mechanical stimuli using a stylus were considered dead. Values of median lethal concentration (LC_{50} mg/L) for ammonia and formalin were estimated with the PROBIT method using a linear regression at 95% confidence. The LC_{50} of ammonia and formalin was used during the final stress tests.

2.4 Stress tests at the end of trials

At the end of trials E1, E2 and E3, ten PL were distributed into 1 L rectangular plastic containers, which were considered as experimental units. For each stress test (ammonia and formalin), ten experimental units and two control groups were used with a total of 120 PL. Aeration was kept constant at saturation. Temperature was maintained by placing the containers in a water bath with thermostats. Two stress tests for formalin (E3 TF1 and E3 TF2) were carry out during trial E3. Stress tests were carried out following the conditions summarized in table 2.

After one hour exposure to formalin and 24 hours to ammonia, the number of living PL was recorded. For the stress test results to be acceptable, survival in controls must be at least 90% USEPA (1993). A survival of 60 % or more was considered the acceptability criteria for good PL quality.

Table 2: Conditions for carrying out final stress test

	Formalin exposure stress test (TF)	Ammonia exposure stress test (TA)
Concentration	600 mg CH_2O/L	30 mg Total ammonia/L
Exposure time	1 h	24 h
Number of organisms	10 PL	10 PL
Replicates	10	
Number of control groups	2	
Feeding	Suspended 24 hours before testing	
Aeration	Constant at saturation	
Temperature °C	25 – 30 °C (Lobão, 1997)	
pH	7.5 – 8.3 (Lobão, 1997)	
Salinity	0 ‰	

The following variables of water quality were evaluated during the stress tests: temperature (digital thermometer Hidance - China or sensor YSI, USA), pH (pHmeter Aceche – China or Oakton® pHTestr®, USA) and salinity (refractometer Ketotec - China or EZODO PCT-407, Taiwan). Total ammonia was registered with colorimetric kit (JBL - Germany) for total ammonia (NH_4^+ /NH_3 detection limit 0.1 to 5.0 mg/L), dilution was made when necessary. Temperature and pH were measured at the end of the formalin test (1 hour), meanwhile total ammonia mg/L, temperature and pH were measured 0h, 12 h, 16 h, 20 h and 24 h in the ammonia test.

2.5 Histological analysis of PL gill tissues from trial E3

Samples of PL from trial E3 were collected during the experimental period and were fixed for 24 hours with Davidson's solution. Post larvae (n = 5) were randomly sampled prior the beginning of the stress tests. Samples of dead and surviving PL from TF1 and TF2 tests as well as dead and surviving PL from test TA were also sampled. All PL sampled were dipped directly into the fixer in a 1:10 ratio (10 times, for the volume of the fixative relative to that of the sample) and after 24 hours they were transferred to 70% ethanol (Morales and Cuéllar-Anjel 2008). PL samples were then sent to the Laboratory of Histology, Embryology and Animal Pathology of the Veterinary Medicine Faculty at UNMSM, where they were prepared for histologic analysis using protocols described by Morales and Cuéllar-Anjel (2008). Histological sections were made and stained with Mayer-Bennett Hematoxylin and Phloxin/Eosin (H&E) for general visualization of the affected tissues. The presence

of histological alterations was determined by the degree of tissue alteration observed under a light microscope (Nikon Eclipse E200, USA).

Histological changes were examined semi quantitatively according to Stalin et al. (2013). The histopathological parameters were classified as normal histological structure (0 %), mild changes (< 10 %), moderate changes (>10–50 %) and extended severe changes (>50 %). Data were represented as the mean of five slides from each sample of PL control (prior the beginning of the stress tests), dead and surviving PL from TF2 tests as well as dead and surviving PL from test TA.

2.6 Statistics

The PROBIT method was used for ammonia and formalin preliminary stress test to estimate the values of median lethal concentration (LC_{50} mg/L) using a linear regression at 95 percent confidence. The survival percentages for all trials as well as histological analysis of tissues from trial E3 were recorded in an Excel spreadsheet and the data processing was carried out in the statistical program R version 3.5.2. Since the different trials (E1, E2 and E3) were run independently, the statistical analysis was performed by TA and TF stress test for each trial. Generalized linear models were made with the binomial family, given that the repetitions were of 10 individuals each, and survival was analyzed. Means and standard errors were compared. In all cases, overdispersion was analyzed by Deviance, that is a measure of error; lower deviance means better fit to data. Deviance/N, whose proportion must have been less than 3. The histological analysis of gill tissues from trial E3 were analyzed qualitatively.

3. RESULTS

3.1 Preliminary test of formalin and ammonia

Data on the mortality percentage of the PL in the preliminary formalin and ammonia exposure stress test is presents in table 3. PL exposed to formalin exhibited 93.3 ± 5.8 % mortality after one hour at concentrations of 800 mg/L. Mortality of 13.3 ± 5.8% was observed at 200 mg/L and increased at higher concentrations. The LC_{50}-1h estimated for formalin was 648.23 mg/L, with a 95 % confidence interval from 710.92 mg/L to 543.45 mg/L. Therefore, the concentration used in the stress tests at the end of the trials was 600 mg/L.

In the stress test of ammonia, mortality of PL started at 20 mg/L and increased at higher concentrations until reaching 90 ± 0 % at 50 mg/L. At the concentration of 50 mg/L, PL presented abnormal behavior and absence of vital signs in the first hours of exposure. The LC_{50}-24h estimated for ammonia was 38,55 mg total ammonia/L with a 95 % confidence interval from 46.6 to 32.5 mg total ammonia/L. Therefore, the concentration used in the stress tests was 30 mg total ammonia/L.

Table 3: Preliminary formalin and ammonia exposure stress test results, mean (± SD) mortality (%) of PL, LC_{50}, and 95 % confidence interval (n=3 for each concentration).

Formalin concentration (mg/L)	Mortality (%)	Total Ammonia concentration (mg/L)	Mortality (%)
Control	0	Control	0
40	0	20	10 ± 0
200	13.3 ± 5.8	30	20 ± 0
400	13.3 ± 5.8	40	26.7 ± 5.8
600	16.7 ± 5.8	50	90 ± 0
800	93.3 ± 5.8		
LC_{50} (mg/L)	648.23		38.55
Confidence interval	543.4 to 710.9		32.5 to 46.6

3.2 Stress test at the end of the trials

The water quality parameters measured during the application of the stress tests are presented in table 4. Total ammonia in the control was under 0.05 mg/L. The mean values obtained here are within the acceptable range for the culture of *M. rosenbergii* PL.

Table 4: Water quality parameters measured during the application of the formalin and ammonia stress tests. Data correspond to the mean ± SD of ten repetitions and two control for formalin test and five measurements (0, 12, 16, 20 and 24 h) of ten repetitions and two control for ammonia test.

Formalin test (TF) 600 mg/L				Ammonia test (TA) 30 mg/L					
	Trial	pH	Temperature °C		Trial	pH	Temperature °C	Total ammonia mg/L	N - NH_3 mg/L
E1	Test	8.0 ± 0.0	28.1 ± 0.0	E1	Test	8.1 ± 0.1	27.3 ± 0.3	37 ± 17.8	2.4 ± 0.91
	Control	8.0 ± 0.0	28.0 ± 0.0		Control	8.0 ± 0.0	28.0 ± 0.0	<0.05	0.003 ± 0
E2	Test	7.8 ± 0.0	27.2 ± 0.0	E2	Test	8.2 ± 0.0	26.6 ± 0.8	28 ± 14.4	2.5 ± 1.3
	Control	7.9 ± 0.0	27.0 ± 0.0		Control	7.9 ± 0.1	27.0 ± 0.1	<0.05	0.003 ± 0
E3-1	Test	7.5 ± 0.0	29.0 ± 0.0	E3	Test	7.8 ± 0.2	27.8 ± 1.7	38 ± 8.3	1.7 ± 0.61
	Control	7.3 ± 0.0	29.0 ± 0.0		Control	7.9 ± 0.2	27.8 ± 1.8	<0.05	0.001 ± 0
E3-2	Test	7.8 ± 0.0	28.2 ± 0.0						
	Control	7.8 ± 0.0	28.2 ± 0.0						

Table 5 and figure 1, show the survival obtained after the application of the formalin and ammonia stress test in E1, E2 and E3 respectively. Deviance was < 3.

As shown in figure 1, the survival percentage (mean ± SD) during the exposure time of TF and TA for each trial did not present significant differences between TF E1 (71 ± 7.3%) and TA E1 (69 ± 9.9%), as well as between TF E2 (61 ± 6.3%) and TA E2 (62 ± 9.1%). Post larvae showed more than 60 % survival in both trials E1 and E2 for the TF and TA stress tests, fulfilling the acceptability criteria for good PL quality.

In the case of the formalin tests applied after the E3 test, TF1 E3 (57 ± 11.6%) and TF2 E3 (48 ± 10.3%) do not differ from each other. However, there were significant differences for the PL survival percentage between TA E3 (75 ± 12.6%) and the formalin stress tests applied for this trial. Being TA the only stress test that obtained a survival greater than 60% after E3 trial, the acceptability criteria of good quality of LP were not met.

In all cases, the post larvae of the control groups (0 mg/L) maintained 100 % survival and showed normal signs of behavior, such as foraging, territorial fighting, movement throughout the water column, and reaction to mechanical stimuli.

Table 5: Mean (± SD), minimum and maximum survival (%) of *Macrobrachium rosenbergii* postlarvae after the application of the formalin and ammonia stress test for each trial using different systems: (E1) RAS with freshly artificial seawater, (E2) static system with natural seawater and (E3) reused artificial seawater adjusted with magnesium ion replacement (n = 10).

Trial	Formalin test (600 mg/L)	Ammonia test (30 mg/L)
E1	71 (± 7.3) 60 – 80	69 (± 9.9) 50 – 80
E2	62 (± 6.3) 50 – 70	62 ± 9.1 % 40 – 70
E3 1	57 (± 11.6) 40 – 80	75 (± 12.6) 50 – 90
E3 2	48 (± 10.3) 30 – 70	

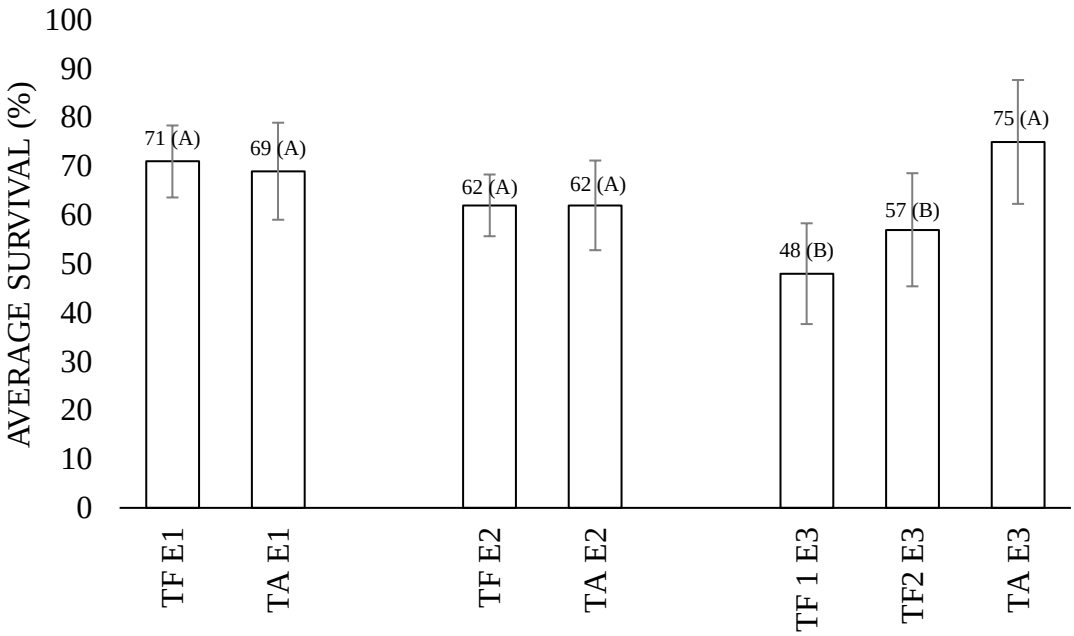

Figure 1: Survival percentage (mean ± SD) of *M. rosenbergii* PL during exposure time of TF and TA for each trial using different systems: E1 RAS with freshly artificial seawater, E2 Static system with natural seawater and E3 reused artificial seawater adjusted with magnesium ion replacement. Means with a common letter are not significantly different ($p > 0.05$) and Deviance/N < 3 for each trial.

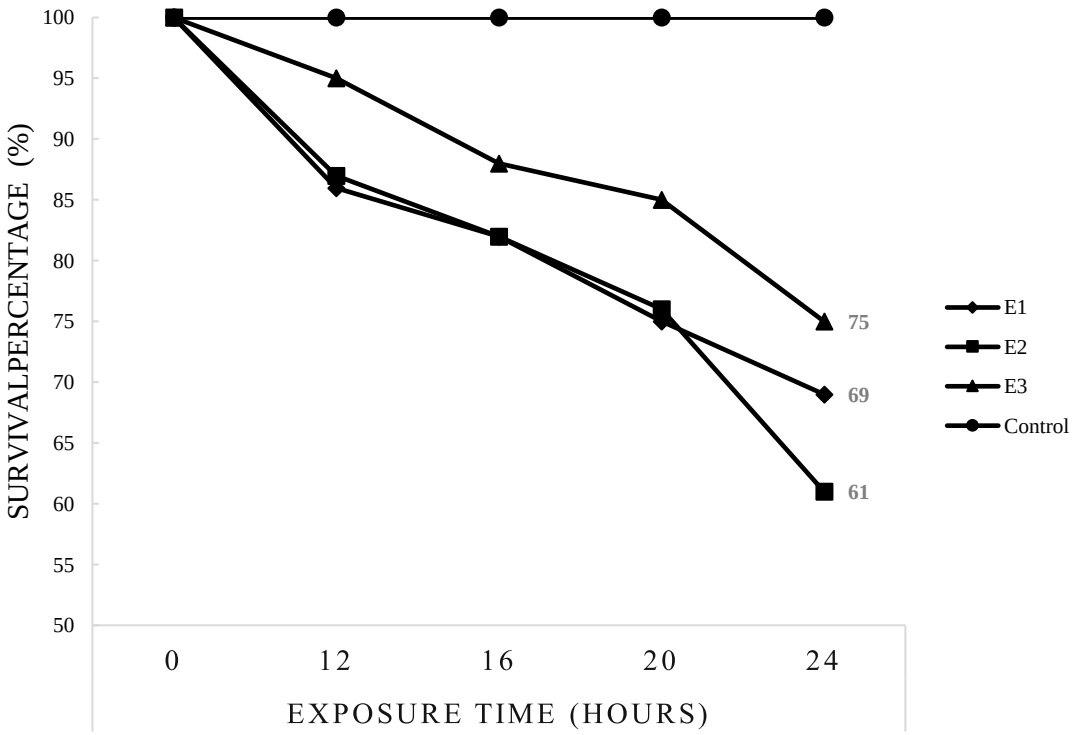

Figure 2: Survival percentage of *M. rosenbergii* PL during exposure time of TA for each trial using different systems, E1 RAS with freshly artificial seawater, E2 Static system with natural seawater, E3 reused artificial seawater adjusted with magnesium ion replacement, and controls.

Figure 2 shows the variations in PL survival during the ammonium stress test, after 12 hours of exposure and then with observations every 4 hours for each trial using different production systems. After the first 12 hours, survival dropped to approximately 85% for the E1 and E2 ammonia tests, and from that time on both tests presented a similar PL survival percentage, reaching a PL final survival of 69 % (E1) and 61% (E2).

In the case of the E3 ammonia test, after the first 12 hours PL survival decreased to 95%, the reduction in survival every 4 hours was less than in E1 and E2, reaching a final survival of 75%.

Figure 3 shows the variations in PL survival percentage when exposed to the formalin tests, the E1 and E2 tests presented a lower standard deviation than the tests carried out after trial E3.

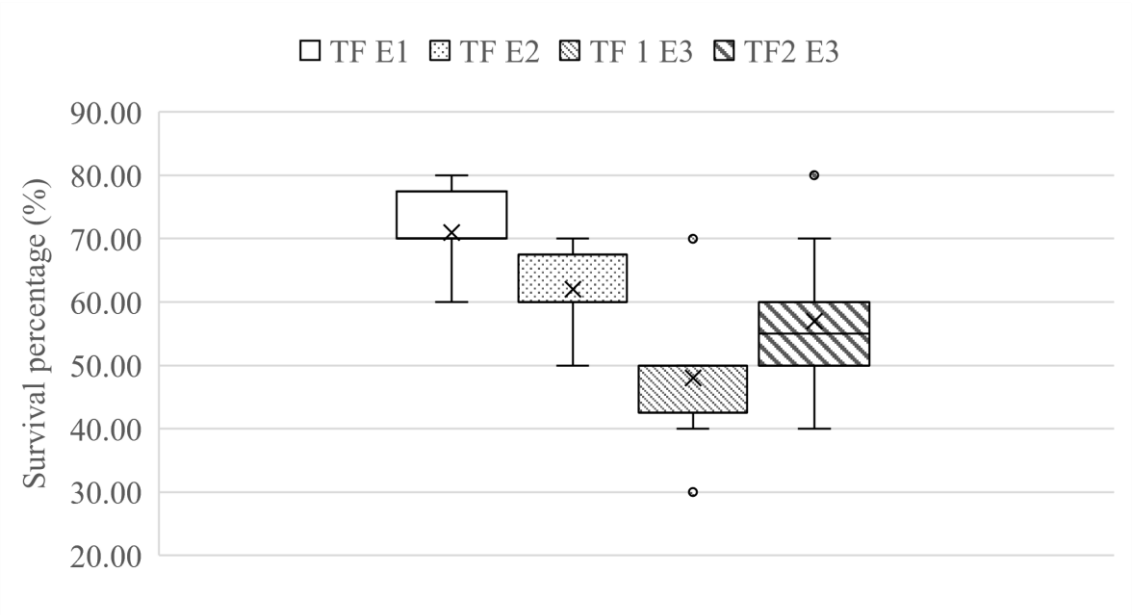

Figure 3: Survival percentage of *M. rosenbergii* PL during exposure time of TF for each trial using different systems, E1 RAS with freshly artificial seawater, E2 Static system with natural seawater, E3 reused artificial seawater adjusted with magnesium ion replacement.

3.3 Histological analysis of PL gills tissues from trial E3

Histological changes that were examined semi quantitatively are summarized in Table 6, the severity and frequency of lesions founded due to the exposure of formalin and ammonia were registered for the PL control and dead and survivals of TF and TA.

Table 6: Histological lesions observed in *M. rosenbergii* PL gills from trial E3 exposed to formalin and ammonia stress test, prior the test (control) and after stress test in PL dead and survivals.

Histological parameters	CONTROL	Dead TF	Survivals TF	Dead TA	Survivals TA
Swollen lamellae (SL)	+	+++	+++	-	++
Lamellae fusion (LFU)	+	++	++	+	++
Thickening of the lamellar epithelium	+	+++	+++	+	+
Haemocytic infiltration	+	+++	+++	+	++
Pyknosis	+	+++	++	+	-
Karyolysis	+	-	-	-	+

(-) None (0 %) (+) mild changes (< 10 %), (++) moderate changes (>10–50 %) and (+++) extended severe changes (>50 %)

The histological analysis of the gill tissues of the PL before being subjected to the stress tests presented some hemocyte infiltration, filament congestion, slight dilation of lymphatic vessels in lamellae hyperplasia and thickening of the lamellar epithelium as it is showed in figure 4.

The gills of PL that died because they were subjected to stress test TF 1 and TF 2 (600 mg/L for 1 hour of exposure), showed that the gill structure suffered harmful effects like severe hemolymph congestion in lamellae and gill filaments, thickening of lamellae and branchial filament, severe dilatation of the lymphatic vessels as it is shown in figure 5. Meanwhile those that survived (48%) test TF1, showed moderate congestion in the branchial filaments, dilatation of the lymphatic vessels and thickening of the lamellae and the branchial filament, but those from TF 2 (57%) showed severe hemolymph congestion in the gill filament and lamellae, and cellular hyperplasia at the apex of the filament as shown in figure 5.

The histological analysis of the gills of PL that died because they were subjected to stress test TA (30 mg/L for 24 hours of exposure) showed that the gill structure suffered harmful effects, like filaments with thin lamellae and slight infiltration of hemocytes, in addition to pyknosis, necrophagy was registered in some individuals, bacillary bacteria were observed around the lamellae, as well as loss of cellular detail and cytoarchitecture as it is shown in figure 6.Those who survived showed filaments of similar sizes with lamellae with slight infiltration of hemocytes and diffuse dilation of lymphatic vessels as shown in figure 6.

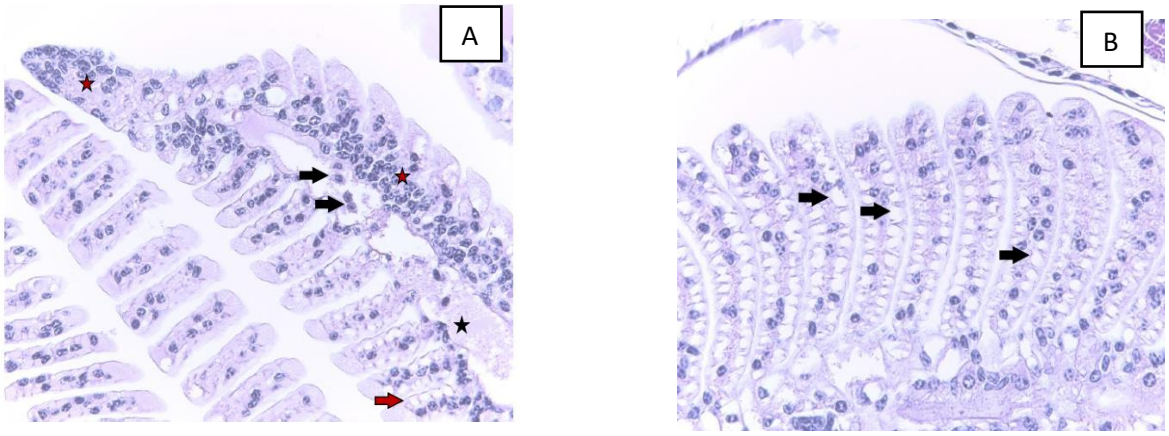

Fig. 4 Microphotography sets from gill histological analysis of *M. rosenbergii* PL exposed to stress test (TF and TA) performed in trial E3. Histological analysis of the gill tissues of the PL prior the beginning of stress tests (CONTROL). A: Hemocyte infiltration (black arrows), filament congestion (black star), slight dilation of lymphatic vessels in lamellae (red arrow) and hyperplasia and thickening (red star) are observed. Plate 1: 5 cuts. 40X, H&E stain B: Thickening of the lamellae with loss of space between them and moderate dilation of the lymphatic vessels in the lamellae (black arrow) is observed. Plate 1: 5 cuts. 40X, H&E stain.

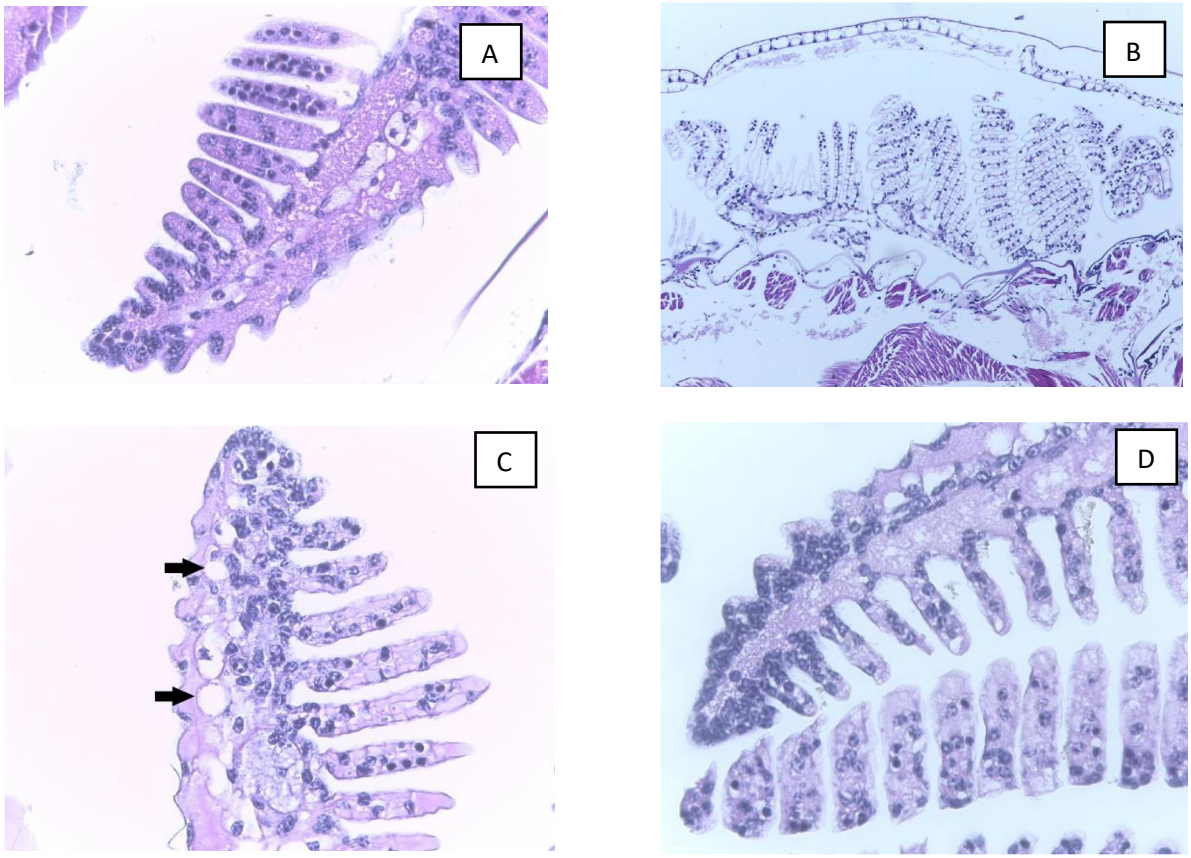

Fig. 5: Microphotography sets from gill histological analysis of *M. rosenbergii* postlarvae exposed to stress test TF performed in trial E3. A: Dead PL from the test TF1: Severe hemolymph congestion in lamellae and gill filaments, thickening of lamellae and branchial filament is observed. Plate 2: 5 cuts. 40X, H&E stain. B: Dead PL from the test TF2: Asymmetry of branchial filaments and lamellae, severe dilation of lymphatic vessels, with thickening of lamellae and branchial filament and loss of space between lamellae is observed. Plate 4: 5 cuts. 20X, H&E stain. C: Surviving PL from the test TF1: A moderate congestion in the branchial filaments, dilatation of the lymphatic vessels (black arrow) and thickening of the lamellae and the branchial filament are observed. Plate 3: 5 cuts. 40X, H&E stain. D: Surviving PL from the test TF2: Severe hemolymph congestion is observed in the gill filament and lamellae, at the apex of the filament a cellular hyperplasia is observed. Plate 5: 5 cuts. 40X, H&E stain.

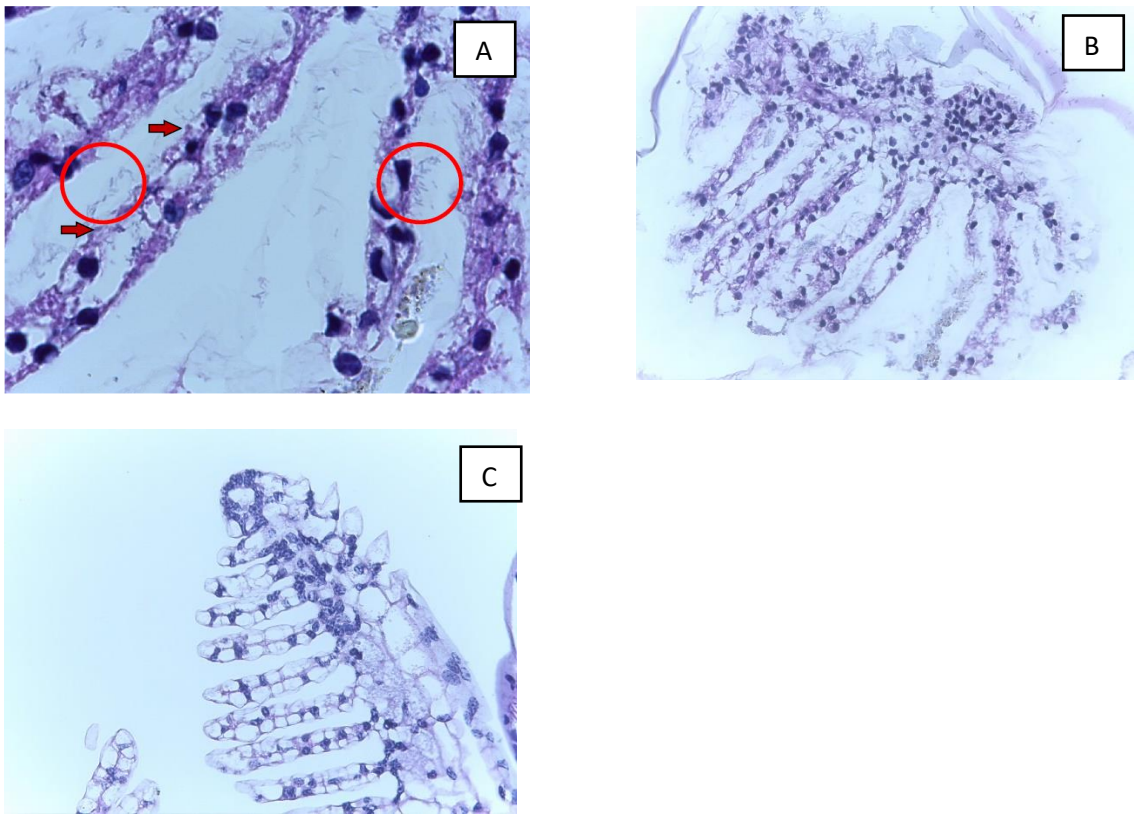

Fig. 6: Microphotography sets from gill histological analysis of *M. rosenbergii* postlarvae exposed to stress test TA performed in trial E3. A: Dead PL from the test TA: Bacillary bacteria are observed around the lamellae (red circles), as well as loss of cellular detail and cytoarchitecture (red arrows). Plate 6: 3 cuts. 100X, H&E stain. B: Dead PL from the test TA: Filaments with thin lamellae and slight infiltration of hemocytes are observed, in addition to pyknosis. C: Surviving PL from the test TA: Filaments of similar sizes can be seen with lamellae with slight infiltration of hemocytes and diffuse dilation of lymphatic vessels, not so open.

4. DISCUSSION

Calcium and magnesium are two macro-elements that are important for larval exoskeleton hardness, ecdysis frequency, haemolymph osmolality and larval survival (Adhikari et al., 2007; Rafiee et al., 2015; Tavabe et al., 2013). As previously described for the larviculture of *M. rosenbergii* using artificial sea water within a RAS (Miglio et al. 2021), it was also possible to observe a decrease in the concentration of the magnesium ion towards the end of the production cycle (trial E3). This could indicate the use of this element by the larvae during the molting process. As a strategy to keep the water adequate for several production cycles, it was suggested a continuous evaluation of the concentrations of magnesium ion in the hatchery RAS culture water. The production conditions in the trials E1 and E3, did not present ion deficiencies for *M. rosenbergii* larval production in terms of

the values suggested by Tavabe et al. (2013), Tavabe et al. (2017) for Ca (240 mg/L); Mg (300 mg/L); K (150 mg/L) and Na (4000 mg/L) and have been similar in terms of ion concentration, by supplementing magnesium ion in the artificial seawater reused in several production campaigns in the RAS. However, the results of larval survival rates in both production cycles differed, being higher (45 – 61 %) in the freshly prepared seawater trial (E1) when compared to E3 RAS system (31 – 40 %). Additionally, the production cycle was completed faster in E1 (21 days) than E3 (25 days). Furthermore, a possible explanation for the low total survival rate in trial E2, (38 %) was an excessive manipulation of larvae, as they were split in multiples tanks during the production cycle, because E2 did not present ion deficiencies as well. The nitrogen components in the trials constantly monitored resulting in appropriate values for the development of *M. rosenbergii* larvae: safety levels of total ammonia nitrogen at 1.2 and 0.8 mg/L (Mallasen and Valenti, 2005); chronic exposure to concentrations as high as 2 mg/L NO_2–N (Mallasen and Valenti, 2006); nitrate, does not represent a toxic element no effect was observed for concentrations up to 180 mg/L NO_3-N (Mallasen et al. 2004).

Wilder et al. (2004) referred that in Vietnam there is poor maturation rate of the *M. rosenbergii* broodstock females in captivity, as well as they began to carrying eggs more precociously in successive generations (e.g., 7-10 g), resulting in poor egg and larval quality. These authors argued that in the wild, female prawns do not usually become gravid until 20-40 g and eggs obtained from these females are of good quality and provide high survival rates after hatching. In the Peruvian hatchery of Las Palmas, broodstocks used in trial E1 and E2, weighed twice as those used in trial E3. Due to a prolongated drought in the northern area of the country, the stocking of ovigerous females taken from the ponds for E3 trial larvae production did not present an adequate size (11 to 15 g), which is probably related with the low PL survival at the final cycle of production in E3. However, the findings of the current study regarding larval quality index (LQI), according to the criteria proposed by Tayamen and Brown (1998) modified considered an LQI of "Good", similarly in the 3 trials.

The lethal concentration values (LC_{50}) for formalin and ammonia stress tests in our study are consistent with the literature. Our estimate for the LC_{50} for formalin (648 mg/L) is in line with Khasani et al. (2017) and Khasani et al. (2022), which applied a formalin concentration ranging from 500 to 750 mg/L in *M. rosenbergii* PL. Similarly, for the ammonia stress test, Dutra (2017) reported that a concentration of 20 mg/L resulted in 48% mortality rate after 96 hours in *M. amazonicum* juveniles, while mortality reached 100% in 40 mg/L during 72 hours, which are within the LC_{50} 24h of 38.55 mg total ammonia/L estimated for *M. rosenbergii* PL in our study.

Another important finding of the present study was reassuring that stress tests of formalin and ammonia are effective tools in detecting weak or stressed PL of *M. rosenbergii*. The survival rates

for both TF and TA in trials E1 and E2 were higher than 60%, whereas in trial E3 TA stress test showed a survival greater than 60 %, and TF resulted in less than 50% survival. This observation may not be supported by the hypothesis that mortalities below 20% (survival greater than 80 %) indicated that the *M. rosenbergii* PL were classified as tolerant to environmental stressors or suboptimal environment conditions (Khasani et al., 2022). Although this interpretation differs from Samocha and Pragnell (2019), who expected survival varying from 40 to 50% for *L.vannamei* PL 1 and PL 7 days, respectively, when exposed to formalin. These authors also reported that tolerance increases with shrimp age, ranging from 300 mg/L at PL1 to 600 mg/L at PL7. Villalon (1991) performed salinity tests for *L. vannamei* and suggested that when the survival is lower than 60 %, the batch of post-larvae should be rejected.

The data reported here seem to support the assumption that 4-day-old PL have already undergone a stress test, consisting of progressive conditioning from brackish to freshwater. This can be evidenced in the histological analysis of the gill tissues of the PL before being subjected to the stress tests which already presented some hemocyte infiltration, filament congestion, slight dilation of lymphatic vessels in lamellae, thickening of the lamellar epithelium, slight alterations that can be characterized as regressive damages (Dutra et al. 2017). Nevertheless, the evaluation of histological sections of dead PL showed that the gill structure suffered harmful effects such as severe hemolymph congestion in lamellae and gill filaments, thickening of lamellae and branchial filament. Those who survived the stress test showed moderate congestion and cellular hyperplasia. Dutra et al., (2017) observed that the higher the concentrations of total ammonia and nitrite, the greater the damages caused to the gill structure of juvenile of *M. amazonicum*, subjected to ammonia and nitrite concentrations. Furthermore, comparative analysis between PL that survived the ammonia and formalin stress test, in our study indicated only a slight damage in TA test and moderate to severe effects in TF stress test, indicating that 4-day-old PL are highly sensitive to the acute formalin test.

5. CONCLUSION

Stress tests of formalin and ammonia proved to be an effective tool to detect weak or stressed PL of *M. rosenbergii*, due to larval origin or rearing system conditions. It is therefore suggested that their implementation and feasibility of application must be evaluated under the conditions of each hatchery center. Although both formalin and ammonia stress tests can be considered as applicable protocols for the evaluation of PL quality, being that 4-day-old PL are highly sensitive to the acute formalin test than to the ammonia test.

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7. CONFLIC T OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

8. FUNDING

The author(s) received no specific funding for this work.

9. AUTHOR CONTRIBUTIONS

Maria Cristina Miglio: conception and design, acquisition of data, analysis and interpretation of data, writing the manuscript; Braulio Zaga: acquisition and preliminary analysis of data; Katia Albines: acquisition and preliminary analysis of data; William Severi: critical review of the content of the manuscript; Silvio Peixoto: critical review of the content of the manuscript.

10. DATA AVAILABILIT Y STATEMENT

The data that support the findings of this study are available from the corresponding author, [MM], upon request from the reviewers.

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4. Consideraciones finales

La evaluación del agua de cultivo tanto reutilizada como recién preparada permite concluir que el SRA en el criadero comercial puede mantener la calidad del agua en condiciones adecuadas para la producción de larvas de *M. rosenbergii*. Sin embargo, el uso continuo de agua en el sistema presenta el inconveniente de acumulación de nitratos y deficiencia de iones de magnesio y potasio, así como acumulación de iones sodio y cloruros luego de 240 días de reúso de agua, en siete ciclos continuos de producción, bajo los protocolos definidos por la operación de la hatchery comercial Las Palmas. La concentración de iones de potasio y magnesio a través del uso del agua en el SRA podría afectar la supervivencia y la metamorfosis de las larvas.

Por lo tanto, la estrategia para mantener el agua preparada en el sistema de recirculación por varios ciclos de producción consistiría en una evaluación continua de las concentraciones de esos iones, abasteciéndolos cuando sea necesario, con productos disponibles localmente. La relación magnesio/calcio también debe elevarse a niveles adecuados, dado su potencial efecto en la metamorfosis.

Para evaluar la calidad de las PL resultantes de ciclos de producción comercial se aplicaron pruebas de estrés por formalina y amonio. Las PL producidas en SRA con agua de mar artificial recién preparada y con agua de mar en un sistema estático con recambio de 50 % de agua cada dos días, fueron sometidas al mismo protocolo de pruebas de estrés logrando superar las pruebas de estrés con un valor mayor al 60 % de sobrevivencia, por lo que se consideraron de buena calidad.

Por otro lado, PL del mismo origen, pero producidas en SRA con agua de mar artificial reutilizada durante varios ciclos de producción, en la que se practicó el balance iónico, añadiendo magnesio, fueron sometidas a pruebas de estrés. Al finalizar los tiempos de exposición a la formalina y al amonio las PL presentaron una supervivencia mayor a 60 % en la prueba de amonio, sin embargo, no superaron la prueba de estrés a la formalina por lo que no se consideraron las PL como de buena calidad.

El análisis histológico de los tejidos branquiales de las PL sometidas a las pruebas de estrés mostró que la estructura branquial sufrió efectos dañinos como congestión severa de hemolinfa, engrosamiento y asimetría en lamelas y filamentos branquiales, así como picnosis nuclear. En la prueba de estrés por amonio, los sobrevivientes mostraron daños leves y efectos moderados de infiltración de hemocitos con respecto a la exposición a la formalina, que presento de moderada a severa congestión de filamentos branquiales y hemolinfa, engrosamiento de lamelas e hiperplasia celular, lo que indica que las PL son más sensibles a la prueba de formalina que a la prueba de amoníaco. Las pruebas de estrés han demostrado ser una herramienta efectiva para detectar poblaciones débiles o estresadas de PL de *M. rosenbergii*, su implementación y factibilidad de aplicación debe ser evaluada bajo las condiciones de cada hatchery.

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