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RESEARCH ARTICLE

Metamorphic Success and Production cost of *Holothuria scabra* Reared on Microalgae Concentrates Compared with Live Microalgae

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ABSTRACT

The production of live microalgae poses challenges for the expansion of sandfish hatcheries, hindered by high costs and limited technical resources. In relation to this, the use of three imported commercial concentrates (Instant Algae®) - TW1200 (Thalassiosira weisflogii), TISO1800 (Isochrysis sp.), and Shellfish1800 (mixed diatom) - were compared with live Chaetoceros calcitrans (CC). The diet efficacy was evaluated based on larval development, growth, and survival to late auricularia (LA) with hyaline spheres (HS), and the number of post-settled juveniles. Larvae reared with TW did not progress beyond LA, while those fed CC exhibited earlier LA development, larger sizes (1028.43 ± 19.38 µm), and significantly more post–settled juveniles (9,268 ± 2,183.79) compared to SHELL and TISO. Although TISO larvae reached a larger size during LA (855.7 ± 62.67 µm), SHELL resulted in a higher number of post-settled juveniles. The better performance of CC and SHELL may be attributed to their higher carbohydrate content. Despite SHELL and TISO having lower juvenile yields and longer feeding durations, the estimated cost per juvenile using SHELL, TISO, and CC were PHP 2.00, PHP 11.77, and PHP 0.52, respectively. Results showed that microalgae concentrates are not a cost-effective option under the studied conditions. The potential use of microalgae concentrates as supplemental feeds and further research to develop the use of local microalgae concentrates to sandfish larval culture are discussed.

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1. INTRODUCTION

Sea cucumbers are important fishery resources in many parts of the world. There are about 70 species that are commercially harvested for the dried sea cucumber, 'trepang,' or 'beche-de-mer' market (Purcell et al. 2016). International trade is rapidly increasing both in diversity and scale, which has been recorded in at least 70 countries (Louw and Bűrgener 2020). The increase in demand for sea cucumbers in the global market led to overexploitation in many countries (Purcell 2010; Conand et al. 2014). Due to the sea cucumber's high value and ease of capture, stocks worldwide are overfished. Among the overfished sea cucumber stocks reported earlier are those in the New Caledonia, Solomon Islands, Galapagos, Indonesia, Papua New Guinea, Australia, Fiji, Madagascar, Red Sea in Egypt, and the Philippines (Uthicke and Conand 2005).

The Philippines is considered a hotspot for sea cucumber fisheries and is one of the major producers and exporters of "trepang" in the world (Choo 2008), harvesting tropical species of sea cucumbers, yielding an average of 2,937 tons annually from 1985 to 1997 (FAO 2022). In at least 60 municipalities in 14 regions of the country, artisanal fishers collect sea cucumbers as an alternative to fishing during off periods (Labe 2009). However, overexploitation led to a shortage of supply from the wild (Akamine 2002). A total of 47 sea cucumber species have been recorded to be commercially valuable, from 16 species in 1987, 25 species in 2000, and 33 species in 2008 (Trinidad-Roa 1987; Schoppe 2000; Choo 2008; Labe 2009). The country's export of sea cucumber in 2008 was about 875 MT that valued at estimated amount of PHP 258 million, which is majorly exported to Hong Kong, China, Japan, Korea, and Singapore (Brown et al. 2010). In 2018, sea cucumber exports decreased to 746 MT (FAO 2020) and 810 MT on average by 2020 (FAO 2022). From being the top producer of sea cucumber

in the Indo-Pacific, the Philippines is now ranked number 14th as the world's sea cucumber producer (FAO 2020).

Holothuria scabra, commonly known as sandfish, is one of the overexploited species throughout the Indo-Pacific region because of its high-value dried product (Purcell et al. 2014; 2018). Dried sandfish commands prices as high as USD 1,898 per kilogram in Hong Kong (Purcell et al. 2018). The international market prices for sea cucumbers have been increasing for years, leading to the continued increase in fishing pressure in many countries (Barclay et al. 2016), including the Philippines. Given this situation, sea cucumber aquaculture has been developed to help rebuild declining stocks and increase fishery production through restocking and sea ranching (Battaglene 1999; Hamel et al. 2001; Asha and Muthiah 2007; Juinio-Meñez et al. 2017). Currently, many countries in the Indo-Pacific and Southern Africa are undertaking sandfish culture at varying scales (Jimmy et al. 2012; Mills et al. 2012; Hamel et al. 2022).

H. scabra is highly suitable for aquaculture due to its short larval phase, and wide temperature and salinity tolerance (Hamel et al. 2001; Pitt and Duy 2004). In the culture of sandfish, hatcheries rely on the mass production of live cultured microalgae to feed the larvae until post-settled juveniles for at least 30 days (Agudo 2006). The first microalgal species used in the larval diet of *H. scabra* were *Chaetoceros calcitrans, Rhodomonas* sp., *Tetraselmis* spp., and *Isochrysis galbana* (Agudo 2006; Asha and Muthiah 2006; Battaglene 1999; Duy 2010; Ivy and Giraspy 2006; Morgan 2001). The use of *C. muelleri* as a single diet was later suggested by Duy (2010) and was then applied in Australia and Vietnam by Knauer (2011) and Duy (2012), respectively. Other studies also reported that a mixed diet of microalgae is superior and important for better larval development (Dabbagh and Sedaghat 2012; Ivy and Giraspy 2006). In the Philippines, *C. calcitrans, Rhodomonas* sp., *Tetraselmis tetrahele,* and *I. galbana* are commonly used as larval food for *H. scabra* (Sibonga et al. 2021; Juinio-Meñez et al. 2012). These species have been administered as single and mixed diet for the larval rearing (Gamboa et al. 2012; Sibonga et al. 2021; Campo et al. 2019).

To date, few hatcheries in Luzon, Visayas, and Mindanao are able to produce tens of thousands of post-settled sandfish juveniles per year by using live microalgae (Australian Centre for International Agricultural Research 2021; Hamel 2022). However, more hatcheries that are able to produce sandfish juveniles for ocean nursery systems are needed to effectively rebuild the wild stocks in the country. Hatchery-produced juveniles that are translocated to other sites could reduce the fitness of the animals and could negatively affect the restocking efforts (Uthicke and Purcell 2004). Moreover, genetically distinct populations from different biogeographic regions in the Philippines have been identified (Ravago-Gotanco and Kim 2019). The conservation of genetic diversity is an essential consideration in scaling culture production in the Philippines (Juinio–Meñez et al. 2017). Thus, hatcheries in different regions that can produce cultured sandfish for restocking and grow-out culture from the local genetic stock are imperative for the responsible and sustainable development of both culture and capture fisheries.

For many marine hatcheries, producing live microalgae can be challenging due to the high production cost and the lack of technical resources and skilled persons (Coutteau and Sorgeloos 1992; Oostlander et al. 2020). Mass production of microalgae is usually cultivated outdoors or in open spaces which are reliant on natural illumination and ambient temperatures that are vulnerable to seasonality and contamination. This can sometimes cause crashed microalgal cultures that result in production loss (e.g., Sales et al. 2022). These issues have led to research investigations of alternative food sources for larvae using highly concentrated phototrophically grown microalgae (Reed and Henry 2014). The products are now commercially available and are considered to have potential use in the hatchery culture of invertebrates (Reed and Henry 2014; Rikard and Warton 2012). Recently, "off-the-shelf" commercially available microalgae from Instant Algae®, Reed Mariculture Inc., USA have been used for sea cucumber culture in Vietnam, Papua New Guinea, and Norway (Duy et al. 2015; Militz et al. 2018; Schagerström et al. 2021). Earlier experiments showed that some microalgae concentrates are readily ingested and efficiently digested by sandfish larvae and thus support their growth, survival, and subsequent settlement as post-settled juveniles (Duy et al. 2015). In terms of nutrient composition, microalgae concentrate generally have consistent composition, while live microalgae can vary according to culture conditions such as culture medium composition, temperature, and growth phase (Reed Mariculture 2021; Pacheco-Vega & Sanchez-Saavedra 2009; Pernet et al. 2003). Therefore, "off-the-shelf" microalgae may support the development of simpler and more reliable culture protocols for sea cucumber hatcheries. Using microalgae concentrates for mass production of sandfish juveniles has been demonstrated with no comparison to live microalgae (Militz et al. 2018). The use of microalgae concentrates

as feeds remain underexplored, warranting additional trials to evaluate operational feasibility, especially in producing sandfish juveniles in the hatchery. There is a need to expand *H. scabra* hatchery culture production in the Philippines (Juinio-Meñez et al. 2017), and alternative or supplemental larval feeds, such as microalgae concentrates, may aid in the expansion of sandfish hatcheries, especially those that lack live microalgae culture facilities. This is potentially a significant factor in expanding the hatchery culture production of sandfish in the Philippines while maintaining genetic diversity since the genetic stock of sandfish is not the same in different regions of the archipelago (Ravago-Gotanco and Kim 2019).

In this study, we compared the larval development, growth, and survival rates using three imported commercial microalgae concentrates (TW 1200®, TISO 1800®, Shellfish 1800®) and live *Chaetoceros calcitrans* as a benchmark for live microalgal feed. The metamorphic success per treatment was evaluated based on the presence and size of hyaline spheres (HS) in late auricularia, and settlement success. The number of post-settled juveniles was used as a basis to estimate the production cost per juvenile in each treatment. We hypothesized that if the metamorphic success of *H. scabra* reared in microalgae concentrates is better, then it could be an alternative feed to live microalgae. In addition, the cost of production of each juvenile will be lower.

2. MATERIALS AND METHODS

2.1 Larval production

Larvae used for this study were produced from *H. scabra* broodstock collected from the Bolinao-Anda Reef Complex in Pangasinan, Philippines (16°16'36.1" N, 120°00'09.0" E) and were reared in the hatchery at the Bolinao Marine Laboratory (BML) of the University of the Philippines. Broodstock was collected and conditioned two weeks prior to spawning induction. These were maintained in a tank with sand as substrate, supplied with mild aeration, and flowed through filtered seawater. The mean weight of the broodstock was 196.6 ± 22.6 g.

Broodstock was induced to spawn by a combination of desiccation, thermal shock, and food shock following the methods modified from Agudo (2006). Spawning was observed in 10 females and 18 males. Each individual was placed in a separate container to collect eggs and sperms. In a separate bin, 10 mL of sperm was added to 40 L of egg concentrate. Fertilized eggs were then stocked in a bin, and samples were taken to assess the success of the fertilization. Fertilized eggs were transferred to twelve 500 L larval rearing tanks (with an effective volume of 400 L) with a stocking density of 0.3 eggs mL^{-1}, supplied with mild aeration sufficient for water and food circulation in the tanks.

2.2 Larval rearing and feeding

Three commercial microalgae concentrate products developed by Instant Algae®, Reed Mariculture Inc., namely: (1) TW 1200 (*Thalassiosira weisflogii*; TW), (2) TISO 1800 (*Isochrysis* sp.; TISO), and (3) Shellfish 1800 (mixed diet of *Isochrysis* sp., *Pavlova* sp., *Tetraselmis* sp., *Thalassiosira pseudonana, T. weissflogii;* SHELL) were used as experimental treatments. The microalgae concentrate treatments were compared with cultured live *Chaetoceros calcitrans* (CC), which is being used at the BML sandfish hatchery, as the positive control treatment. Each diet treatment had three replicates and was randomly assigned to larval-rearing tanks. Feeding started two days after fertilization with a ration of 20,000 cells mL^{-1} per day, partitioned into three feeding times daily (9 AM, 3 PM, and 8 PM). Although the cell size differed among the microalgal feeds, the uniform ration was adopted to standardize microalgal cell density among treatments. Feeding was stopped when the majority of the larvae monitored in the water column were in the doliolaria stage.

Microalgae concentrates were kept in the original containers and stored at 4°C for the duration of the study. Prior to feeding, the average cell density mL^{-1} of each microalgae concentrate was determined. A 1 mL aliquot of the concentrate was added to 1 L of filtered seawater and was mixed thoroughly to evenly distribute the microalgae cells. The suspension was poured through a 60 µm mesh screen to remove any clumps before counting the cells. For each type of algal concentrate, the cell counts from a 0.1 mL aliquot were determined using a hemocytometer to calculate the volume needed for the required ration in larval tanks. Prior to adding the concentrates to the tanks, the suspension was first poured onto a 60 µm mesh screen to remove any clumps.

Approximately 50–70% of the water was changed in larval rearing tanks every other day throughout the study for 30 days in live treatment (Agudo 2006) and 35 days for microalgae concentrate treatments until post-settlement. Tank cleaning, together with the water change, was done every week to regularly remove or siphon out the excess food. The duration of larval rearing on the microalgae

concentrate treatments was five days longer than in the live microalgae due to the late settlement of the larvae in concentrate treatments. Water quality parameters were also assessed twice daily at 9 AM and 3 PM for the duration of the study. The mean water temperature was 25.83 ± 0.92°C, salinity at 34.1 ± 0.96 ppt, pH at 8.05 ± 0.15, and dissolved oxygen at 6.84 ± 0.71 mg/L during the duration of the experiment.

Once more than 50 % of competent doliolariae were observed, the settlement was induced on Day 16 (D16) for CC live treatments and D25 for microalgae concentrates treatments by adding polycarbonate corrugated settlement plates (41 x 38 cm) coated with *Spirulina* (Duy 2010) at the bottom of the tanks.

2.3 Larval development, growth, and survival

Larval stages and length were monitored every other day, starting D2 until D20. Samples were collected using a 60 µm nylon mesh net. The larval development and size of 50 larvae per tank were recorded while the development stages were assessed following Ramofafia et al. (2003) and Agudo (2006). Abnormal larvae were also noted, indicated by the decrease in size, distorted or unextended lateral processes, bent posterior projection, and unformed or shapeless stomach (Sibonga et al. 2021).

Larval survival was then determined every other day, starting D3 until D23. Three replicates of 50 mL water sample from each tank were collected and the average number of larvae per mL was determined for the larval density. Percent survival over time was estimated from the difference in the average density of larvae per sampling period relative to the initial stocking density of fertilized eggs (0.3 eggs mL^{-1}).

2.4 Metamorphic and settlement success

Metamorphic competency was based on the percentage of late-stage auricularia with hyaline spheres (HS) and its average size. HS were used as indicators of nutritional status and metamorphic success (Duy et al. 2016; Peters-Didier and Sewell 2019). From the 50 larvae samples per tank, the number of late auricularia with HS was counted from D10 to D20 to get the percentage of larvae with HS. The average sizes of HS per treatment were then measured. The total length at each development stage, presence, and sizes of HS were measured using a microscope with a digital Dino-Lite eyepiece.

Settlement success was determined by the number of post-settled juveniles counted after the experiment. The number of post-settled juveniles in each tank was determined from photographs of juveniles in 3 replicate representative 26 cm^2 areas where settled juveniles were observed (6 settlement plates, side, and bottom of tank). In the CC treatment tanks, juveniles settled on the settlement plates, the sides, and the bottom of the tanks, while juveniles only settled on the bottom of the tank in the microalgae concentrate treatment tanks. The surface areas (SA) of the side and bottom of the tanks were 2.13 m^2 and 1.04 m^2, respectively, and 0.31 m^2 for each settlement plate. Since juveniles did not settle on all tank surfaces and settlement plates, the juvenile number was determined by taking 10% of the surface areas as a conservative estimate of juveniles in each replicate tank. Photographs were taken on D30 for CC treatment and on D35 for TW, TISO, and SHELL treatments. Juveniles were counted from photographs using ImageJ software. To obtain the total estimate of the number of juveniles using the representative 26 cm^2 area (RA) in each tank, juveniles on surfaces of tanks and settlement plates were estimated separately using their respective surface areas. This formula was used:

$$\text{Number of juveniles} = \frac{SA}{RA}\ x\ 0.10\ x\ no.of\ juveniles\ in\ RA$$

where: SA is the surface area of either part of the tank or settlement plate and RA is the representative area.

The percent survival from egg to post-settled juveniles per treatment was then calculated by dividing the number of post-settled juveniles by the number of stocked larvae with the stocking density of 0.3 eggs mL^{-1} in 400 L volume.

2.5 Production cost

The costs of producing juveniles for each treatment for the whole duration of the experiment were determined. The estimated costs in the larval rearing up to post-settlement of juveniles include electricity, water, *Spirulina*, labor for feeding and maintenance, and separate additional costs for live and microalgae concentrate treatments (Supplemental Table 3). To estimate the price per juvenile for each treatment, the total cost for larval rearing is divided by the number of post-settled juveniles produced.

Estimated daily electricity and water (seawater and freshwater pump usage) costs for larval rearing per treatment were determined from daily electrical usage multiplied by the number of rearing days. In the live CC treatment, the cost was calculated for 20 days of feeding days and an additional 10 days for the maintenance of tanks before

estimating the number of post-settled juveniles. Additional electricity and water costs for the culture of live microalgae were also estimated. These included added costs for the air conditioner, refrigerator, and LED lamp used in the algal room and additional use of seawater for the mass culture of microalgae. In microalgae concentrate treatments, the electrical and water costs were calculated for 35 days as well as the respective total feeding and maintenance days until post-settlement. Electrical consumption for the refrigeration of microalgae concentrates was also added to the estimated cost.

Other estimated costs for live microalgae include fertilizers and chemicals for microalgae culture. For microalgae concentrates, the cost of each bottle and the logistical fees were noted. The costs of daily hatchery labor and *Spirulina* for settlement induction were the same for all treatments.

2.6 Data analysis

For each monitoring period, mean ± standard deviation for larval sizes, survival, presence, and diameter of hyaline spheres in late auricularia, and number of post-settled juveniles were obtained per treatment. The number of late auricularia with hyaline spheres and survival of post-settled juveniles from the larval stage were expressed in percentages. All data were tested for normality and heterogeneity using the Shapiro–Wilk test and Levene's test, respectively, and those that did not fit the assumptions were transformed using the log, square root, and arcsine functions. Normally distributed data in larval length and survival were tested with One Way Analysis of Variance (ANOVA). Pairwise comparisons between groups were done using Tukey's HSD post-hoc test. Data on larval length, hyaline sphere presence and diameter, and the data set in post-settled juveniles that did not conform to normality even after transformation were analyzed using the Mann-Whitney U test and Kruskal-Wallis test. A Pearson correlation coefficient test was performed for the relationship between the presence and diameter of hyaline spheres in late auricularia. Significant differences among treatments were considered if $p < 0.05$. All statistical analyses were executed in Statistica 14.0 (Statsoft, Inc., USA).

Relative abundance (%) of different larval developmental stages was visualized using a stacked bar chart to identify dominant larval stages between different treatments. The size distributions of all larvae sampled per treatment were summarized in box plots per day to show variability within and among treatments over time. Since larval development within each treatment was not synchronous, the proportion of different stages during each sampling period and the sizes of the dominant larval stages were compared to evaluate relative larval growth and development rates across treatments.

3. RESULTS

3.1 Larval development and growth

The rate of development (i.e., progression of the dominant larval stages) in microalgae concentrate treatments and live treatment differed considerably. Figure 1 shows the percentage of early, middle, and late auricularia larval stages at different monitoring points. Based on the distribution of larval stages per day, larval development was almost similar between TISO and SHELL. LA was present by D6, and doliolaria were first observed on D12 and D14. However, the majority of the larvae were still at LA on D20, while doliolaria never dominated on any day in TISO and SHELL treatments. The slowest development was observed in the TW treatment. There were few LA on D8, but most of the larvae did not progress beyond middle LA and no doliolaria larvae were observed (Figure 1). Meanwhile, larval development was much faster in CC than in the microalgae concentrate treatments. LA were already observed on D6, while doliolaria larvae were observed starting D10 and was the majority stage of larvae until D20. Feeding was stopped in CC at D20 since no LA larvae were already observed, while it was continued in the microalgal treatments until D35.

Larvae with morphological abnormalities were mostly observed in the three microalgae concentrate treatments (1–18.3%) but not in the live microalgae, starting with D12 with high numbers from D18 to D20 (Figure 2a–2c). In comparison, larvae in the CC treatment on D10 were well-developed LA (Figure 2d).

The size profiles of all larvae sampled in the diet treatments during different monitoring days are shown in boxplots in Supplemental Figure 1. On D2, body lengths of the early auricularia in all treatments had similar median sizes ranging from 524.5 µm to 536 µm. This was also evident in short and centered boxes between the first and third quartiles. By D4, larvae in the middle auricularia (MA) stage significantly increased in size from D2 among treatments based on the non-overlapping box plots. Differences in sizes among treatments were first evident in D6 with the development of late auricularia (Figure 1). In the three microalgae concentrate treatments, the composition of the larval stages was variable, and the dominant

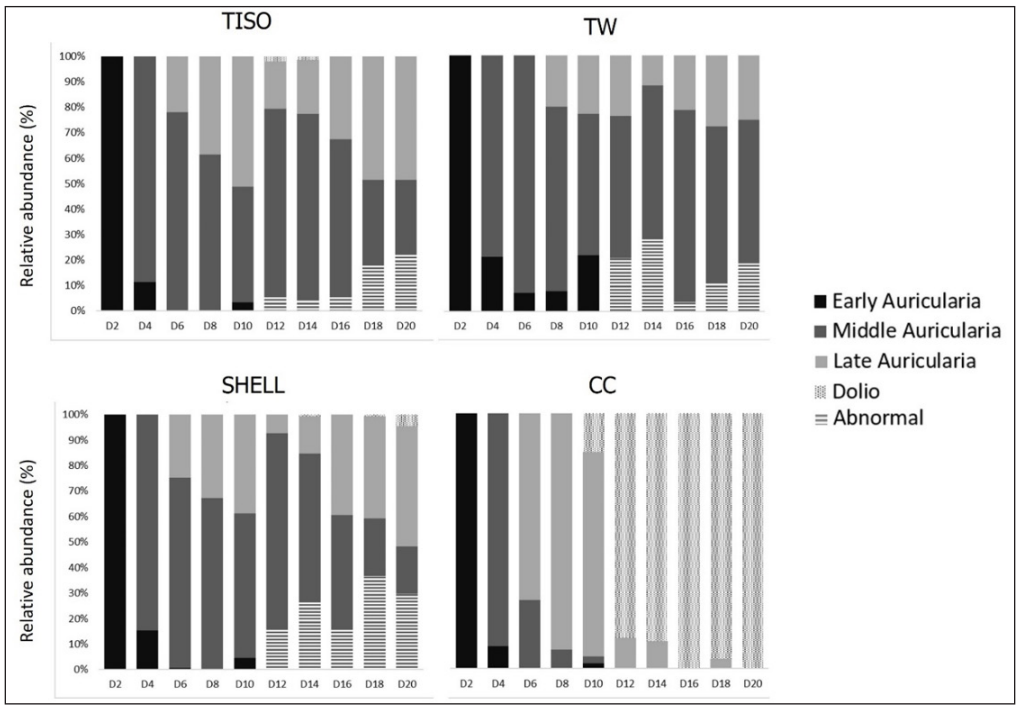

Figure 1. Relative abundance (percentage) of early, middle, late auricularia; doliolaria, and abnormal larvae of *Holothuria scabra* fed with microalgae concentrates (TISO, TW, SHELL) and live microalgae (CC) over the 20-day rearing period.

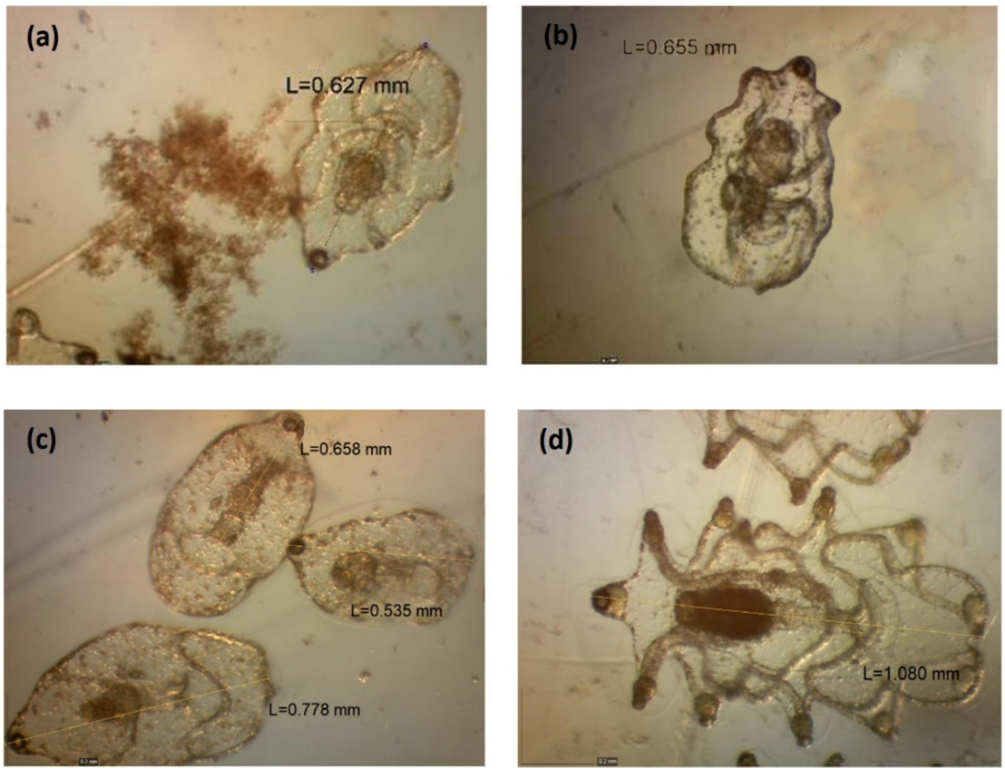

Figure 2. Abnormal early auricularia-like larvae in microalgae concentrate treatments at particular days (D); (a) TW (D20), (b) TISO (D18), (c) SHELL (D19); compared with well-developed late-stage auricularia in the **(d)** CC (D10).

stage sampled was not consistent from D6 to D20 (Supplemental Figure 1). On the other hand, the body length sizes in CC were significantly higher than in the microalgae concentrate treatments (Table 1). This was observed from D6 to D10 when the dominant stage was LA, as indicated by the non-overlapping boxplots from other treatments (Supplemental Figure 1). On D6, D8, and D10, CC treatment had median sizes of 916.5 µm, 1,045 µm, and 1,015 µm, respectively, which decreased to 591 µm and 520.5 µm on D12 to D14 when larvae metamorphosed into the non-feeding doliolaria stage.

In all treatments, larval length increased from early to LA (Table 1, Supplemental Fig. 2). The average length of EA on D2 (Table 1) was not significantly different among treatments. In the MA stage on D4, among the microalgae concentrate treatments, TISO had a significantly greater mean length (646.2 ± 19.6 µm) than with larvae fed with SHELL being the lowest (607 ± 4.67 µm). In the live treatment, the average size of larvae in CC was significantly larger than SHELL and TW, but not with TISO.

Development to LA among the microalgae concentrate treatments was dominant only on D20 in TISO and SHELL (Table 1). Moreover, the larvae were smaller, with the highest mean body lengths at only 855.73 ± 62.69 µm and 806.6 ± 80.57 µm, respectively. Notably, very few larvae developed to LA stage in TW. Thus, LA was never dominant on any day throughout the experimental period in TW. In contrast, the fastest development was observed in CC, with the observed presence of LA as early as D6 (Figure 1). These larvae also attained the longest body length by D8 (1028 ± 19.38 µm) compared to those microalgae concentrate treatments (806.6 ± 80.57–855.73 ± 62.69) (Supplemental Figure 2).

3.2 Survival of larvae

Survival of larvae varied in different treatments as the experiment progressed (Figure 3, Supplementary Table 1). There was no significant difference between TISO and SHELL, with survival of 97.7 % and 87.6%, respectively, on D13 and survival of 48.9% and 49.6%, respectively, on D21. Meanwhile, live CC had higher survival during the LA stage on D7 and D9 but no significant difference with the TISO and SHELL. Average survival in TW was significantly lowest (ANOVA, $p < 0.05$), starting at D13 with 31.7% until D21 with 23.4%. A sharp decrease in

Table 1. Mean body length (means ± SD) of different auricularia stages of *Holothuria scabra* fed with microalgae concentrates and live microalgae. Early auricularia stage on D2, middle auricularia stage on D4, Late auricularia stage on D8 for CC using ANOVA and D20 for TISO and SHELL using Kruskal-Wallis test.

Treatments	Larval Size of different Auricularia stages (µm)		
	Early (D2)	Middle (D4)	Late
TISO	523.07 ± 11.1[a]	646.2 ± 19.6[a]	855.73 ± 62.69[b] (D20)
SHELL	525.93 ± 4.42[a]	607 ± 4.67[b]	806.6 ± 80.57[c] (D20)
TW	517.71 ± 5.23[a]	623.22 ± 8[b]	*Not dominant on any day*
CC	525.7 ± 9.14[a]	663.71 ± 13.89[a]	1028.43 ± 19.38[a] (D8)

Note: Values with different superscripts indicate significant differences across treatments ($p < 0.05$).

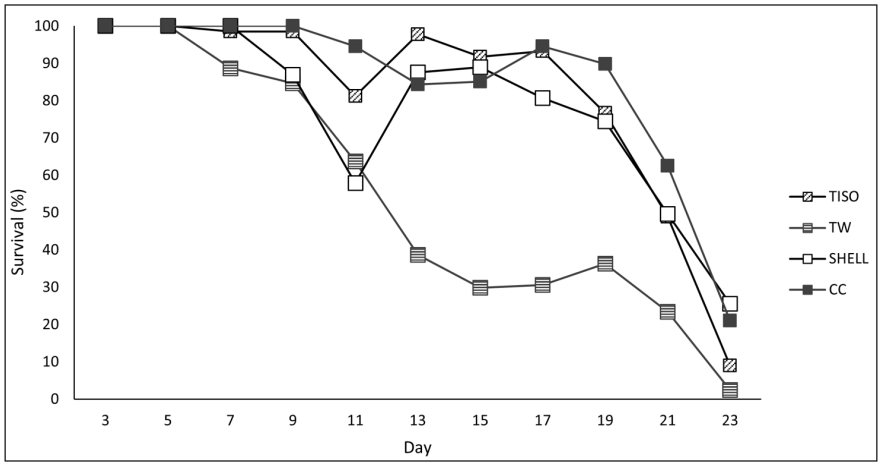

Figure 3. Percent survival of *Holothuria scabra* larvae in different treatments.

larval density occurred in one of the TW replicate tanks on D12 and in two replicate tanks of the SHELL treatment on D21 and D23, respectively. The decrease in larval densities observed on D10 in CC was mainly attributed to larval settlement.

3.3 Metamorphic and settlement success

Among the microalgae concentrate treatments, larvae in TISO and SHELL started to metamorphose into doliolaria on D12 and D14, respectively. In TW**,** LA appeared on D8 but did not dominate on any day (Figure 1), and no doliolaria larvae were observed in this treatment. Larvae in CC metamorphosed to LA starting D6 and comprised the highest percentage of larval stages by D8. In the same treatment, larvae that metamorphosed to doliolaria were first observed on D10 and were predominant on D12.

Due to the varying development rate of larvae in the different treatments, there were no hyaline spheres on other late-stage auricularia during other sampling dates (Figure 4). LA with hyaline spheres was mostly sampled on D10 to D20 in the microalgae concentrate treatments. The number of LA larvae with hyaline spheres and the size of the hyaline spheres were relatively higher in SHELL and TISO (Figure 4, Table 2). No significant differences were observed in the development of hyaline spheres (HS) in SHELL and TISO, but it was significantly lowest in TW (Table 2). In contrast, larvae in CC were the first to develop HS, with the significantly highest number of larvae with HS and the largest HS among all treatments (Table 2). As shown in Figure 1, most of the larvae fed with live CC settled by D16, and the remaining larvae were mostly in the doliolaria stage. Therefore, no additional larvae were sampled in this treatment for hyaline spheres monitoring beyond this date. Moreover, treatments with a higher percentage of LA with large hyaline spheres were observed to have a positive correlation ($p < 0.05$) with the number of settled juveniles at the end of the experiment.

The average number of post-settled juveniles was estimated in the CC tanks at D30 and D35 for the microalgae concentrate treatment tanks. Notably, while TISO performed better than the SHELL diet in terms of larval development, post–settled juveniles were higher in the latter treatment. No post-settled

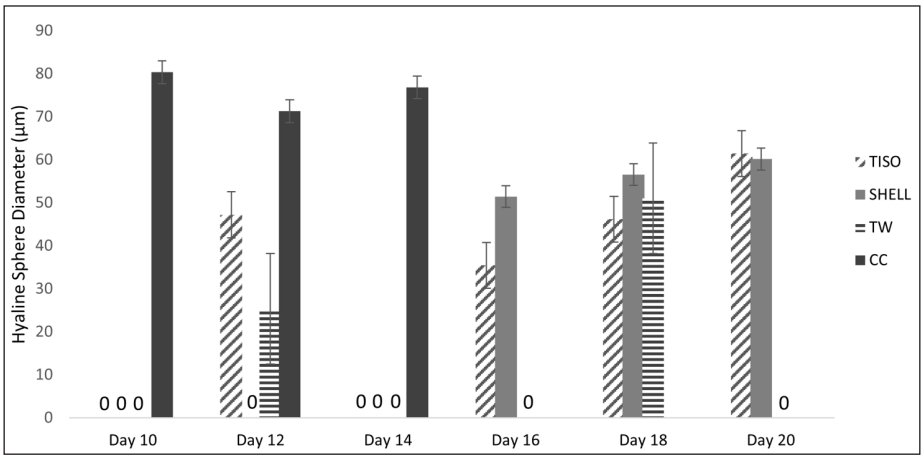

Figure 4. Size of hyaline spheres in late auricularia larvae sampled during daily monitoring in the different treatments with error bars indicating standard deviations. Absence of late auricularia with hyaline spheres in samples is denoted by 0.

Table 2. Percentage of late auricularia larvae with hyaline spheres and their sizes and estimated average number of post-settled juveniles by D30 for CC and D35 for other microalgae concentrate treatments.

Treatment	Percentage of late Auricularia with hyaline spheres (%)	Size of Hyaline spheres (µm)	Average no. of settled juveniles in settlement plates and surface area of tanks
TISO	14 (D20)^{b}	61.42 ± 6.53^{b}	415 ± 270.6^{c}
SHELL	16.67 (D20)^{b}	60.14 ± 3.77^{b}	2,414 ± 1,968.44^{b}
TW	4 (D12)^{b}	25.33 ± 19.57^{c}	0
CC	76.67 (D10)^{a}	80.33 ± 1.63^{a}	9,268 ± 2,183.79^{a}

Note: Values with different superscripts indicate significant differences across treatments ($p < 0.05$).

juvenile was observed in TW. Meanwhile, settled juveniles in CC were three-fold higher than SHELL, and 20 times greater than TISO (Table 2). The average survival from egg to post-settled juveniles is 7.72% for CC, 2.01% for SHELL, and 0.35% for TISO.

3.4 Production cost

Overall, the cost for the use of live microalgae (CC) was lower when compared with TISO and SHELL but not with TW (Supplemental Table 3). Despite the higher cost of electricity and labor due to the culture of microalgae, the cost of microalgae concentrate bottles, along with the logistics fees and the labor, including feeding until D35, contributed to a larger expense in the microalgae concentrate treatments. Since the yield of the post-settled juveniles in CC was much higher, the estimated price per juvenile is only PHP 0.52 per juvenile, while it is PHP 2.00 and PHP 11.77 for the SHELL and TISO treatment, respectively (Table 3). No juvenile was produced in the TW treatment.

4. DISCUSSION

The viability of using some imported microalgae concentrates was validated. Moreover, the results of the study provided an initial basis for the evaluation of the relative efficacy and production efficiency of the different microalgae concentrates. Larval performance, metamorphic and settlement success, and estimated production cost of post-settled juveniles were better in the live microalgae treatment than any of the microalgae concentrates under the rearing condition in this study. Consequently, the cost of production was higher for microalgae concentrates. Further studies on the potential use of microalgae concentrates as supplemental or alternative feed are discussed.

4.1 Larval development and survival

Several factors affect the development of larvae until post-settlement. Larval quality is assessed based on the larval size, progression to larval stages, and larval survival. The results of CC treatment in this study were considered a benchmark for current larval rearing practice under similar rearing conditions to compare the three microalgae concentrate treatments. In general, the length of larvae and progression to developmental stages in all the treatments excluding TW are comparable with other studies on sandfish larval development (Knauer 2011; Agudo 2006; Duy 2010; Juinio-Meñez et al. 2017). However, the results are not directly comparable since the rearing conditions in the other studies were different. In addition, studies on sandfish larval feed until settlement did not compare microalgae concentrates and live microalgae (Militz et al. 2018; Duy et al. 2016).

The normal development and survival of larvae are prerequisites for successful metamorphosis to post-settled juveniles and could be attributed to nutritional content. Results of this study suggest that larval growth and survival were better in diets with higher carbohydrate content. In other studies, animal and plant feed diets with high carbohydrate content also supported rapid growth in larger *H. scabra* juveniles (Orozco et al. 2014) as well as in *Stichopus (Apostichopus) japonicus* and *Australostichopus mollis* (Zhou et al. 2006; Slater et al. 2009). Carbohydrate content is reported to be more important than dietary protein and lipid in determining the nutritional value of microalgae (Duy et al. 2016). Among the microalgae concentrate treatments, TISO has the highest percent carbohydrate component, with 24% in dry weight, followed by SHELL, with 22%, and the least TW, with 12% (Reed Mariculture 2021). Notably, larvae in the TISO diet performed better from middle to late larval stages compared with the SHELL diet. Meanwhile, larvae in TW consistently had poor development and survival throughout all the larval stages resulting in no post-settled juveniles produced. This is contrary to the study of Duy (2016) that used progressive feeding of microalgae concentrates from Reed Mariculture, such as TISO and TW, for larval rearing of sandfish. Poor health during the early stages may be carried over to the later stages, precluding the accumulation of sufficient nutrients in the doliolaria stage. As the experiment progressed, stunted and abnormal growth, and mass mortalities among larvae were observed in the TW treatment. The incidence of bacterial growth

Table 3. Estimated cost per juvenile reared in different treatments. Calculations are from the total post-settled juveniles in three replicate tanks and the total cost estimate of hatchery operations (breakdown in Supplementary Table 3) during the rearing period.

	CC	TISO	SHELL	TW
Total post-settled juveniles	27,804	1,245	7,243	0
Total cost estimate for rearing (PHP)	14,350.75	14,647.02	14,502.93	11,773.77
Estimated cost per juvenile (PHP)	**0.52**	**11.77**	**2.00**	~

may be related to the greater amount of uneaten food. Thus, aside from the poor nutrition of the diet, poor water quality could have also contributed to mass mortalities in the TW treatment.

Live *Chaetoceros calcitrans* is also high in carbohydrate content (23.4–26.1%) (Brown et al. 1997), which supports the development of larvae. In addition, the better growth performance in CC may also be related to the motility of the cells. The cells may be more accessible to the larvae than the microalgae cells in the concentrates, which readily settle on the bottom of the tanks, thus reducing the availability of cells for the larvae in the water column (Duy et al. 2015).

4.2 Metamorphic and settlement success

Late auricularia is a competent stage of sandfish larvae that is physiologically ready to metamorphose if stimulated properly (Hamel et al. 2022). Further, transformation to doliolaria larvae until pentactula or the post-settled stage is an indication of larvae competence to metamorphose. Transition to this stage, as well as the development of hyaline spheres, can be an indication of metamorphic success (Battaglene 1999). These spheres function as nutritional reserves providing nutritive support for the non-feeding perimetamorphic period before the settled juveniles are competent to feed (Chen et al. 1991; Smiley et al. 1991). Ramofafia et al. (2003) reported that competent and healthy late auricularia *H. scabra* fed with live microalgae have an HS diameter of 50–70 µm, and those poorly developed have < 30 µm diameter. Moreover, HS diameters of 25–40 µm in larvae that are fed with microalgae concentrates are considered acceptable levels to support subsequent survival and growth (Duy et al. 2016). In this study, the late auricularia in CC had a larger HS diameter (80.33 ± 1.63 µm on D10) while the average diameters of HS in TISO and SHELL on D20 (61.42 ± 6.53 µm and 60.14 ± 3.77 µm, respectively) are within what is expected for competent and healthy larvae. In TW, a few late auricularia had very small HS (25.33 ± 19.57), and no larvae developed into doliolaria. According to Duy et al. (2016), there is a positive correlation between HS formation and dietary levels of carbohydrates. The results of this study further indicate that the feeds with higher carbohydrate content promote better larval growth and development.

While there was no significant difference in the average HS diameter in the TISO (mono species) and SHELL (mixed species) treatments, larval development to middle and late auricularia was better in the former. The number of post-settled juveniles produced in the SHELL treatment was also five times higher than in TISO. This clearly indicates that *H. scabra* larvae were able to ingest the SHELL diet efficiently and assimilated more nutrients compared to the mono species concentrates TISO and TW. This is contrary to the report of Duy et al. (2015) which states that the efficacy of the SHELL mixed diet is lower compared with other algal concentrates. The higher post-settlement success in the SHELL diet compared to TW and TISO is consistent with studies that show that mixed diets are more nutritious than mono-species diets (Brown et al. 1989; Knauer and Southgate 1999). Nevertheless, post-settlement success in the live mono species *Chaetoceros calcitrans* treatment was over three times greater than in SHELL and an order of magnitude greater than for TISO. This indicates that the live CC diet is more nutritious than any microalgae concentrate. Based on all performance indicators in this study, the nutritional value of live CC is evidently far better than the three microalgae concentrates. Determining the most suitable feed during different larval stages is another method to improve the production of larvae and post-settled juveniles using microalgae concentrates (Militz et al., 2018; Duy et al., 2016).

The number of juveniles produced in this study in CC and SHELL is comparable to the 2.5% survival rate from egg to post–settled juveniles in other studies that used live microalgae (Mills et al. 2012). The total juvenile yield in the same two treatments is also higher than the total post–settled juveniles produced in the study of Militz et al. (2018), which used microalgae concentrates. TISO, on the other hand, has a lower survival rate and juvenile yield compared to the aforementioned studies.

4.3 Prospective use of microalgae concentrates

Under the single diet and fixed standard feeding regimen in this study, the use of commercial microalgae concentrates was not a lower-cost option for the production of post-settled *H. scabra* juveniles. Based on the estimated price per juvenile (Table 3) reared in SHELL and TISO, the costs were about 284.6% and 2163.5% higher compared to the CC live treatment, respectively. The higher cost was due to the significantly lower yield of post-settled juveniles, the longer feeding duration, and the logistical cost from the international supplier. Despite the higher production cost, the ability of microalgae concentrates to produce post-settled juveniles indicates that it can be used as an alternative food for larvae in the

absence of live feeds. This will be advantageous to hatcheries that do not have the capacity to produce live microalgae. The use of microalgae concentrates in these hatcheries will still be a lower-cost option relative to putting up a new culture facility. Economic viability is the key factor in determining the appropriateness of utilizing microalgae concentrates. If incorporating microalgae concentrates as food input proves to be more financially advantageous for hatcheries, the adoption of this low-cost feed for many sandfish hatcheries would follow. High operational expenses associated with sandfish larval rearing can be further improved by utilizing microalgae concentrates as food input in many local hatcheries.

Various studies have shown that live mixed cultures are better in larval development (Brown et al., 1989; Knauer and Southgate, 1999). Among the microalgae concentrates, the mixed species SHELL (mixed diet of *Isochrysis sp., Pavlova* sp., *Tetraselmis* sp., *Thalassiosira pseudonana*, *T. weissflogii*) may potentially be used as a supplementary larval feed during the late auricularia stage when more larvae are competent to settle. During this time, the settlement of the microalgae concentrate on the bottom may enhance post-settlement success (Duy et al. 2016). The cost per juvenile produced was lowest when using SHELL, and this is particularly important for hatcheries that are not capable of producing live microalgae. In lieu of live mixed microalgal feeds, using mixed microalgae concentrates as supplemental feed may help to improve the cost-efficient production of quality larvae and juveniles in the hatchery (Militz et al. 2018). SHELL can supplement the single-species diet of live microalgae currently used in local hatcheries.

Microalgae concentrates used in this study were from an international supplier; however, the potential of using local microalgae feeds for hatchery production of sandfish post-settled juveniles warrants investigation. Locally-produced commercial microalgae concentrates (e.g., Zinaya Algal Paste from Kamino Algae Technologies Inc. and Juan Algae from Algacon Aquafeed) have been developed. The concentrate species produced by the two Philippine brands are *Nannochloropsis* sp., *Thalassiosira* sp., *Tetraselmis* sp., *Chaetoceros calcitrans, Chaetoceros muelleri,* and *Chlorella vulgaris* that are commonly used for shrimp, milkfish and other high-value finfish, and mollusk. These products can potentially increase the percent survival on their production and lessen their monthly operating expenses (Quiambao 2022; Atienza 2022). The availability of suitable local microalgae concentrates provides an opportunity to evaluate the use for rearing sea cucumber larvae and reduce the cost of hatchery production. Live *C. calcitrans* has been widely used in the mass production of sandfish post-settled juveniles (Pitt 2001; Agudo 2006). *C. muelleri* is also used as a single diet in Vietnam and is considered a highly nutritious feed for sandfish larvae (Duy 2010; Knauer 2011). Both species are routinely used in the production of sea cucumber juveniles at the University of the Philippines Marine Science Institute Bolinao Marine Laboratory. The viability of these local *Chaetoceros* concentrates on improving metamorphic and settlement success can accelerate the expansion of sandfish hatcheries that are not able to produce live microalgae.

5. CONCLUSIONS AND RECOMMENDATIONS

Based on the metamorphic and settlement success and production costs of *H. scabra* juveniles, none of the microalgae concentrates was better than live microalgal feeds. However, for cases where live microalgae are unavailable in a hatchery, concentrates could be alternative diets, although juvenile production will be lower than that for live microalgae.

Utilizing microalgae concentrates in hatchery systems is a recent innovative approach aimed at overcoming logistical issues linked to the continuous cultivation of live microalgae. This study validated the potential use of the imported commercial microalgae concentrate for the hatchery production of juvenile sandfish, as shown in other studies conducted under different rearing conditions. However, the use of these imported concentrates is not a lower-cost option compared to the use of live *Chaetoceros calcitrans*. Among the microalgae concentrates tested, SHELL, a mixed diet concentrate, may be used as a supplementary feed during the late auricularia stage to improve settlement success. This is indicated by the higher number of juveniles produced compared to the monospecific concentrates in the study.

Additional logistical costs incurred in importing microalgae concentrates coupled with the lower yield of post-settled juveniles accounted for the higher estimated cost of juveniles. The availability of local microalgae concentrates presents opportunities to reduce the cost of production using suitable feeds. In particular, the use of *Chaetoceros* spp. concentrates should be evaluated and optimized. This will enable more hatcheries to produce sandfish post-settled juveniles. Increasing the number of hatcheries that can produce *H. scabra* juveniles can accelerate the integration of sandfish post-settled juvenile production

in marine hatcheries throughout the archipelago. The increased supply of juveniles produced from local genetic stocks will increase culture production while conserving the population genetic diversity of this high-value species in the country.

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SUPPLEMENTARY MATERIAL

Below is the link to the electronic supplementary material.
Supplementary file

AUTHORS CONTRIBUTIONS

Garpa TJS: Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing-Original Draft, Writing- Review & Editing, Visualization. **Caasi OJC**: Methodology, Investigation, Formal analysis, Writing-Review & Editing, Visualization. **Juinio–Meñez MA**: Conceptualization, Methodology, Resources, Writing-Original Draft, Writing-Review & Editing, Supervision, Project administration, Funding acquisition.

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CONFLICTS OF INTERESTS

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

ETHICS STATEMENT

The authors confirm that they have adhered to the journal's ethical policies. No ethical approval is required.

AVAILABILITY OF DATA AND MATERIALS

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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