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Effect of density, temperature and diet on the growth, survival and development of larvae and juveniles of *Isostichopus* sp.

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Abstract

Isostichopus sp. is a variety of *Isostichopus badionotus*, proposed as a new species, which has been intensely fished in the Colombian Caribbean, arousing interest for its aquaculture. This study evaluated the effect of two culture densities (1–3 larvae ml^{-1}), two temperatures (23°C and 26°C) and two microalgae diets (*Isochrysis galbana*, *Chaetoceros calcitrans*, *Nannochloropsis oculata* 1:1:1 and *I. galbana*, *C. calcitrans* 1:1) on the survival, development and growth of its larvae. Larval culture lasted 22 days until metamorphosis to doliolaria larvae, and 7 days later, the first juveniles were observed with a size of 621.8 ± 12.7 μm (±*SE*). The highest growth rates, survival and percentage of doliolaria larvae were obtained with 1 larva ml^{-1} (29.2 μm/day and 31.5% doliolaria larvae) and 26°C (28.4 μm/day and 10% doliolaria larvae). However, in the two diets examined, the larvae showed low growth rates (between 1.3 and 8.5 μm/day), stagnation in development and high mortality. Our results indicate that it is feasible to culture larvae from *Isostichopus* sp. to juveniles, recommending the use of 1 larva ml^{-1} and 26°C. However, to meet the nutritional needs of the larvae the inclusion of microalgae *Pavlova* sp. and *Tetraselmis chuii* in the diet is recommended. This paper reports for the first time the successful production of sea cucumber juveniles of this species in Colombia.

KEYWORDS

aquaculture, density, diet, *Isostichopus* sp., larval development, temperature

1 | INTRODUCTION

The growing demand for sea cucumbers by Asian markets has led to overfishing of this resource in various parts of the world, putting many species at risk (Lovatelli et al., 2004; Purcell et al., 2010; Uthicke & Conand, 2005). Faced with this situation, aquaculture is seen as a viable alternative for the protection of natural populations subjected to high fishing pressure and at the same time satisfies the growing market demand for this resource (Asha & Muthiah, 2006; Battaglene & Bell, 1999; Conand & Byrne, 1993; Hu et al., 2013; Mercier et al., 2004; Purcell et al., 2012). However, the success of sea cucumber culture depends to a large extent on the production of seed or juveniles in hatcheries that can supply marine farms or the development of restocking programmes (Asha & Muthiah, 2005; Li & Li, 2010). In countries such as China, Philippines, Vietnam, Australia and Madagascar, where there is an established industry around the consumption and/or fishing of sea cucumbers, successful techniques have been developed to obtain seeds of high value and high demand species such as *Apostichopus japonicus* and *Holothuria scabra* (Purcell et al., 2012; Robinson & Pascal, 2009).

In the Colombian Caribbean, where there is illegal, unquantified and unregulated fishing of several species of sea cucumbers (Ortega-Lara et al., 2015; Puentes et al., 2014; Reyes-Sánchez et al., 2011; Rodríguez et al., 2013), one of the species that arouses greater interest for its conservation and culture is *Isostichopus badionotus*, not only because it is one of the most intensely fished due to its high economic value (Ortiz et al., 2011; Puentes et al., 2014; Purcell et al., 2018; Rodríguez et al., 2013), but also for its potential for aquaculture (Purcell et al., 2012; Sánchez, 2012; Rodríguez-Serna et al., 2012; Zacarías-Soto et al., 2013; Zacarías-Soto & Olvera-Novoa, 2015).

Despite presenting several morphotypes with a wide variation in its coloration and external morphology, this species has been recognized as a single species based on ossicle morphology, which is the main taxonomic character in sea cucumbers (Borrero-Pérez et al., 2012, 2017). However, recently Vergara et al. (2018), based on DNA analysis, morphology and habitat preferences demonstrated that one of the most fished morphotypes in this region is a new species close to *I. badionotus*, which they named *Isostichopus* sp. In this manuscript, we will refer to this morphotype, which is characterized by a thin and rigid body, with colouring patterns in the form of patches, combining the colours dark brown, light brown and/ or red and sharp pointed dermal papillae also as *Isostichopus* sp. (Figure 1).

Isostichopus sp. similar to *I. badionotus* is gonochoric, with external fertilization, without external sexual dimorphism and shows a sex ratio of generally 1:1. Sexually mature organisms are characterized by an annual reproductive cycle, with a reproductive season (July–December) beginning in the warmest months, coinciding with the rainy season in the region and extending until the beginning of the dry season, when water temperatures drop due to a local phenomenon of upwelling (Agudelo-Martínez & Rodríguez-Forero, 2017; Díaz et al., 2000; Guzmán et al., 2003; Mancera-Pineda et al., 2013).

The first experiments in Colombia on sea cucumber reproduction under controlled conditions were carried out by Agudelo-Martínez and Rodríguez-Forero (2015, 2017) with *Isostichopus* sp., obtaining spontaneous spawning in captivity and larval culture up to the late auricularia stage. However, despite the progress achieved in terms of knowledge of reproduction and larval development, the obtaining of seed remains a challenge, mainly due to the high mortality that occurs during larval culture. According to authors as Asha and Muthiah (2005, 2006), Agudo (2006), Knauer (2011), Liu et al. (2010), and Li and Li, (2010), the correct management of exogenous factors such as culture density, temperature and the quantity and type of food are determining in the survival, growth, settlement and metamorphosis of sea cucumber larvae. These same authors have reported the larval culture and the obtaining of seed in species like *H. scabra*, *Holothuria spinifera* and *A. japonicus* using densities with ranges between 0.5 and 3 larvae ml^{-1}, temperatures between 21°C and 28°C and monoalgal diets or combinations of different microalgae such as *Isochrysis galbana*, *Chaetoceros calcitrans*, *C. moelleri*, *Nanochlorosis salina*, *Phaeodactylum tricornutum* and *Dunaliella salina*.

However, in *Isostichopus* sp. the influence of these variables on larval culture is unknown. In this work, it is reported for the first time how sea cucumber juveniles of *Isostichopus* sp., native to the Colombian Caribbean were obtained, also describing the effects of density, temperature and type of diet on the growth, development and survival of larvae and juveniles. This is a contribution to the culture technology of this species with greater commercial importance in the tropical Caribbean.

FIGURE 1 Dorsal view of *Isostichopus* sp. specimens with variations in their coloration

2 | METHODOLOGY

This study was conducted from November 2016 to February 2017 in the laboratories of the Aquaculture Technology Research and Development Group (GIDTA) at the Universidad del Magdalena, Santa Marta Colombia.

2.1 | Collection of animals and spawning

In October 2016 during the peak of the breeding season (Agudelo-Martínez & Rodríguez-Forero, 2017), a group of 20 adult specimens of *Isostichopus* sp. with a body drained weight average of 172.4 ± 49.6 g (±*SD*; weight of the body wall plus internal organs without the water contained in the respiratory tree) and length average of 16.0 ± 2.9 cm (measured with a tape measure that contoured the body of the sea cucumber from the mouth to the anus) were collected by snorkeling (0.5–3 m depth) in Rodadero Bay, (11°12′27,40″N–74°13′47,44″W) and transported to the laboratory.

All collected individuals were placed in a 500-L flat bottom tank, provided with filtered sea water at 1 μm (FSW) and irradiated with ultraviolet light, at 26°C and salinity of 34‰, with water changes of 50% daily and fed once daily with a mixture of *Spirulina* and *Sargassum* powder equivalent to 6% of the dry weight of sea cucumbers, in a proportion of 20:80 of food: sand. Since these individuals do not show sexual dimorphism, it was assumed that the samples collected presented the same proportion of females and males as reported in the wild 1:1 (Agudelo-Martínez & Rodríguez-Forero, 2017).

The spawning occurred 3 weeks later spontaneously in the same acclimatization tank at night and without any type of stimulation or intervention on the part of the researchers, coinciding in the lunar calendar with the phase of the new moon.

After spawning, the broodstock animals were removed from the tank, and approximately 12 hr after spawning, the tank was drained; the oocytes were collected on a 40 μm sieve and washed with seawater filtered at 1 μm to remove excess sperm and then seeded in 200-L tanks at a density of 10 oocytes ml^{-1} to continue their embryonic development. 48 hr after spawning, the tanks were drained and the larvae obtained were used in density, temperature and feeding trials.

2.2 | Larval density trials

In order to evaluate the effect of density on the survival, development and growth of *Isostichopus* sp. larvae, an experiment was designed in which two culture densities were evaluated, one low of 1 larva ml^{-1}, normally recommended in the culture of tropical species larvae such as *I. badionotus* and *H. scabra* (Agudo, 2006;

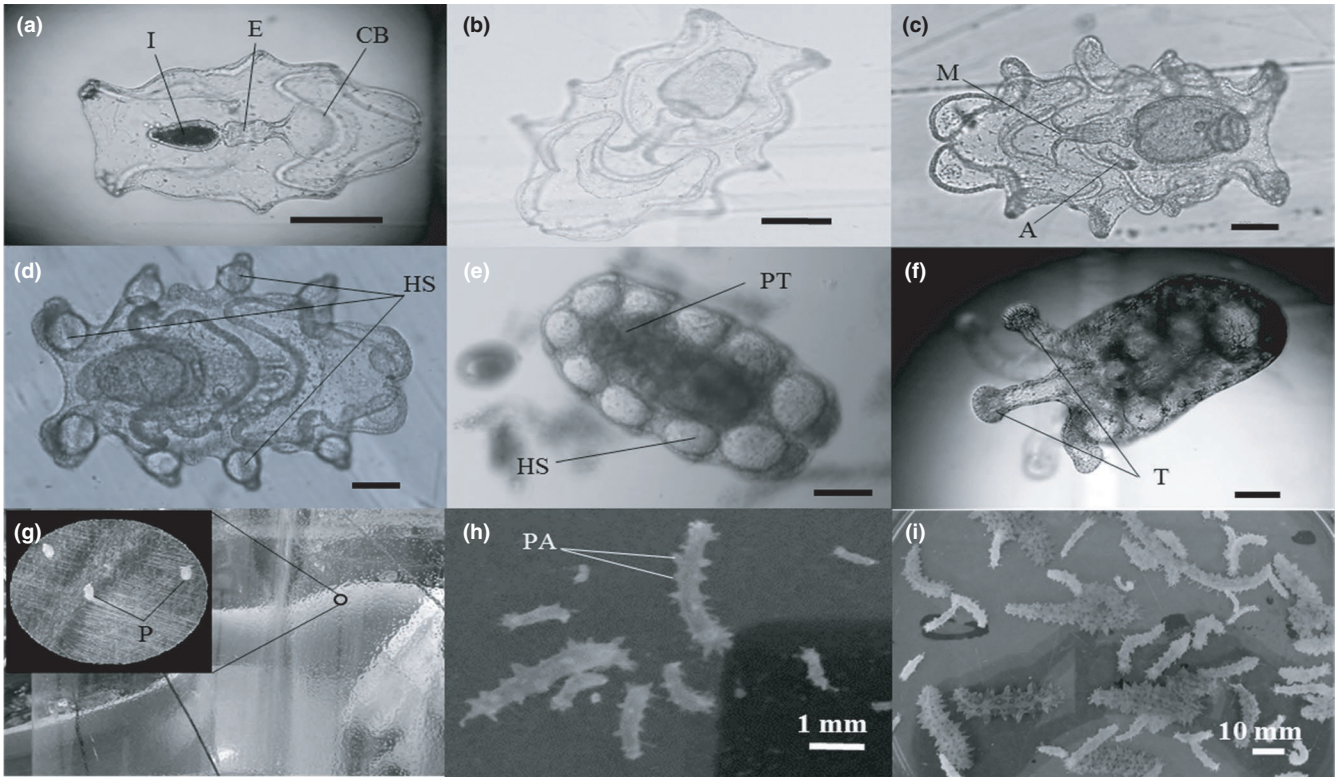

FIGURE 2 Larval development of *Isostichopus* sp. (a) Early auricularia; intestine (I), oesophagus (E), buccal cavity (BC). (b) Mid auricularia, (c) late auricularia; mouth (M), axohydrocoel (A). (d) Late auricularia with hyaline spheres; hyaline spheres (HS). (e) Doliolaria; primary tentacles (PT), hyaline spheres (HS). (f) Pentactula; buccal tentacles (T). (g) Pentactula larva on settlement plates; pentactula (P). (h) Juveniles 64 days; ambulacral podia (AP). (i) Juveniles 95 days old. The bar represents 100 μm except in the figures (h) and (i)

Zacarías-Soto et al., 2013) and another high of 3 larvae ml^{-1}, that has been reported as the maximum density with which species as *A. japonicus* larvae have culminated their development (Liu et al., 2010).

Each treatment was made up of three replicates, each one made up of a tank calibrated at 190 L, provided with FSW, soft aeration, thermo-regulated at 25 ± 1°C; and the photoperiod was maintained at 12:12 hr (light:darkness). During the larval culture every 2 days, the tanks were drained and the larvae were retained in a sieve of 120 μm and transferred to a plastic bucket gauged to 5 L. After homogenizing, three sub-samples of 1 ml of each replicate were taken to (a) estimate the number of larvae present by means of direct count under the microscope; (b) determine the rate of survival and at the same time; (c) record the degree of development of the larvae. Larvae pass through three stages of auricularia (early, mid and late), and then, the hyaline spheres appear and metamorphosis to the doliolaria larvae occurs (Figure 2). The remaining larvae were transferred to a clean tank with the same characteristics as described above. The lengths of 20 randomly selected larvae from each replicate were measured daily using a microscope with AxioVision Rel software 4.8. The physicochemical parameters temperature and salinity were recorded daily.

The larvae were fed 1 time per day using a mixture of the microalgae *I. galbana*, *C. calcitrans*, *Nannochloropsis oculata*, *Pavlova* sp. and *Tetraselmis chuii*, in a proportion according to the stage of development in which the larvae were found (Table 1). The microalgae used in this study were provided by the microalgae production laboratory of the GIDTA research group and cultured in Guillard F/2 medium (Guillard, 1975).

2.3 | Temperature trials

Based on the average seawater temperature during the maximum spawning peaks of *Isostichopus* sp. (August–September) and the end of its reproductive season (November–December) (Agudelo-Martínez & Rodríguez-Forero, 2017; Díaz et al., 2000; Mancera-Pineda et al., 2013), two experimental temperatures 26°C and 23°C were established to examine the survival, development and growth of larvae. Each treatment consisted of three replicates, each made up of a tank calibrated to 100 L provided with FSW, soft aeration and at a culture density of 1 larva ml^{-1}. In the 26°C treatment tanks, an electric heater was added, equipped with a thermostat that allowed a variation in temperature of ±0.5°C, while the 23°C treatment tanks were placed directly under the air current of an air conditioning system, with a variation in temperature of ±1°C.

The methodology for water exchange, counting and measurement of larvae, as well as the concentration and type of microalgae diet provided (Table 1) was the same as used in the density trial.

2.4 | Feeding trials

The effect of two microalgae diets on survival, development and growth of the larvae of *Isostichopus* sp. was evaluated. Diet 1. *I. galbana* and *C. calcitrans* in relation 1:1 (I:C) and diet 2. *I. galbana*, *C. calcitrans* and *N. oculata* in relation 1:1:1 (I:C:N). Both microalgae mixtures are reported as appropriate for larval culture in several tropical sea cucumbers (Agudelo-Martínez & Rodríguez-Forero, 2017; Agudo, 2006; Asha & Muthiah, 2002, 2006; Sánchez, 2012). Each treatment consisted of three replicates, each made up of a tank calibrated at 130 L provided with FSW, soft aeration, thermo-regulated at 25 ± 1°C at a culture density of 1 larva ml^{-1}.

The methodology for water changes, counting and measurement of larvae was the same as used in the density trial. The concentration of the diet supplied varies according to the developmental phase of the larvae (Table 1).

2.5 | Settlement of larvae and cultivation of juveniles

In the treatments in which the larvae reached the doliolaria stage, polycarbonate plates (30 × 40 cm) were added to serve as substrate during the settlement, which were pre-incubated for approximately 10 days in a tank that had been inoculated with the microalgae *I. galbana*, *Pavlova* sp., *T. chuii*, *C. calcitrans* and *N. oculata*.

A total of 20 plates, corresponding to a settlement area of 48.000 cm^2, were distributed between the water column and the bottom of each culture tank and in addition the culture tanks were covered with a shadow mesh to reduce the penetration of light and to favour the settlement, because the larvae have positive phototropism (Agudelo et al., 2016; Agudo, 2006). The settling tanks had a 150% daily water change, always maintaining their water level during the process through the entry at the top of the tanks of a constant flow

TABLE 1 Microalgal feeding rate for *Isostichopus* sp. larvae

Larval stage	Feeding rate (cells ml^{-1})	Density trials	Temperature trials	Diet trials	
		Microalgae	Microalgae	Microalgae I:C:N	Microalgae I:C
Early auricularia	20,000	*Isochrysis galbana*: *Chaetoceros calcitrans*: *Nannochloropsis oculata*: *Tetraselmis chuii*: *Pavlova* sp. (1:1: 1:1: 1)	*I. galbana*: *C. calcitrans*: *N. oculata*: *T. chuii*: *Pavlova* sp. (1:1: 1:1: 1)	*I. galbana*: *C. calcitrans*: *N. oculata* (1:1: 1)	*I. galbana*: *C. calcitrans* (1:1)
Mid-auricularia	30,000				
Late auricularia	40,000				
Pentactula	40,000				
Juveniles	60,000 cells ml^{-1} of *I. galbana*: *C. calcitrans* (1:1) + Algamac-2000 (Bio-Marine, Inc.) (0.5 g/m) + *Spirulina* (0.25 g/m)				

of FSW and a similar outflow at the bottom of the tanks, retaining on a 40 μm sieve the unsettled larvae and then returning them to the tanks. Six days after the start of observation of first settlement, the plates were removed from the tanks and replaced by new plates, which remained in the settlement tanks until the presence of larvae on the 40 μm sieve was no longer observed during the water change process.

The plates containing settled larvae were transferred to 250-L (250 × 30 × 40 cm) rectangular tanks for the lifting of juveniles with FSW and soft aeration. The concentration and type of food supplied to the settlement larvae and juveniles are described in Table 1. The size and total number of juveniles produced in each treatment were evaluated on day 64 of the culture.

2.6 | Statistical analyses

The survival and larval growth (response variables) of *Isostichopus* sp. in each of the trials conducted were analysed by a generalized linear mixed model (GLMM) (using an 'identity' linkage function), with tank nested into the treatment (either density, temperature or feeding) as an interactive random effect, the treatment (density, temperature or feeding) as a main fixed effect and the age of larvae (culture days) as a fixed effect of categoric order. Where significant effects were detected, multiple range comparisons were employed using Tukey's post hoc honest significant difference (HSD) test. The differences in the percentage of larval development between treatments were analysed for each day using a one-way ANOVA. Differences among treatment means were tested for significance by a post hoc multiple comparisons (Tukey's HSD) test. Prior to data analysis, normality assumptions were checked using the Kolmogorov–Smirnov test and variance homogeneity using Cochran's C-test. In the case of survival in the larval density and feeding experiments, the data were transformed with square root and in the temperature experiment they were transformed with Rank to meet these assumptions. The growth data in the feeding experiment were transformed with square root. Statements of significant differences were based on accepting $p \leq 0.05$. All analyses were carried out with the statistical program Statgraphics XVII.

3 | RESULTS

3.1 | Larval density trials

During the first 18 days of culture, the larvae maintained at a density of 1 larva ml^{-1} showed higher survival rates compared to those maintained at 3 larvae ml^{-1} (32.2 ± 2.8% and 26.5 ± 1.3% [±*SE*]) respectively. However, from day 20 of culture a decrease in survival was observed, recording for day 22 a final survival of 7.9 ± 1.0% and 9.1 ± 1.2% respectively (Figure 3a).

The GLMM showed a significant effect of density on the survival of the larvae ($F = 10.88$; $df = 1$; $p = 0.03$) and post hoc tests revealed that the highest survival occurred at the density of 1 ml^{-1} larva. The random effect of the tank was not significant on the survival of the larvae ($F = 2.46$; $df = 4$; $p = 0.057$). The information relevant to the model is described in Table 2.

All larvae remained in the early auricularia stage during the first 6 days of culture. The presence of mid auricularia larvae was recorded on day 8 of culture in both densities with 89.3% and 89.6%, respectively, and from the 10th day of culture the presence of all three stages of auricularia larvae (early, mid and late) was observed at both densities (1 larva ml^{-1} 8.3%, 27.3% and 64.4%, respectively, and 3 larvae ml^{-1} 3.6%, 30.9% and 65.5% respectively; Figure 4a). The three stages of auricularia larvae was maintained until day 18 of culture, characterized by the gradual increase in the percentages of late auricularia larvae and the formation of hyaline spheres, sign of the proximity of their metamorphosis to doliolaria larva (Figure 2d). In the case of larvae cultivated at the density of 1 larva ml^{-1}, the hyaline spheres were identified for the first time on day 16 of culture in 18.3% of the larvae observed, while for the density of 3 larvae ml^{-1}, this was only detected on day 18 of culture in 8.3% of the larvae observed.

From day 20 of culture, only larvae in mid and late auricularia stage were observed in the density of 1 larva ml^{-1} (16.0% and 84.0% respectively) and 3 larvae ml^{-1} (with 15.7% and 84.3% respectively). In the same way, it was observed that 66.7% of the larvae in the density of 1 larva ml^{-1} and 33.3% in the density of 3 larvae ml^{-1} had hyaline spheres. At the end of the larval culture on day 22, the presence of larvae that metamorphosed to the doliolaria stage was observed. For this day, the percentages of mid, late auricularia and doliolaria larvae at the density of 1 larva ml^{-1} were 9.8%, 58.7% and 31.5%, respectively, while at the density of 3 larvae ml^{-1} they were 12.0%, 83.3% and 4.7% respectively (Figure 4a). The average length and duration of each larval development stage is described in Table 3.

One-way ANOVA results showed significant differences only on culture day 22, where the density of 1 larva ml^{-1} recorded a significantly lower percentage of late auricularia larvae ($F = 13.18$; $df = 1$; $p = 0.022$) and at the same time a significantly higher percentage of doliolaria larvae ($F = 13.77$; $df = 1$; $p = 0.021$) compared to the density of 3 larvae ml^{-1} (Figure 4a).

The larvae cultivated at a density of 1 larva ml^{-1} showed a growth of 29.2 μm/day and an average length at the end of the larval culture of 913.2 ± 14.7 μm (±*SE*), which was significantly higher than that obtained by the density of 3 larvae ml^{-1} with a growth of 27.3 μm/day and an average length at the end of the culture of 874.7 ± 12.8 μm (Figure 3b; Table 3). The GLMM showed a significant effect of density on larval growth ($F = 32.02$; $df = 1$; $p = 0.005$) and post hoc tests revealed a higher average growth in larval density of 1 larva ml^{-1}. Also, the random effect of the tank was significant ($F = 4.70$; $df = 4$; $p = 0.001$). The information relevant to the model is described in Table 2.

3.2 | Temperature trial

Survival at both temperatures showed a similar behaviour, observing a strong decrease of approximately 60% in the number of

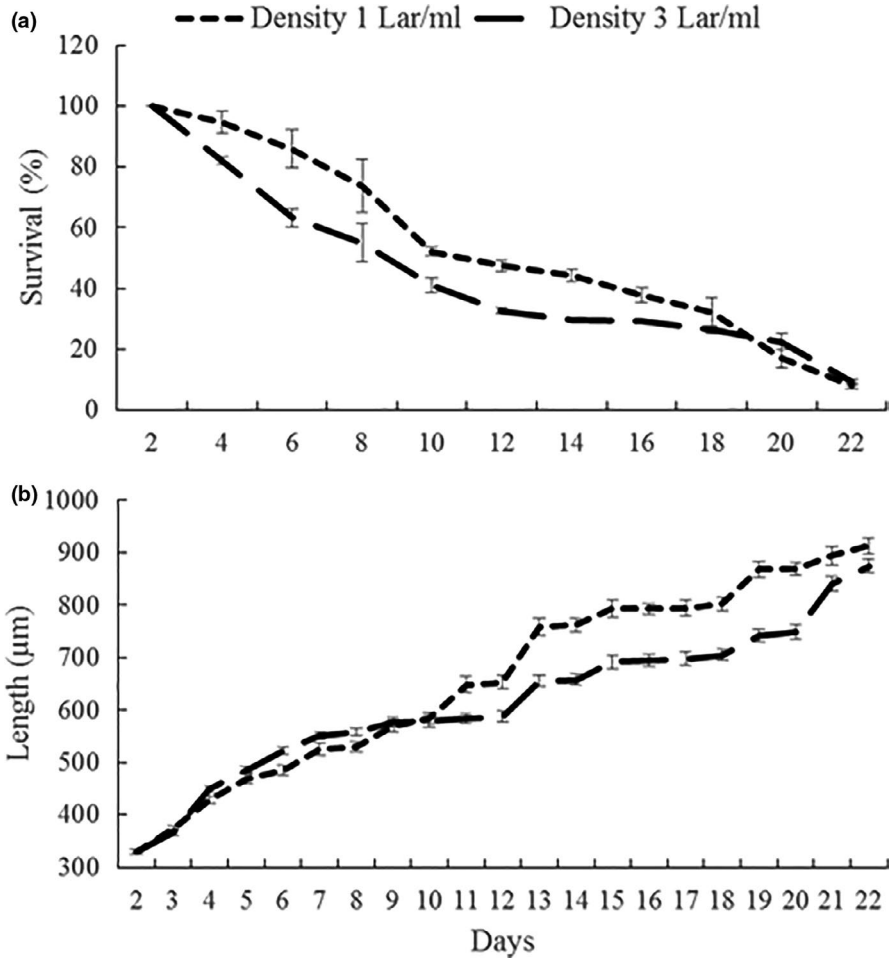

FIGURE 3 (a) Survival of *Isostichopus* sp. larvae ($n = 3$), and (b) growth of *Isostichopus* sp. larvae ($n = 60$) under different culture densities. Bars indicate standard error

TABLE 2 Model information for larval culture density, culture temperature and larval feeding trials

Elements of the model	Larval density trials	Temperature trial	Feeding trials
Analysis object	*Isostichopus* sp. larvae	*Isostichopus* sp. larvae	*Isostichopus* sp. larvae
Distribution	Normal	Normal	Normal
Link function	Identity: $g(\mu) = \mu$	Identity: $g(\mu) = \mu$	Identity: $g(\mu) = \mu$
Response variables	Percentage of live larvae Increase of size in microns (µm)	Percentage of live larvae Increase of size in microns (µm)	Percentage of live larvae Increase of size in microns (µm)
Explanatory variable	Density of larval culture used	Culture temperature used	Larval feeding diet used
Random effect	Culture tank	Culture tank	Culture tank

larvae between days 6 and 10 of culture in both the treatments of 23°C and 26°C (Figure 5a), period of time that coincides with the passage of larvae from early auricularia to mid and late auricularia stage (Figure 4b), recording a final survival of 2.2% and 3.3% respectively. No significant difference in larval survival was observed under any of the temperatures tested, nor was there a significant influence from the random effect of the tank (GLMM, $F = 0.14$; $df = 1$; $p = 0.727$ and $F = 2.48$; $df = 4$; $p = 0.056$ respectively). The information relevant to the model is described in Table 2.

Regarding the larval development at both temperatures, 100% of the larvae remained in early auricularia stage until day 6 of culture. At day 8 of culture, the three larval stages early, mid and late auricularia were present (at 23°C 22.6%, 51.0% and 26.4%, respectively, and at 26°C 26.3%, 45.2% and 28.5% respectively). For day 10 of culture, a decrease in the percentages of early and mid-auricularia and an increase in the percentage of late auricularia was noted in both temperatures, a trend that was maintained until day 18 of the culture. First larvae with presence of hyaline spheres appeared on day 14 of culture, initially only at 26°C in 7% of the larvae observed and later on day 16 also at 23°C in 8% of the larvae, while the temperature of 26°C for the same day recorded a 17%.

On the 20th day of culture at 23°C, only mid- and late auricularia larvae were observed (66.7% and 33.3%, respectively) and of these 23.3% presented hyaline spheres, while at 26°C 96.4% of the larvae were in late auricularia (and of these 41.7% presented hyaline spheres) and 3.6% had already metamorphosed to doliolaria larva. On day 22

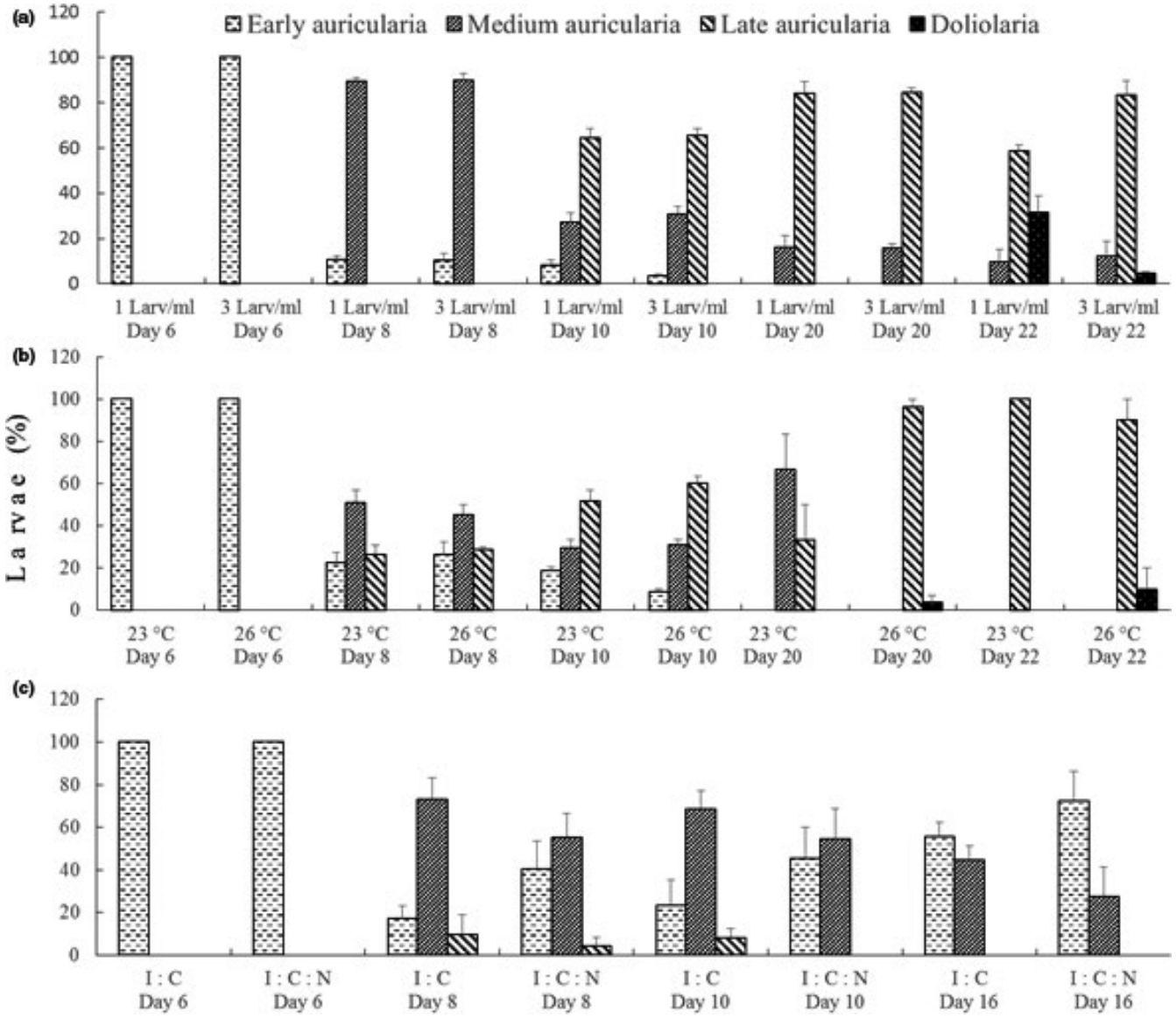

FIGURE 4 Percentages of early-, mid-, and late auricularia and doliolaria larvae of *Isostichopus* sp. on days 6, 8, 10, 20, 22. (a) Density trials; (b) temperature trials; (c) diet trials. The larvae from the diet trials survived until day 16. Bars indicate standard error ($n = 3$)

at 23°C 100% of the larvae were in late auricularia stage, of which 28.3% had hyaline spheres while at 26°C 90% of the larvae were in late auricularia (with 60% with presence of hyaline spheres) and 10% had already metamorphosed into doliolaria larvae. The average length and duration of each larval development stage is described in Table 3.

One-way ANOVA results showed on day 10 a significantly lower percentage of early auricularia larvae at 26°C compared to 23°C ($F = 18.85$; $df = 1$; $p = 0.012$) and on days 12 and 20 at 26°C a significantly higher percentage of late auricularia larvae was recorded ($F = 73.47$; $df = 1$; $p = 0.001$ and $F = 169.0$; $df = 1$; $p = 0.006$, respectively, Figure 4b).

Larvae cultivated at 26C° had an average growth of 28.4 μm/day and an average length at the end of the culture of 895.5 ± 15.3 μm, which was significantly higher than that obtained at 23°C with a growth of 22.8 μm/day and an average length at the end of the culture of 784.9 ± 17.7 μm (Figure 5b; Table 3). The GLMM showed a significant effect of temperature on larval growth ($F = 44.43$; $df = 1$; $p = 0.003$) and the post hoc test revealed a higher average growth at 26°C; similarly, the random effect of the tank was significant ($F = 3.46$; $df = 4$; $p = 0.0079$). The information relevant to the model is described in Table 2.

3.3 | Feeding trials

The feeding trial presented the shortest survival times, recording diet I:C live larvae until day 16 and diet I:C:N until day 18, with a final survival of 8.5% and 5.3% respectively. The highest mortality occurred between days 6 and 8 of culture, with 62.4% and 76.9% for diets I:C and I:C:N, respectively (Figure 6a), coinciding with the formation of mid- and late auricularia larvae. No significant differences in larval survival were observed under any of the diets

TABLE 3 Length (±*SE*) of *Isostichopus* sp. larvae at the beginning and end of each stage of development in density, temperature and diet trials $n = 20$

	Length (μm)						
Larval stage	Day	1 Larv. ml^{-1}	3 Larv. ml^{-1}	*T*—23°C	*T*—26°C	I:C	I:C:N
Early auricularia							
Start	2	328.77 ± 4.66	329.65 ± 4.10	328.77 ± 5.11	328.03 ± 3.47	330.58 ± 4.31	331.52 ± 5.43
End	6	485.57 ± 10.12	522.55 ± 7.65	454.37 ± 8.12	533.7 ± 11.01	415.81 ± 8.50	396.80 ± 6.58
Medium auricularia							
Start	7	525.65 ± 11.15	551.03 ± 7.99	510.58 ± 9.70	543.70 ± 12.17	433.62 ± 10.55	381.55 ± 6.64
End	9	570.15 ± 11.60	577.03 ± 9.27	587.28 ± 11.46	624.78 ± 14.39	435.68 ± 9.43	363.18 ± 7.74
Late auricularia							
Start	10	585.18 ± 10.86	579.63 ± 11.43	597.15 ± 11.30	627.78 ± 12.59	435.28 ± 10.47	362.57 ± 8.52
End	22	913.24 ± 14.70	874.65 ± 12.84	784.95 ± 17.71	895.45 ± 15.32	–	–
Doliolaria	22	700.50 ± 8.50	690.50 ± 12.20	–	698.30 ± 16.40	–	–
Pentactula	25	510.70 ± 11.9	508.50 ± 10.6	–	512.20 ± 9.70	–	–
Juvenile	29	621.80 ± 12.7	625.30 ± 11.2	–	620.50 ± 12.5	–	–
Juvenile 64th[a]	64	5.10 ± 0.10 mm	4.30 ± 0.20 mm	–	4.70 ± 0.30 mm	–	–

[a]The length of the 64-day juveniles is expressed in millimetres.

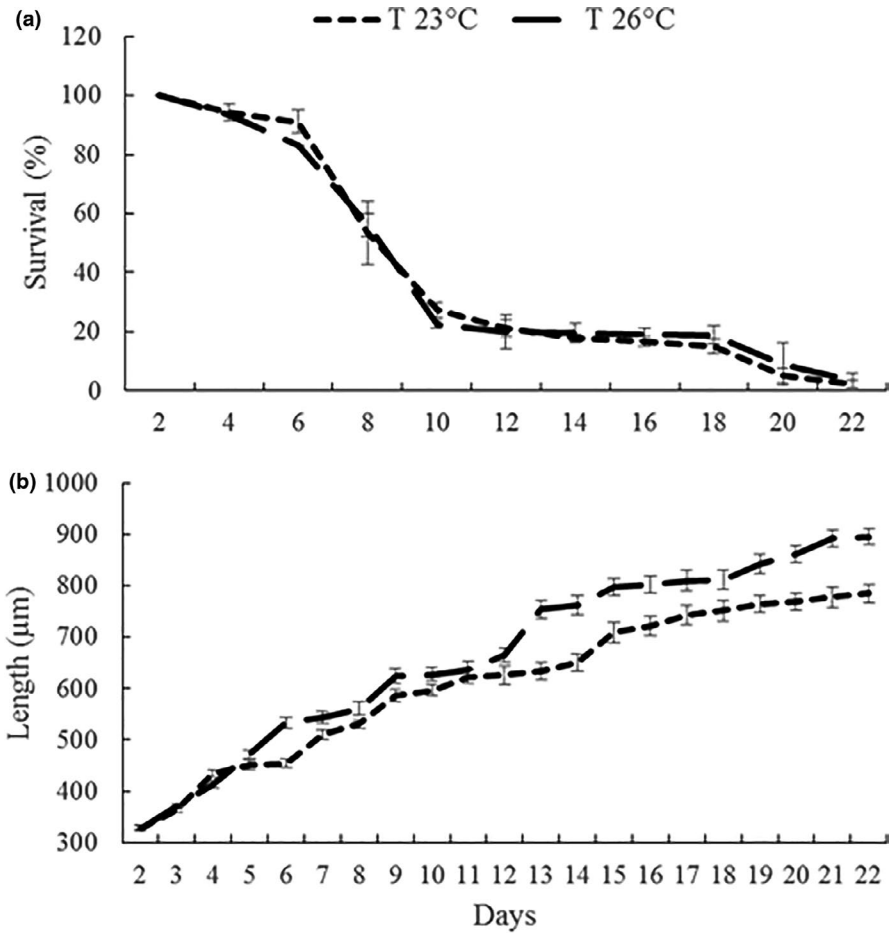

FIGURE 5 (a) Larval survival of *Isostichopus* sp. larvae under two different culture temperatures ($n = 3$), (b) larval growth of *Isostichopus* sp. under different culture temperatures. Bars indicate standard error ($n = 60$)

tested (GLMM, $F = 1.29$; $df = 1$; $p = 0.319$); however, the random effect of the tank was significant (GLMM, $F = 3.77$; $df = 4$; $p = 0.012$). The information relevant to the model is described in Table 2.

100% of the larvae of both diets remained in early auricularia stage until day 6 of culture. The presence of larvae in early-, mid- and late auricularia stage was observed from the eighth day, in diet I: C with 17.2%, 73.3% and 9.5%, respectively, and in diet I:C:N with 40.5%

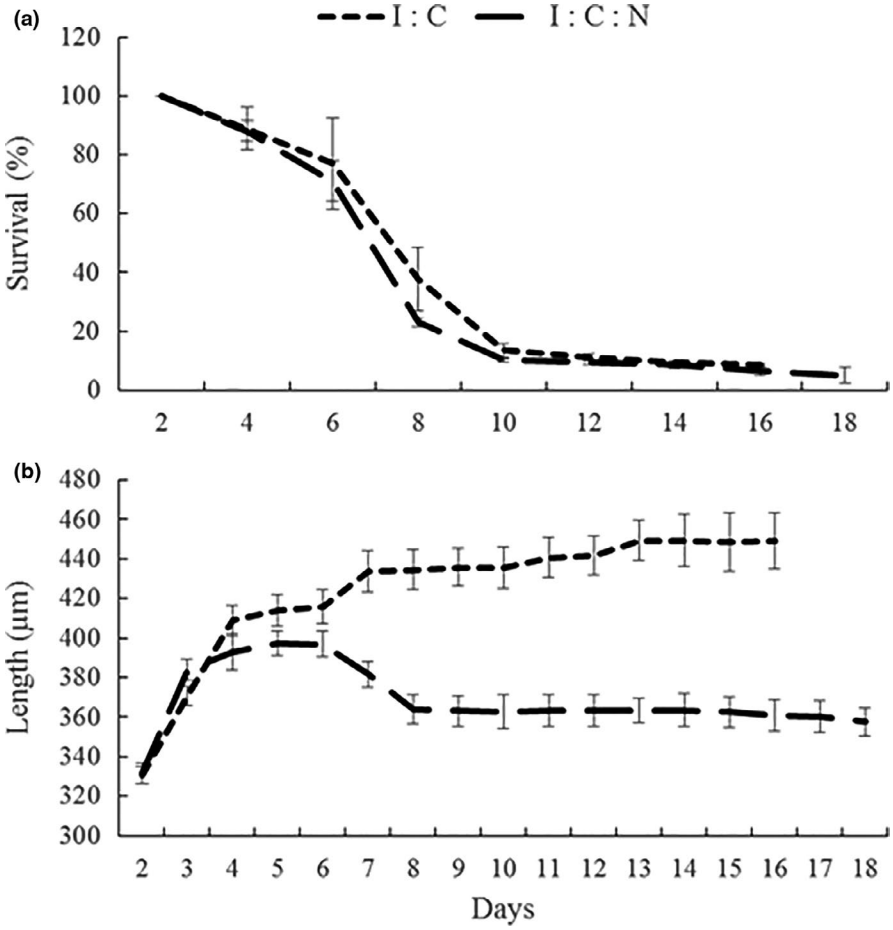

FIGURE 6 (a) Survival of *Isostichopus* sp. larvae with two different diets. ($n = 3$), (b) larval growth of *Isostichopus* sp. with different diets. *Isochrysis galbana* and *Chaetoceros calcitrans* (I:C); *I. galbana*, *C. calcitrans* and *Nannochloropsis oculata* (I:C:N). Bars indicate standard error ($n = 60$)

55.3% and 4.2% respectively. However, at the end of the culture on day 16, a presence of larvae in late auricularia stage was not observed and only the early and mid stages were recorded (diet I:C 55.5% and 44.5% and diet I:C:N 72.4% and 27.6% respectively). No significant differences in larval development were observed with respect to the diets tested, nor were they recorded in any of the larval diets with the presence of hyaline spheres (Figure 4c). The average length and duration of each larval development stage is described in Table 3.

The larvae fed with the I:C diet presented an average growth of 8.5 μm/day, reaching at the end of the culture (16 day) an average length of 449.2 ± 14.2 μm, which was significantly greater than that recorded by the larvae fed with the I:C:N diet with an average growth of 1.3 μm/day and an average length at the end of the culture (day 18) of 361.0 ± 8.0 μm (Figure 6b; Table 3). The GLMM showed a significant effect of the diet on larval growth ($F = 12.46$; $df = 1$; $p = 0.024$) and the post hoc test revealed a greater average growth in the diet I:C, similarly the random effect of the tank was significant ($F = 22.48$; $df = 4$; $p = 0.001$). The information relevant to the model is described in Table 2.

3.4 | Settlement of larvae and cultivation of juveniles

On the 23rd day of culture, the settlement substrates were placed in the 26°C, 1 and 3 larvae ml^{-1} treatments, as these were the only ones that presented larvae in doliolaria stage. Two days later, the first pentactula larvae settled with an average size of 510.7 ± 11.9 μm (±*SE*) (Figure 2f). For the 27th day of culture, the substrates were densely populated with pentactula larvae with an average of 17 ± 2.6 individuals cm^{-2} (±*SE*) (Figure 2g).

The presence of the first juveniles with a size of 621.8 ± 12.7 μm (±*SE*) was observed on the 29th day of culture. For day 64 of culture in the treatment of 1 larva ml^{-1}, a total of 455 juveniles were obtained with an average size of 5.1 ± 0.1 mm (±*SE*), with a maximum and minimum length of 13.4 and 1.2 mm respectively. The treatment of 3 larvae ml^{-1} yielded 165 juveniles with an average size of 4.3 ± 0.2 mm and a maximum and minimum length of 15.4 and 1.6 mm respectively. Finally, the treatment of 26°C yielded 60 juveniles with an average length of 4.7 ± 0.3 mm and a maximum and minimum length of 13.6 and 1.4 mm respectively (Figure 2h).

4 | DISCUSSION

This study determined the effects of density, temperature and type of diet on the growth, development and survival of the larvae of the sea cucumber *Isostichopus* sp. and reports for the first time the successful production of sea cucumber juveniles of this species in Colombia.

4.1 | Larval density trial

In the present study, it is observed that larvae cultivated at higher density (3 larvae ml^{-1}), showed a lower survival and growth rate compared to those kept at low density (1 larva ml^{-1}). In the same way in the density of 3 larvae ml^{-1}, a smaller percentage of the larvae developed hyaline spheres, requiring a greater time for the formation of these spheres and produced a significantly smaller percentage of larvae that metamorphosed to the doliolaria stage.

These results suggest that the increase in density in *Isostichopus* sp. negatively affects the survival, growth and development of the larvae and therefore the obtaining of "competent" larvae, which according to authors such as Chia (1977), Dautov and Kashenko, (1994), Dautov, (1997) and Ramofafia et al. (2003) are defined as those larvae that have sufficient nutritional and energy reserves to complete the metamorphosis, which is intimately linked to the formation of hyaline spheres.

These results are consistent with those of Li and Li (2010), Liu et al. (2010) in *A. japonicas* larvae and Ren et al. (2017) in *Parastichopus californicus* larvae, who evaluated a wide range of larval culture densities (from 0.2 to 10 larvae ml^{-1}), observing that as the density increased the survival decreased and the larvae experienced a slow growth and development, showing a reduction in the percentage of larvae that metamorphose to doliolaria, which besides having a smaller size, require more time to reach this stage.

Authors such as Avila et al. (1997), Ito and Kitamura (1998), Li and Li (2010), Liu et al. (2010), MacDonald (1988), Orensanz et al. (1991), and Velasco and Barros (2008) indicate that the use of high densities in marine invertebrate larval culture increases the possibility of collisions between larvae. Collisions that cause the abrupt cessation of movement of the ciliary bands that the larvae use to swim and feed also cause a reduction in the rate of ingestion, which can lead to nutritional deficiencies. In the same way, the increased density can generate competition for food, which would also affect the rate of ingestion.

Although the same amount of food was used in this work for both low and high density, the concentrations used were the highest recommended by Agudo (2006) to supply the nutritional needs of sea cucumber larvae. Similarly, Liu et al. (2010), within their research with *A. japonicus* comparing densities of 0.8 and 1.6 larvae ml^{-1}, provided to their treatment of higher density twice the recommended food ration of Agudo (2006), but obtained as in the present research, less growth, survival and development at higher density.

These same authors point out that the use of high densities leads to an increase in excretion products such as faeces and ammonium that deteriorate the quality of the water, conditions that negatively affect the larval growth, survival and metamorphosis of the larvae. In this sense, despite the fact that in the present research the concentrations of ammonium in the water were not measured, it was possible to observe during water changes a greater amount of faeces and conglomerates of dead algae on the bottom of those tanks that handled a higher larval density compared to those of low density.

By day 20 of culture, about 70% of the larvae at a density of 1 larvae ml^{-1} presented well-developed hyaline spheres, which are indicative of the end of the auricularia stage and the beginning of metamorphosis (Ramofafia et al., 2003; Ren et al., 2017). Therefore, it is plausible to suppose that the sudden decrease in survival in this density from day 20 of culture is a consequence of the beginning of the metamorphic processes that lead to the formation of the doliolaria larva, going via pentactula to juveniles (Figure 2). The increase in mortality could be due to stress generated by the changes that the larva undergoes during this period, in which it does not feed and depends exclusively on the lipid reserves stored in the hyaline spheres (Lacalli & West, 2005; Rakaj et al., 2017; Ramofafia et al., 2003; Ren et al., 2017).

The variability in larval growth produced by the effect of the culture tank observed in all the trials is difficult to explain, since being a random variable it can have different origins like the position of the tank, the amount of light it receives etc. However, taking into account that water replacement (which involves drainage of the tank, retention of larvae and resuspension in a new tank) is one of the processes that generates greater stress to the larvae, we assumed that the variation in drainage time between tanks (although brief) may be one of the main causes of the effect of variability produced by the tanks.

4.2 | Temperature trial

Despite not finding significant differences in the survival of the larvae between the two tested temperatures, it was possible to establish a greater growth and development in the larvae kept at 26°C, of which more than 50% at the end of the larval culture presented well-developed hyaline spheres and 10% had metamorphosed to doliolaria, indicating that an increase in temperature positively affects the growth and development of the larvae of *Isostichopus* sp.

These results are consistent with those reported by Asha and Muthiah (2005) in the tropical species *H. spinifera*, who evaluated different temperature ranges between 20°C and 32°C, reporting fast growth and optimal development of the larvae followed by early metamorphosis to doliolaria when temperatures were above 28°C. Similarly, authors such as Li and Li (2010), Liu et al. (2010), Li et al. (2011) and Ren et al. (2017), who evaluated different temperatures between 10°C and 24°C in temperate species such as *A. japonicas* and *P. californicus*, observed a positive effect on the growth, development and metamorphosis of the larvae when the temperature increased.

All these authors agree on the positive effect of the increase in temperature, but also indicate that it should be maintained within certain ranges to avoid negative effects on the growth and survival of larvae, suggesting that the optimal temperature for the correct development of any sea cucumber larvae often occurs within the

temperature ranges found during the breeding season in the natural habitat.

In this regard, Agudelo-Martínez and Rodríguez-Forero (2015, 2017) point out that the reproductive season of *Isostichopus* sp. in the region of Santa Marta extends from late July to early December, a period of time that according to Díaz et al. (2000) and Mancera-Pineda et al. (2013) is characterized by a drastic change in seawater temperature, with temperatures between the months of July and October of 29°C to 26°C and a rapid decrease to 24°C/23°C between the months of November and January due to a local phenomenon of upwelling of cold water from the sea. This would suggest that the temperature range within which the reproductive season of *Isostichopus* sp. occurs would be between 27°C and 23°C.

The increased growth and development presented by larvae kept at a higher temperature (26°C) could be a consequence of an increase in the metabolism of the larva. In this respect, Beiras et al. (1994) and Lu and Blake (1997), observed that in bivalve larvae an increase in temperature generates an increase in activity and ingestion rate. On the other hand, Bayne (1983) mentions that the cell walls of microalgae are better digested, because the enzymes that digest them are completely activated under these conditions and also the ciliary activity of the stomach in the larvae is higher under these conditions.

4.3 | Feeding trials

The low rates of growth and survival observed in both diet I:C and diet I:C:N suggest that the mixtures of these microalgae do not meet the nutritional needs of the larvae of *Isostichopus* sp., either because the concentrations of microalgae in relation to the mixture of 1:1 were not adequate to meet the nutritional requirements of the larvae and/or because one or more of the microalgae used in these diets offered poor nutritional value to the larvae of *Isostichopus* sp. In this regard, Knauer (2011) notes that the size and dry weight of microalgae varies depending on the species and therefore also varies its organic content and energy and, therefore, recommends calculating the mixture of microalgae based on an equal dry weight as a correction factor.

In relation to the proportion of microalgae used in trials, Velasco (2007) indicates that the dry weight of *I. galbana* (size range 3–6 μm) is four times higher than that of *C. calcitrans* (size range 3–7 μm) and five times higher than that of *N. oculata* (size range 2–3 μm), which in turn is 0.5 times lighter than *C. calcitrans*. Likewise, the organic contents among these species vary (74.8%, 77.3% and 49.2% respectively). This would indicate that the I:C:N diet (whose ratio is 1:1:1 based on the number of cells) by including *N. oculata* would offer lower dry weight and organic content compared to the I: C (1:1) diet, which may explain why the latter diet has better growth.

On the other hand, Velasco (2007) when evaluating the energetic physiology of two scallops fed with different microalgae, points out that the microalga *N. oculata* stores its energy in complex chemical bonds that require a high metabolic expenditure for their utilization, thus difficult to assimilate. In this same sense, Asha and Muthiah (2006), when evaluating the use of different microalgae for the culture of larvae of *H. spinifera*, found that a mono-algae diet composed by *N. salina* produces a stagnation in the growth of larvae from day 7 after fertilization, reduces larval survival and increases the percentages of deformed larvae. Although the larvae presenting a greater growth in diet I:C did not complete their development, this indicates that neither of the two tested diets satisfies the nutritional requirements of the larvae of *Isostichopus* sp. These results contrast with those obtained in the density and temperature trials, where the larvae fed with a diet composed of *I. galbana*, *C. calcitrans*, *N. oculata*, *Pavlova* sp. and *T. chuii* were achieving complete development up to the juveniles, suggesting that the inclusion of these last two microalgae in the diet would provide the nutritional and energetic requirements necessary for the larvae to complete their development.

On the other hand, rather than the physical characteristics of micro algae, it is probably the differences in biochemical composition, such as the total amounts and proportions of essential nutrients that explain the greater effectiveness of a diet. Duy et al. (2016) maintain that obtaining competent larvae depends to a great extent on the use of microalgae with relatively high proportions of arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), since these polyunsaturated fatty acids together with a diet rich in carbohydrates significantly favour the development of hyaline spheres and the subsequent metamorphosis to doliolaria larva.

With respect to the microalgae used in this trial, Pernet et al., (2003) and Volkman et al. (1989) point out that *I. galbana* has a high DHA content, but has low EPA values and does not contain ARA, while *C. calcitrans* has a high EPA and ARA, but low DHA content. James et al. (1989), mentions that *N. oculata* has medium EPA values and does not contain DHA. This would indicate that of the two diets evaluated, the one that would offer a better balance in relation to the content of polyunsaturated fatty acids EPA, DHA and ARA would be diet I:C; however, the stagnation in the development of larvae suggests that the amount of nutrients provided by the diet were insufficient. As observed in the experiment with inclusion of the microalgae *Pavlova* sp. and *T. chuii* in the diet, additional algae are needed to avoid these deficiencies.

The positive effect can be due on the one hand to a greater contribution of polyunsaturated fatty acids in the diet, since *Pavlova* has high EPA and DHA contents (Volkman et al., 1989) and *T. chuii* has high EPA contents (Dunstan et al., 1992) and on the other to an increase in the amount of food available in terms of dry weight, because *Pavlova* sp has a dry weight similar to that of *I. galbana* and *T. chuii* is a larger microalgae (size range 10–15 μm), with a dry weight three times greater than *I. galbana* (Velasco, 2007).

The results obtained in this trial contrast with those reported by Asha and Muthiah (2002, 2006) who obtained successful larval development and juveniles of *H. spinifera* either using a diet composed of *I. galbana*, *C. calcitrans* and *N. oculata* in relation 1:1:1 or a diet composed of *I. galbana* and *C. calcitrans* in relation 1:1. It is also opposed to what has been reported by authors such as

Knauer (2011) and Ren et al. (2015), who maintain that the use of *Chaetoceros* either alone or as a dominant part in a mixture improves the growth, development and survival of larvae of *H. scabra* and *P. californicus*. However, they are consistent with those reported by Sánchez (2012) and Zacarías-Soto et al. (2013), who found that a diet consisting of *I. galbana* and *Tetraselmis* sp. (1:1) favours the development of larvae and the obtaining of juveniles of *I. badionotus*.

Based on the results of the density and temperature experiments of the present study, it was established that the larval cycle duration of *Isostichopus* sp. from fertilization to the formation of the doliolaria larva is 22 days, less time than that reported by Agudelo-Martínez and Rodríguez-Forero (2017) for the same species in the same region, who obtained the doliolaria stage between 28 and 30 days after fertilization; however, it was similar to that reported by Sánchez (2012) and Zacarías-Soto et al. (2013), in *I. badionotus*, who obtained Doliolaria larvae between days 18 and 20 after fertilization.

In relation to juveniles, the average maximum length obtained in this research after 64 days of cultivation (5.1 ± 0.1 mm) was lower compared to those reported by Zacarías-Soto et al. (2013), who indicate that juveniles of *I. badionotus* at 65 days after fertilization presented an average length of 10.1 ± 1.3 mm. Similarly, Mercier et al. (2004) report that juveniles of *I. fuscus* at 63 days after fertilization presented an average length of 25 mm. On the other hand, the low number of juveniles obtained in this study and their wide variation in size could have been influenced by the scarce surface area offered during the settlement period, which led to a high concentration of pentactula larvae settled on a small space, which could have influenced their survival and size.

In this sense, in species such as scallops (bivalves) whose larvae show developmental phases similar to those of the sea cucumber (a period of swimming larvae, settlement and metamorphosis, culminating in the abandonment of the settlement substrate), it has been demonstrated that a high density of larvae settled in the substrates negatively affects the growth and survival of the juveniles (Bourne et al., 1989; Velasco & Barros, 2008).

5 | CONCLUSIONS

Isostichopus sp. reproduced successfully under controlled conditions, a new achievement that added to the great demand and value of the genus *Isostichopus* and makes it a species with a high potential for aquaculture in Colombia. The larval development of this species lasted 22 days, passing through three stages of auricularia larva (early, mid and late) until reaching the doliolaria stage. The best results in growth, development and metamorphosis to doliolaria larvae were obtained maintaining a culture density of 1 larva ml^{-1} and a temperature of 26°C in the water. The larvae of *Isostichopus* sp. are not satisfied with a diet of I:C and I:C:N only, and they require a wide choice of microalgae to complete their development until juveniles.

The settlement stage started after obtaining the doliolaria larva, which when reaching the pentactula stage settled on the offered substrate. Although this process lasted 9 days in total, most of the larvae settled in the first 5 days; therefore, it is advisable to replace the plates of settlement every 3 days to avoid a high density of pentactula larvae on the substrates.

Although the technology for sea cucumber seed production in Colombia is still at an early stage of development, the results obtained in this research constitute an important contribution to the protection, conservation and sustainable use of this valuable resource.

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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.

AUTHOR CONTRIBUTIONS

E.A., A.R., B.W. and A.K. contributed to conception and design of the study; E.A. performed the statistical analysis and wrote the first draft of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. All applicable international, national and/or institutional guidelines for the care and use of animals were followed (Bremen Senate Authority 2016/7). This manuscript has not been published and is not under consideration for publication elsewhere.

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