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Optimizing Growth and Rearing Techniques for Larvae and Juveniles of the Sea Cucumber *Holothuria arguinensis*

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Abstract: The ever-growing demand for sea cucumbers is a threat to these echinoderms and their habitats; however, a way to relieve stock pressure lies in meeting demand through aquaculture. As such, this study aimed to improve the growth and survival percentage of *Holothuria arguinensis* during larval development, settlement and juvenile growth. Three diets of microalgae (Diet RbPt = *Rhodomonas baltica* + *Phaeodactylum tricornutum*; Diet RbSm = *Rhodomonas baltica* + *Skeletonema marinoi*; Diet RbCc = *Rhodomonas baltica* + *Chaetoceros calcitrans*) were administered daily to the larval phase. Additionally, three substrates were supplied (pvc rolls, wavy pvc plaques and tile) at three different depths in order to determine the most favourable for settlement. Lastly, the potential benefits on growth and survival from introducing macroalgae (*Sacchoriza polyschides*) in the juvenile diet were assessed over a period of 6 months. Despite larvae under diet RbPt having presented a larger mean width, *H. arguinensis* fed with Diet RbCc presented a higher settlement survival at the end of the trial. No preferences were noted among the tested substrates, regardless of diet. Lastly, juveniles with added *S. polyschides* in their diet showed increased growth in mean weight and length when compared to individuals that were not fed with this seaweed.

Keywords: aquaculture; Holothuroidea; settlement structures; macroalgal diet; *Sacchoriza polyschides*; microalgae diets; *Rhodomonas baltica*; Skeletonema marinoi; Chaetoceros calcitrans; hatchery techniques

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1. Introduction

Sea cucumbers possess an important ecological role in the ecosystems they inhabit, from bioturbation of sediments to nutrient recycling [1]. However, they are most notably procured for their reputation as a nutritionally rich food resource as well as a very coveted medicinal supplement. With firm roots in traditional Eastern medicine, sea cucumbers across the Indo-Pacific have been extensively exploited to satisfy the rising demand of Asian markets [2]. The resulting illegal, unreported and unregulated exploitations have led to the collapse of several natural stocks and, as a result of local stock depletion, fishing areas have expanded to other regions [3–6]. Considering how demand for sea cucumbers is showing no signs of slowing down, maneuvering past barriers imposed by management, the next best alternative is to increase the supply. As such, aquaculture presents itself as a viable candidate to meet the increasing demand [7].

In eastern Asia, sea cucumber aquaculture has a long history, with breakthroughs such as the implementation of artificial reproduction in the 1930s, later improved in the l980s with optimized conditions for spawning, such as temperature manipulation [8,9]. Once these holothuroid spawning techniques were refined, there was a sharp rise in

production in the aquaculture sector, which kept expanding as natural stocks began to collapse [8,10]. In Europe, however, sea cucumber aquaculture techniques still have much room for optimization, as sea cucumber rearing is non-existent, and research on the topic is taking its first steps. Despite this, successful artificial spawning attempts have been demonstrated in recent studies, focusing on different species such as *Holothuria leucospilota*, *Holothuria polii* and *Holothuria forskali,* among others [11–13]. With the implementation of artificial spawning in aquaculture rearing, more studies have since focused on juvenile rearing in pond culture due to their high yield and relatively fast growth, on top of their low maintenance cost [14,15]. However, considering the ontogenic steps holothuroids take from spawning until juvenile and the high associated mortality [16], more emphasis needs to be placed on maximizing survival percentages during the larval development, settlement and early juvenile stages.

A promising species for future European aquaculture production is *Holothuria arguinensis* Koehler and Vaney, 1906, possessing high commercial interest and prices for consumption, reaching up to EUR 350 *per* dried kg [17]. With a natural distribution along the North-Eastern Atlantic, from the Canary Islands through Morocco and Algeria to the coasts of Portugal and Spain [18], this species is already showing a decrease in wild populations due to unregulated exploitation [19], particularly in regions in the south of Spain such as Andalucía and Málaga, and as far as the Canary Islands [20].

Previous studies have already paved the starting point for growth trials with this species, highlighting the nutritional benefits of certain microalgae during the larval stages, such as *Chaetoceros calcitrans* [21]. Some authors have focused on factors relating to settlement but with other species. Gianasi et al. [22], for example, highlighted the preference that settled sea cucumber *Cucumaria frondosa* larvae tend to have for darker surfaces. This type of behaviour could be a defence mechanism, making juveniles less conspicuous and, thus, less vulnerable to predation. Aside from the fact that aspects such as dietary requirements and behavioural traits are species-specific and are scarce or non-existent for *H. arguinensis*, all previously established trials took place within a limited time frame during the sea cucumber's life cycle. These are partially or entirely conducted during the larval development phase or the early juvenile growth phase independently. Additionally, sea cucumbers grown and maintained in aquaculture still present a slow development time, regardless of the life-cycle stage, on top of an accentuated heterogeneity between individuals from the same batch [13,23,24].

The present study aimed to improve the survival and growth performance of *H. arguinensis* larvae and hatchery-reared juveniles throughout all three of the previously mentioned stages: (1) during larval development, using diets composed of three different mixes of microalgae aimed to assess the influence of diet in the growth performance and survival throughout all larval stages; (2) at settlement, using different structures supplied to the culture tanks at different depths aimed to determine settlement preferences in captivity, regarding structure type and depth; and, lastly, (3) three new diets were supplied to juveniles, testing the introduction of macroalgae (*Sacchoriza polyschides*) to determine its effects on juvenile survival, as well as weight and length gain.

2. Materials and Methods

2.1. Spawning Induction

Wild *H. arguinensis* (mean wet weight ± SD = 352.49 ± 179.74 g, N = 15) were collected by hand with resort to SCUBA diving during low tide, from the coast of Arrábida, Portugal, and allocated to the aquaculture facilities of MARE Polytechnic of Leiria (in Peniche, Portugal) to induce spawning via thermal shock. Water quality parameters at the collection site presented a temperature of 17 °C, salinity of 34, dissolved oxygen (DO) levels of 97.67% and a pH of 7.97. Collected individuals were stored in 46 L containers, filled with seawater from the collection site and provided with constant aeration. Transport lasted for a little over one hour, with no sea cucumbers showing signs of stress or evisceration. Based on the optimized protocol by Venâncio et al. [16], individuals were rinsed with filtered saltwater

(through filters of reducing size, of 50 µm, 20 µm, 5 µm and 1 µm, before passing through a UV light for sterilization) and evenly distributed through 3 tanks of 600 L. From this point onwards, all seawater used was treated as such. The tanks were maintained at a temperature of 18 °C for a fasting period of 48 h for the holothuroids empty their guts. Afterward, they were transferred to a single 600 L pool with a water temperature 5 °C higher for a period of 2 h to induce spawning [16]. Males released their gametes first, followed by females approximately 30 min later.

Fertilization occurred for a period of 1 h, after which the fertilized eggs were siphoned and collected into a 4 L glass container. During the first 6 h after fertilization, 1 mL samples were collected once every 30 min, in replicates of 6, in order to monitor embryonic development. Once the majority of individuals observed reached the morula stage, samples started to be collected at hourly intervals until reaching 24 h post-fertilization. Observed embryos at this time were at the early gastrula stage and, to avoid a rapid increase of nitrogen compounds (NH_3, NH_4^+ and NO_2^-), embryos at this stage were transferred from the 4 L containers to 50 L white fiberglass cylinder-conical tanks. The increased volume of the new tanks mitigated the effects of ammonia concentration by dilution [25]. This, in turn, allowed for fewer interventions in the medium, such as frequent water changes, further reducing the probability of stress-induced mortality [26].

2.2. Larval Development and Settlement

The 50 L tanks used for this trial were cylinder-conical, with 42 cm in diameter and 47 cm in height. These tanks were supplied with settling structures at the trial onset so that biofilm would form naturally with each feeding, minimizing the need for medium intervention with the insertion of settling structures while larval development is occurring. These settling structures were composed of 3 different substrates, namely, black pvc rolls with u-shaped grooves (surface area= 178.50 cm^2), wavy transparent pvc plaques (surface area = 137.40 cm^2) and rough red clay tile (surface area = 47.37 cm^2), as illustrated in Figure 1. One of each substratum was placed at three different depths in the tanks (upper = 9 cm from the surface, middle = 18 cm from the surface and low = 27 cm from the surface) in order to determine which is the most suitable for *H. arguinensis* larvae to settle, as further detailed at the end of this section.

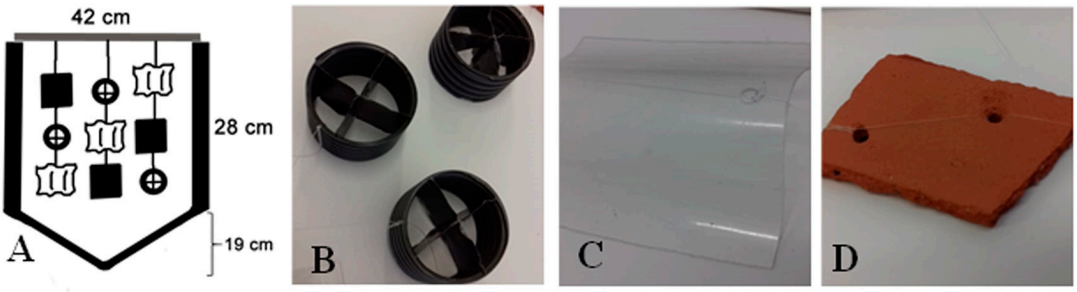


Figure 1. Illustration of the different substrates used for the settlement trials, including (**A**) Design and dimensions of the rearing tanks and the substrate structures within (**B**) PVC roll; (**C**) Wavy PVC plaque and (**D**) Tile fragment.

All tanks were provided with light aeration through diffusing stones so that air bubbles would not damage the developing larvae. Water temperature during larval rearing was higher than at the collection spot to increase embryo and larval survival [21,22], and water quality parameters were measured daily with a multiparametric sonde (YSI-Pro Plus, Yellow Springs, Ohio, USA), followed by water changes of up to 50% to assure stable levels of dissolved oxygen and pH, as well as to maintain nitrogen compounds at untraceable levels, whenever needed. These nitrogen compounds (NH_3, NH_4^+ and NO_2^- in $mg{\cdot}L^{-1}$) were measured with colour tests (API FishCare, UK), with a detection capacity between 0.25 and 5.00 $mg.L^{-1}$. As such, the tanks were maintained at a mean temperature (±SD) of 24.4 ± 0.4 °C, a salinity of 33 ± 1, DO levels at 90.7 ± 1.8 % and mean pH levels of

8.14 ± 0.05 throughout this trial (N = 16), with a photoperiod of 12 h of light, with low intensity fluorescent and natural lighting, based on the previous literature [16,18].

To account for early mortality during embryonic stages [11,27], each tank (n = 9) was stocked with an initial concentration of 4 gastrula·mL^{-1}. Daily determination of the concentration of growing individuals per tank was carried out during the first 16 days after spawning by taking 3 samples of 10 mL per tank, using a graduated pipette after lightly stirring the water to homogenize the spatial distribution of the pelagic larvae. The pipette was placed initially near the middle of the tank's depth and slowly brought upwards as the pipette filled to ensure homogeneity in sampling. Samples were examined with a binocular microscope (Zeiss Stemi DV4, Germany). On the 3rd day after fertilization, with the reveal of the early auricularia stage, larvae from all tanks (n ≈ 5 per tank) had their mean length and width measured (to the nearest 0.001 µm) to establish a starting point (t_0) for a growth trial. Considering how this is the first stage in the larval development phase with a buccal cavity capable of ingesting food [28,29], feeding began immediately after.

In order to determine the most suitable diet for larval development and settlement, all diets of the growth trial were composed of *Rhodomonas baltica* (Cell diameter = 13.391 ± 1.357 µm, N = 100), due to the success of this genus in previous works with congeneric species [30,31] and a complementary-microalgae to better satisfy the developing larvae's nutritional needs [32]. The chosen were *Skeletonema marinoi* (Cell diameter = 4.526 ± 1.281 µm, N = 100) due to their natural occurrence at the collection site [33] and two other species of diatoms *Phaeodactylum tricornutum* (Cell width = 2.391 ± 1.703 µm, N = 100) and *Chaetoceros calcitrans* (Cell diameter = 3.248 ± 1.181, N = 100) with registered success in previous works [16,29]. These microalgae were maintained in flasks of 1–5 L at a mean temperature (±SD) of 18 ± 1 °C with a mean salinity (±SD) of 33 ± 1 (N = 220) and a photoperiod of 12 h of fluorescent light per day. Microalgae concentrations were determined daily, in triplicate, using a Neubauer chamber, and each feeding was supplied with microalgae at their stationary phase. The microalgae measurements were conducted at 1000× amplification in samples of 30 cells *per* species, using the same binocular microscope and software as the larvae samples. The protein and lipid contents of the diets provided, as well as a portion of fatty acids important for larval development, are in Table 1, according to a previously established bibliography. These include Eicosapentaenoic acid (EPA), Docosahexaenoic acid (DHA), Arachidonic acid (ARA) and the sum of polyunsaturated fatty acids (PUFA). As such, each set of three tanks (replicates) was supplied with one of three diets (Diet RbPt = *Rhodomonas baltica* + *Phaeodactylum tricornutum*; Diet RbSm = *Rhodomonas baltica* + *Skeletonema marinoi* and Diet RbCc = *Rhodomonas baltica* + *Chaetoceros calcitrans*), added in equal concentrations of 2500 cells·mL^{-1} (in a 1:1 ratio), so that each tank would be provided a concentration of 5000 cells·mL^{-1} *per* day, throughout the experiment. These microalgae can adhere to substrates and form biofilm, which is, in short, the result of cell aggregation in surfaces as "microcolonies" and releasing a slime-like matrix composed of Extracellular Polymeric Substances (EPS). This matrix is made up of polysaccharides, proteins and nucleic acids, which grant the biofilm's architectural integrity [34,35]. This biofilm will allow settled individuals to feed on it until after reaching their final stage of development.

To assess and compare the effect of the diets on the somatic growth and development performance of *H. arguinensis*, 15 larvae *per* replicate were collected randomly from the daily samples whenever a turnover of a new developmental stage (mid auricularia, late auricularia, doliolaria, pentactula, juvenile) was observed. Each individual had their developmental stage determined and was then measured in length and width under the microscope (Leica® DM 2000 LED light optical microscope, equipped with a Leica® MC170 5MP HD Microscope Camera and the combined Leica Application Suite V4.4.0 software from Leica Microsystems GmbH, Wetzlar, Germany).

Once most individuals reached the juvenile stage, all settling structures described earlier in this section were carefully inspected, and settled individuals were counted to ascertain possible preferences for a particular structure at any particular depth. Afterward,

the tanks were drained, and juveniles were identified with a magnifying glass and carefully extracted with a soft paintbrush to be placed in trays filled with filtered seawater. A final count of all juveniles settled in the tank walls was made, allowing us to determine and compare their relative density (ind·cm^{-2}) in each of the settling structures and the tank walls themselves. Survival percentage was determined based on the total number of collected juveniles and the initial embryo distribution of 4 gastrulas·mL^{-1}.

Table 1. Protein and lipidic content, as well as relevant fatty acid proximal composition, in dry weight%, of the supplied diets based on previous studies. * Dry weight% of total fatty acid composition. (RbPt: *Rhodomonas baltica* + *Phaeodactylum tricornutum*; RbSm: *Rhodomonas baltica* + *Skeletonema marinoi*; RbCc: *Rhodomonas baltica* + *Chaetoceros calcitrans*).

Diet	RbPt	RbSm	RbCc	Source
Protein	65%	49%	72%	[36–38]
Lipids	28%	16%	24%	[36–39]
EPA *	12%	7%	14%	[36–41]
DHA *	3%	3%	3%	[36–41]
ARA *	2%	1%	<1%	[36–41]
∑PUFA *	36%	41%	47%	[36–41]

Note: EPA—Eicosapentaenoic acid; DHA—Docosahexaenoic acid; ARA—Arachidonic acid (ARA); PUFA—polyunsaturated fatty acids.

2.3. Juvenile Growth

In order to maximize the number of available juveniles for this trial, all hatchery-reared juveniles (F1 generation) from the previous larval trial were pooled together and equally distributed over three 550 L tanks (N = 2250 juvenile per tanks) to begin grow-out. Each tank was its own Recirculating Aquaculture System (RAS) with a flow rate of 180 L·h^{-1} and 100% recirculated water. The tanks were kept at a mean temperature (±SD) of 22 ± 0.58 °C and mean salinity (±SD) of 33 ± 1, N = 86. All individuals were fed daily with *Rhodomonas baltica* and *Chaetoceros calcitrans* in equal concentrations of 2500 cells·mL^{-1} (in a 1:1 ratio), placed in the water column and left to deposit at the bottom of the tanks and also forming biofilm across hard surfaces. This growth period lasted 3 months in order to curtail whatever lag, or difference, in growth performance between any of the diets. After a period of 3 months, where juvenile *H. arguinensis* were left in the growing tanks to reach a measurable size outside of a microscope, all holothuroids were collected once again with a soft paintbrush (N = 324; ≤1 mm). Juveniles were then weighed inside a tared water droplet on top of a petri dish on a precision scale (0.0001 g). Juveniles presented no significant differences between treatments during this initial weighing and were evenly distributed in 3 RAS, one *per* treatment and each with three 50 L glass tanks with a flat bottom (N = 9), in order to begin a 6-month growth trial. All tanks were supplied with a sandy substrate, water recirculation and aeration. They were maintained with the same photoperiod and lighting conditions as the larval development trials, water quality parameters were monitored daily and water changes were performed (between 20–50%) to maintain ammonia and nitrites at untraceable levels whenever any of these parameters presented concentrations ≥0.5 mg·L^{-1}. As such, tanks presented a mean (±SD) temperature of 20.55 ± 1.02 °C, with a mean salinity level of 33.93 ± 0.59, mean DO levels of 95.32 ± 2.41% and a mean pH of 8.36 ± 0.16 during the trial (N = 168).

Juvenile H. arguinensis were only weighed at the start and end of this trial to reduce stress via exposure to air and risking an increased mortality rate. Individuals in this trial had their specific growth rate (SGR) determined according to the following equation:

$$\text{Specific Growth rate}\left(\%\ \text{day}^{-1}\right) = 100\ (F_s - I_s)/t$$

where F_s is the sampled weight/length registered at the end of the trial, I_s is the sampled weight/length registered at the beginning of the trial and t is the time (days) registered in between samplings.

Full body length measurements were taken while the individuals were submerged, from the third month onward, to determine the SGR pertaining to *H. argueninsis* juveniles' length. With no significant differences in the starting length measurement between tanks, standardized measurements of 15 random individuals *per* tank took place every month until the end of the trial. Individuals had their whole-body length measured underwater, without manipulation, to avoid the risk of muscle contraction, which would underestimate their mean body length [42].

Throughout this period, 3 diets were tested based on the diet with the lowest mortality rate during the larval growth and settlement phases (Diet RbCc); each of these treatments was administered to a single RAS with 3 tanks each so that feed particulates or dissolved feeding attractants from one treatment would not carry over to another treatment via a shared sump. These diets aimed to evaluate the possible success of implementing powdered macroalgae in the juvenile diet. The chosen macroalgae were *Saccorhiza polyschides*, given their abundance around the shores of Peniche and Berlenga and their positive effect on *H. arguinensis* juvenile growth [43]. The macroalga was collected from the shore and dried in an oven between 45–60 °C for up to 4 h before being minced into a fine powder using an electronic blender. Each microalgae's cellular weight was taken into consideration when calculating the number of cells to administer so diets would be supplied with the same amount of biomass; as such, the provided diets per replicate were: Diet RbCc = *Rhodomonas baltica* (6.6×10^8 cells·mL^{-1}) + *Chatoceros calcitrans* (6.6×10^8 cells·mL^{-1}); Diet RbSp = *Rhodomonas baltica* (6.6×10^8 cells·mL^{-1}) + *Saccorhiza polyschides* (0.03 g) and Diet RbCcSp = *Rhodomonas baltica* (5.0×10^8 cells·mL^{-1}) + *Chaetoceros calcitrans* (5.0×10^8 cells·mL^{-1}) + *Saccorhiza polyschides* (0.02 g). All diets provided were also reinforced with 0.03 g of dry microalgae *per* tank (Algamac 3050 ®) as a nutritional supplement, administered 3 times a week. During feeding, all water circulation would halt for a period of at least 8 h to allow for the algae to settle on the bottom and, in the case of the provided microalgae, adhere to the tank walls, therefore minimizing loss of food through the system's recirculation, leading them to deposit in the sump.

2.4. Statistical Analyses

The length and weight data were analysed with a one-way analysis of variance (ANOVA) in order to determine any differences during the larval development and growth phase at each sampling stage. The same was applied during the juvenile stage, as there was one treatment per RAS, with each tank within serving as a sample unit (n = 3 replicates/tanks per treatment). Assumptions were tested, resorting to Shapiro–Wilk's normality test and the modified Levene's test, Brown–Forsythe, for homogeneity of variances. Whenever any statistically significant difference occurred ($p < 0.05$), the results were further analysed using a Pairwise Multiple Comparison Procedure with a Tukey's test. The substrate preference for settlement was determined with a General Linear Model, with Tweedie distribution and log-link functions, as the response variable, the density of the individuals in the settling structures had a zero-inflated distribution, depicting a point-mass at zero (21% zeros), a positive tail and non-negative values [44]. The tweedie family was implemented directly into a *gam()* function without smooth terms, via the maximum likelihood method, with the mgcv R package. The model was conducted with three categorical factors: Diet (levels RbPt; RbSm; RbCc), Substrate (levels tile; plaque; roll), and Depth (levels low; mid; upper) and tested all interactions. The final model output represents the comparisons between each factor level and the respective reference level, as these are categorical factors. Partial plots were produced with the visreg package under type conditional. Thus, "RbPt" is the reference level for Diet, "tile" is the reference level for Substrate and "low" is the reference level for Depth. Considering the various phases sea cucumbers go through during their early development, there were four distinct survival percentages considered:

Embryonic survival percentage (between the 1st and 3rd days of development), Larval survival percentage (between the 3rd and 16th days of development), settlement survival percentage (between the 16th and 21st days of development) and lastly, total survival percentage (between 3rd and 21st days of development). Due to the nonparametric nature of the data, survival percentages were determined with a resort to a Kruskal–Wallis, followed by Dunn's test for Pairwise Multiple Comparison Procedure. All statistical analyses were performed with SigmaStat v4.0.0.37 and R software v4.2.3 [45]. All data are presented, when applicable, as mean ± standard deviation (SD).

3. Results

3.1. Larval Development

After the first 48 h post-fertilization, there was a considerable reduction in density, regardless of diet (Figure 2). This sharp decrease in individuals is indicative of the high number of embryos that do not make it to the larval stage.

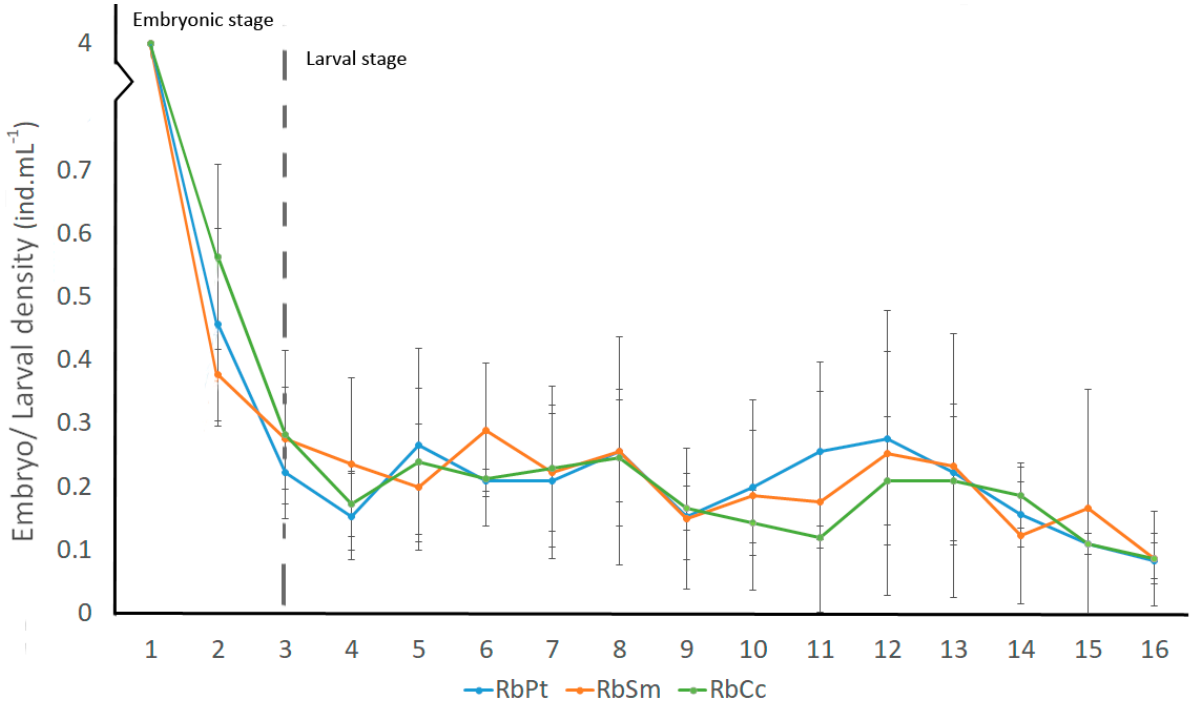


Figure 2. Mean Embryo and Larval density ± (SD), as ind·mL^{-1} registered over the first 16 days of development, according to their respective diets (Diet RbPt = *Rhodomonas baltica* + *Phaeodactylum tricornutum*; Diet RbSm = *Rhodomonas baltica* + *Skeletonema marinoi* and Diet RbCc = *Rhodomonas baltica* + *Chaetoceros calcitrans*, *n* = 3 tanks *per* treatment).

On the 3rd day after fertilization, there were no significant differences in terms of embryo survival percentages between the three treatments (RbPt = 5.60 ± 1.26%; RbSm = 6.92 ± 2.02% and RbCc = 7.08 ± 3.32%), according to a Kruskal–Wallis [$H_{(2)}$ = 1.633; p = 0.652]. As such, the larval development and growth trial began on this day when the first individuals at the early auricularia stage were first observed (Figure 3a). This stage is characterized by the appearance of a functional digestive tract, including the oral cavity, oesophagus, and stomach, enabling feeding on suspended microalgae [11]. On the 7th day after fertilization, all sampled individuals, regardless of diet, were in the mid auricularia stage, characterized by the cilia and functional structures becoming clearly distinguished from the coelom (Figure 3b). On the 11th day, the first sighting of individuals in the late auricularia stage took place. This stage is characterized by its larger size and the first appearance of hyaline spheres, where reserves begin to accumulate (Figure 3c). At this time, while larvae fed with diets RbPt and RbCc already presented most individuals at this

stage (with some sampled individuals from diet RbPt already in the subsequent stage), the larvae fed with diet RbSm still had some individuals in the mid auricularia stage (Figure 4). On the 13th day, tanks supplied with diets RbPt and RbCc already showed individuals in the doliolaria stage, characterized by its more compact size and larger hyaline spheres relative to body size (Figure 3d), while larvae fed with diet RbSm still only presented individuals in the late auricularia stage. Despite a single pentactula individual being detected among those fed with diet RbCc on the 13th day, it was only on the 16th day that this stage was observed in the other diets. This stage, while similar in shape to the doliolaria stage, possesses a protruding oral tentacle with which the holothuroid feeds, the first major step in resembling a fully formed sea cucumber (Figure 3e). Lastly, on the 21st day, juvenile individuals (Figure 3f) were observed in the settling structures for the first time, regardless of diet.

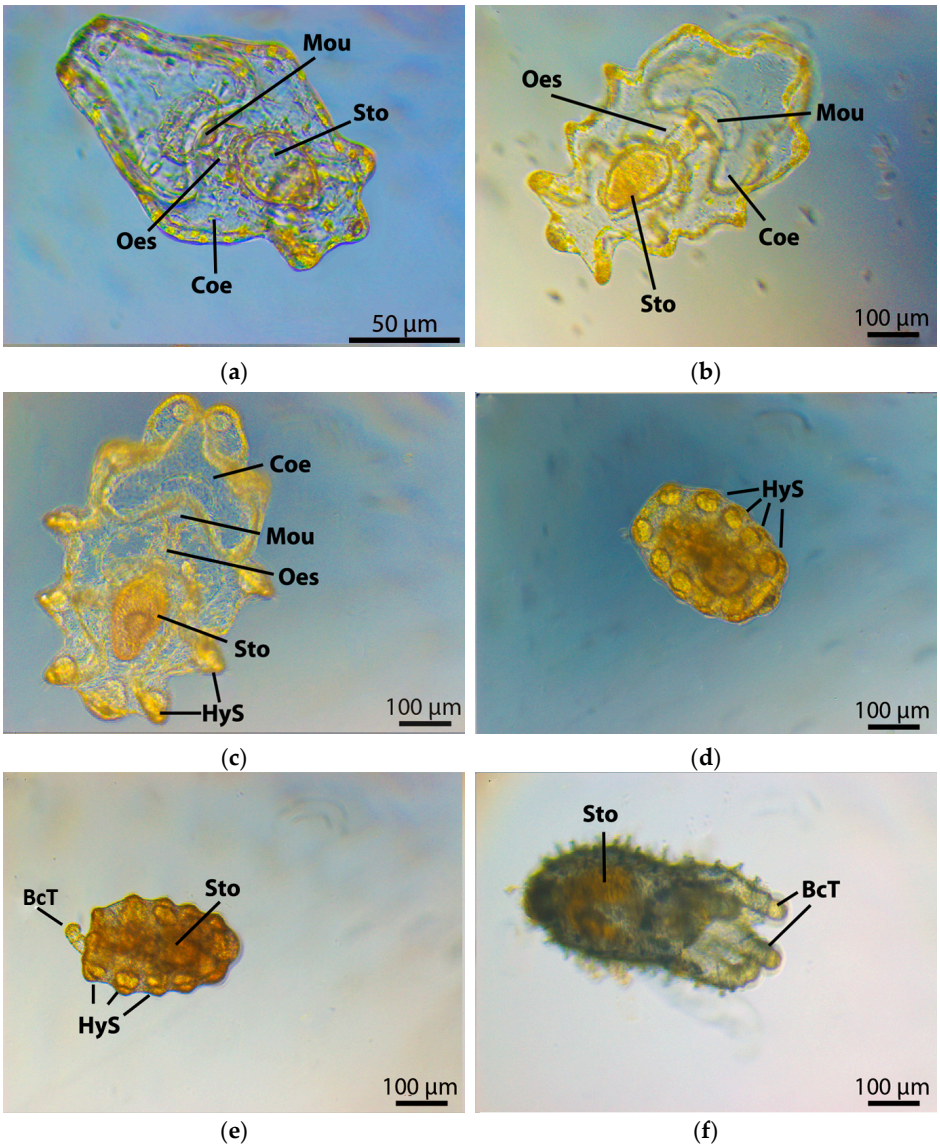


Figure 3. Different stages across Larval development of *Holothuria arguinensis*, including (**a**) early auricularia; (**b**) mid auricularia; (**c**) late auricularia; (**d**) doliolaria; (**e**) pentactula and (**f**) juvenile and their structures, namely (Mou) mouth; (Oes) oesophagus; (Sto) stomach; (Coe) coelom; (HyS) hyaline spheres and (BcT) buccal tentacle.

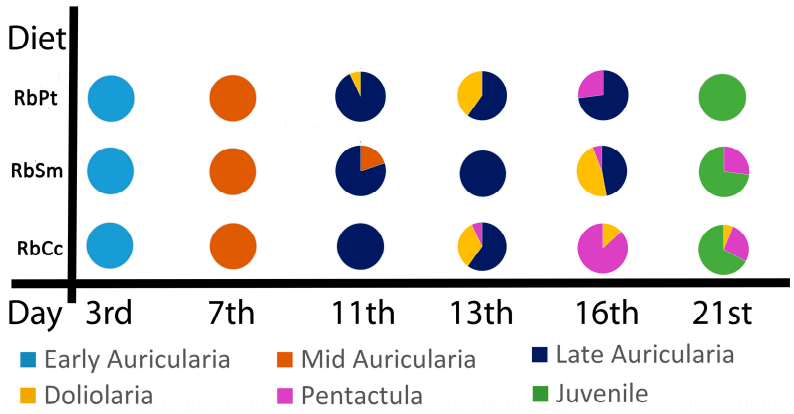


Figure 4. Relative abundance (%) of each developmental stage of *Holothuria arguinensis* at each sampling time (n = 5 individuals *per* tank per day) post-fertilization according to different diets (Diet RbPt = *Rhodomonas baltica* + *Phaeodactylum tricornutum*; Diet RbSm = *Rhodomonas baltica* + *Skeletonema marinoi* and Diet RbCc = *Rhodomonas baltica* + *Chaetoceros calcitrans)*.

Comparing the size pertaining to the mid auricularia stage, the first stage of development after the early auricularia stage (t_0 = 159 ± 18 µm in length; 80 ± 15 µm in width), individuals fed with diet RbCc had significantly higher mean length and width than individuals fed with diet RbSm. Individuals fed with diet RbPt, however, showed no significant differences with any of the other diets at this stage, according to the same tests (Table 2).

Table 2. Mean length and width, in µm (±SD), of developing larval stages across different treatment groups. Different superscript letters indicate significant differences ($p < 0.05$), according to a Tukey test after a One-way ANOVA (n = 3 tanks).

Larval Stage	Measurement	Diet RbPt	Diet RbSm	Diet RbCc	Statistical Analysis
Mid Auricularia	Length	468 ± 74 ^{ab}	376 ± 179 ^{a}	543 ± 84 ^{b}	[$F_{(2, 46)}$ = 7.1906; p = 0.0020; Tukey < 0.01]
	Width	255 ± 57 ^{ab}	217 ± 105 ^{a}	301 ± 75 ^{b}	[$F_{(2, 46)}$ = 4.1700; p = 0.0218; Tukey < 0.05]
Late Auricularia	Length	628 ± 91	672 ± 104	655 ± 82	[$F_{(2, 83)}$ = 1.8811; p = 0.1589]
	Width	407 ± 70	451 ± 72	409 ± 61	[$F_{(2, 83)}$ = 3.9863; p = 0.2230]
Doliolaria	Length	471 ± 78	369 ± 40	520 ± 155	[$F_{(2, 27)}$ = 3.2094; p = 0.0568]
	Width	291 ± 56	253 ± 32	327 ± 93	[$F_{(2, 27)}$ = 2.1315; p = 0.1389]
Pentactula	Length	317 ± 11	312 ± 27	294 ± 25	[$F_{(2, 24)}$ = 2.2184; p = 0.1315]
	Width	180 ± 17 ^{ab}	202 ± 21 ^{b}	171 ± 17 ^{a}	[$F_{(2, 24)}$ = 5.3836; p = 0.0121; Tukey < 0.01]
Juvenile	Length	356 ± 52	299 ± 33	319 ± 86	[$F_{(2, 34)}$ = 3.0223; p = 0.0623]
	Width	264 ± 43 ^{b}	208 ± 20 ^{a}	201 ± 52 ^{a}	[$F_{(2, 34)}$ = 9.0518; p = 0.0007; Tukey < 0.01]

Note: Diet RbPt = Rhodomonas baltica + Phaeodactylum tricornutum; Diet RbSm = Rhodomonas baltica + Skeletonema marinoi and Diet RbCc = Rhodomonas baltica + Chaetoceros calcitrans).

Regarding the late auricularia stage, no treatments presented any differences in terms of length or width. The same happened regarding the doliolaria stage, where no treatments presented any significant differences in mean length or width. Moving on to the pentactula stage, although there were no significant differences regarding mean length, treatments supplied with diet RbSm presented significantly larger widths than individuals fed with diet RbCc. Larvae fed with diet RbPt showed no significant differences with any other group according to the same test. Lastly, no individuals presented any significant differences regarding their mean length at the juvenile stage; however, individuals fed with diet RbPt presented a significantly larger mean width than both other diets.

3.2. Larval Settlement and Survival

Once the larval development trial reached its conclusion on the 21st day after fertilization, all individuals from the F1 generation were counted. All surviving individuals and substrate types were tallied, and results showed an unexpected preference for settlement on the tank walls for larvae fed with RbCc (Figure 5).

The GLM used to explain the settlement distribution of *H. arguinensis* exclusively within provided substrata (Table 3) indicates a lower density of settled individuals from Diet RbCc when compared to Diet RbPt (reference level), as seen in Figure 6a. The model also depicts a decreasing trend in density with increasing depth (Figure 6b). Using the low depth (27 cm) as the reference level, the upper depth (9 cm) presents a significant negative effect (Table 3). Additionally, while there were no significant preferences for any particular substrate in terms of the settlement, the upper depth shows a different settlement pattern. The plaques depict the highest average settlement preference, unlike the other depths in which the plaques have the lowest density (Figure 7).

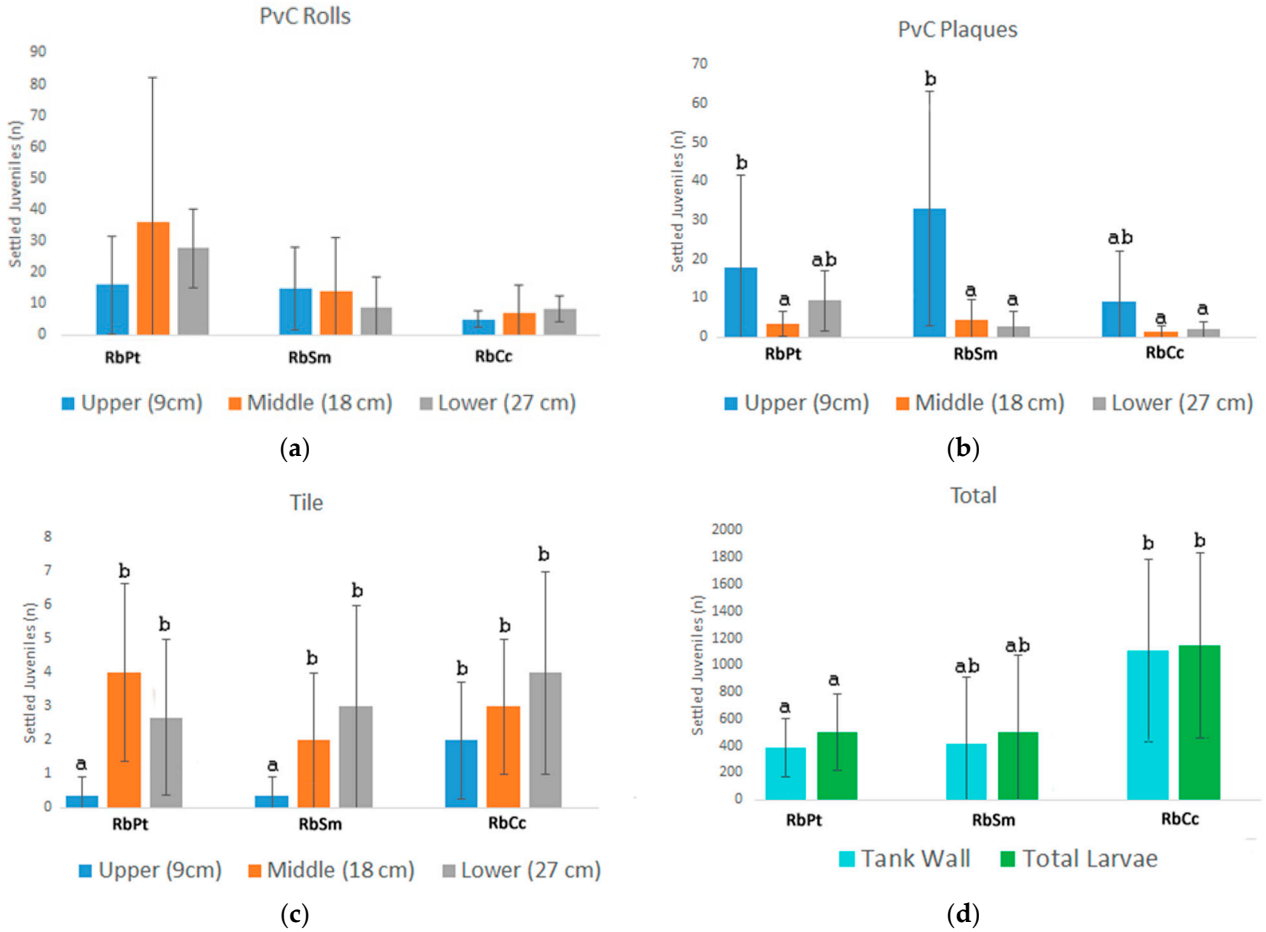


Figure 5. *Cont.*

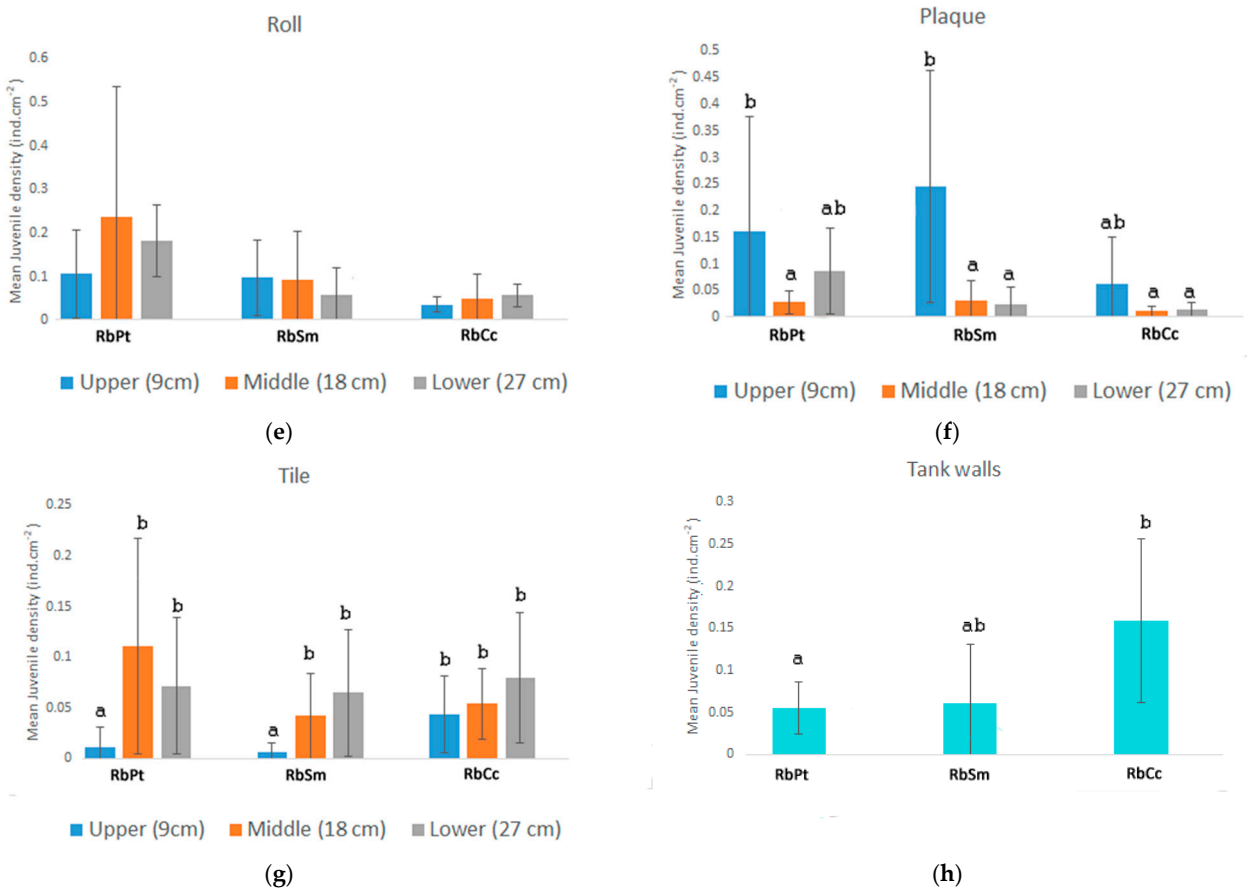
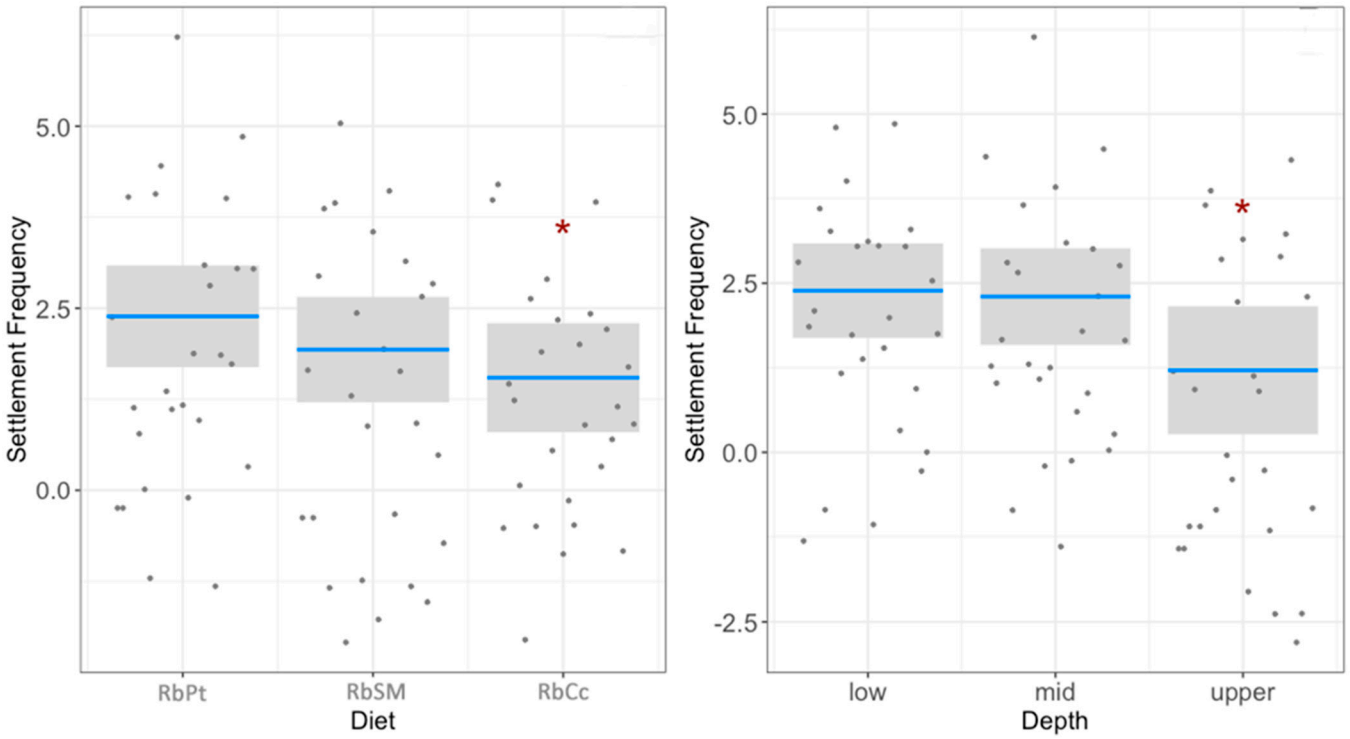


Figure 5. Mean (±SD) number (**a**–**d**) and density (ind·cm^{-2}) (**e**–**h**) of *Holothuria arguinensis* juveniles settled in different substrates according to each diet (Diet RbPt = *Rhodomonas baltica* + *Phaeodactylum tricornutum*; Diet RbSm = *Rhodomonas baltica* + *Skeletonema marinoi* and Diet RbCc = *Rhodomonas baltica* + *Chaetoceros calcitrans*), n = 3 tanks. Different letters indicate statistically significant differences ($p < 0.05$) between substrates at each depth as well as juveniles between diets.

Table 3. Fixed-effect GLM model results with a Tweedie distribution (adj R^2 = 15.3% and explained deviance of 28.3%), explaining density settlement patterns of *Holothuria arguinensis* based on diet provided, substrate type and substrate depth.

	β	Std. Error	*t*-Value	*p*-Value
(Intercept)	2.38863	0.35585	6.713	4.16×10^9 ***
Depth mid (18 cm)	−0.08683	0.47292	−0.184	0.854848
Depth upper (9 cm)	−1.17600	0.56863	−2.068	0.042323 *
Diet RbSm	−0.45669	0.26391	−1.730	0.087951
Diet RbCc	−0.84246	0.28181	−2.990	0.003853 **
Substrate Plaque	−0.69056	0.52075	−1.326	0.189121
Substrate Roll	0.22293	0.45273	0.492	0.623973
Depth mid: Substrate Plaque	−0.46939	0.78038	−0.601	0.549456
Depth upper: Substrate Plaque	2.59900	0.74636	3.482	0.000861 ***
Depth mid: Substrate Roll	0.31581	0.63537	0.497	0.620711
Depth upper: Substrate Roll	0.97460	0.72592	1.343	0.183750

p-values significance: 0 "***" 0.001; "**" 0.01; "*" 0.05; "." 0.1; " " 1.



(**a**) (**b**)

Figure 6. Partial plots of the density model for all significant predictors—Fixed-effect GLM with a Tweedie distribution—explaining the settlement patterns *Holothuria arguinensis* larvae for the factor Diet (**a**) and factor Depth (**b**). The * notation indicates the significant differences with the reference level analysed in this GLM.

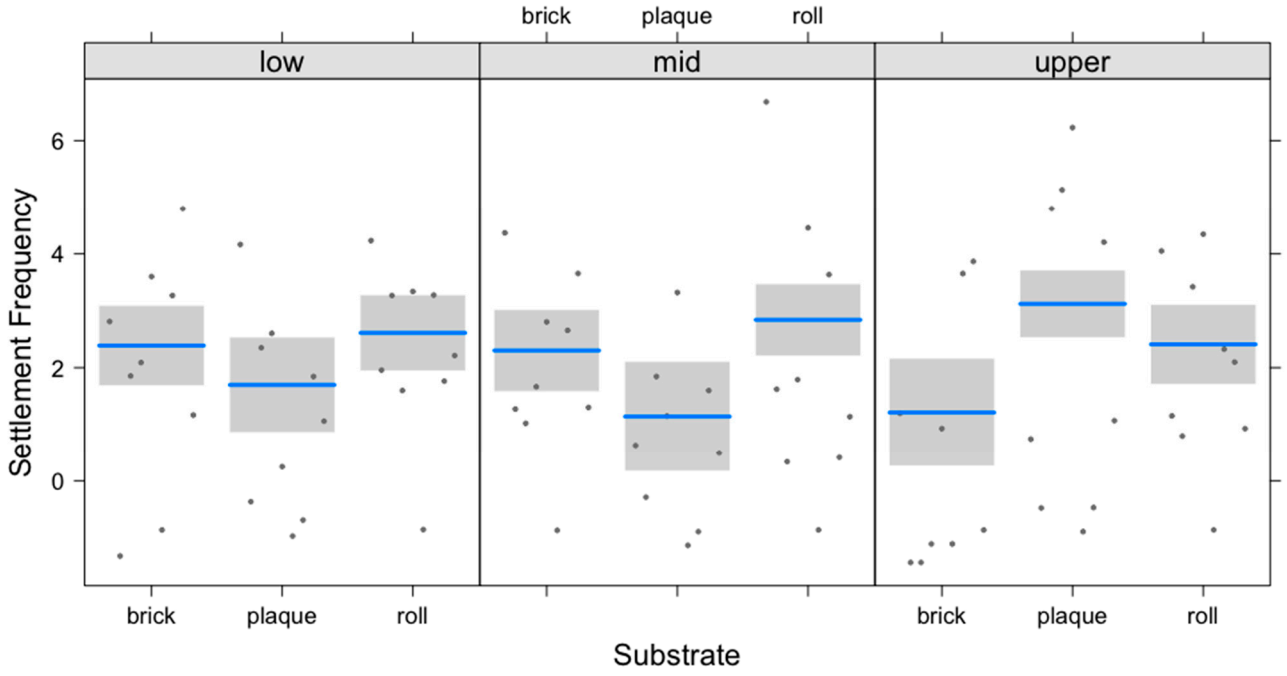


Figure 7. Partial plots of the density model for all significant predictors—Fixed-effect GLM with a Tweedie distribution—explaining the settlement patterns *Holothuria arguinensis* larvae for the factor substrate according to depth.

However, while there were no registered differences in larval survival percentages (Kruskal–Wallis [$H_{(2)} = 2.480$; $p = 0.289$]) on the 16th day of the trial (Figure 8a), there was a significantly higher rate of total settled individuals (settlement structures + tank walls) from diet RbCc than any of the other two diets (Kruskal–Wallis [$H_{(2)} = 10.571$; $p < 0.001$; Dunn; $p < 0.05$]) at the 21st day of trial, as seen in Figure 8b.

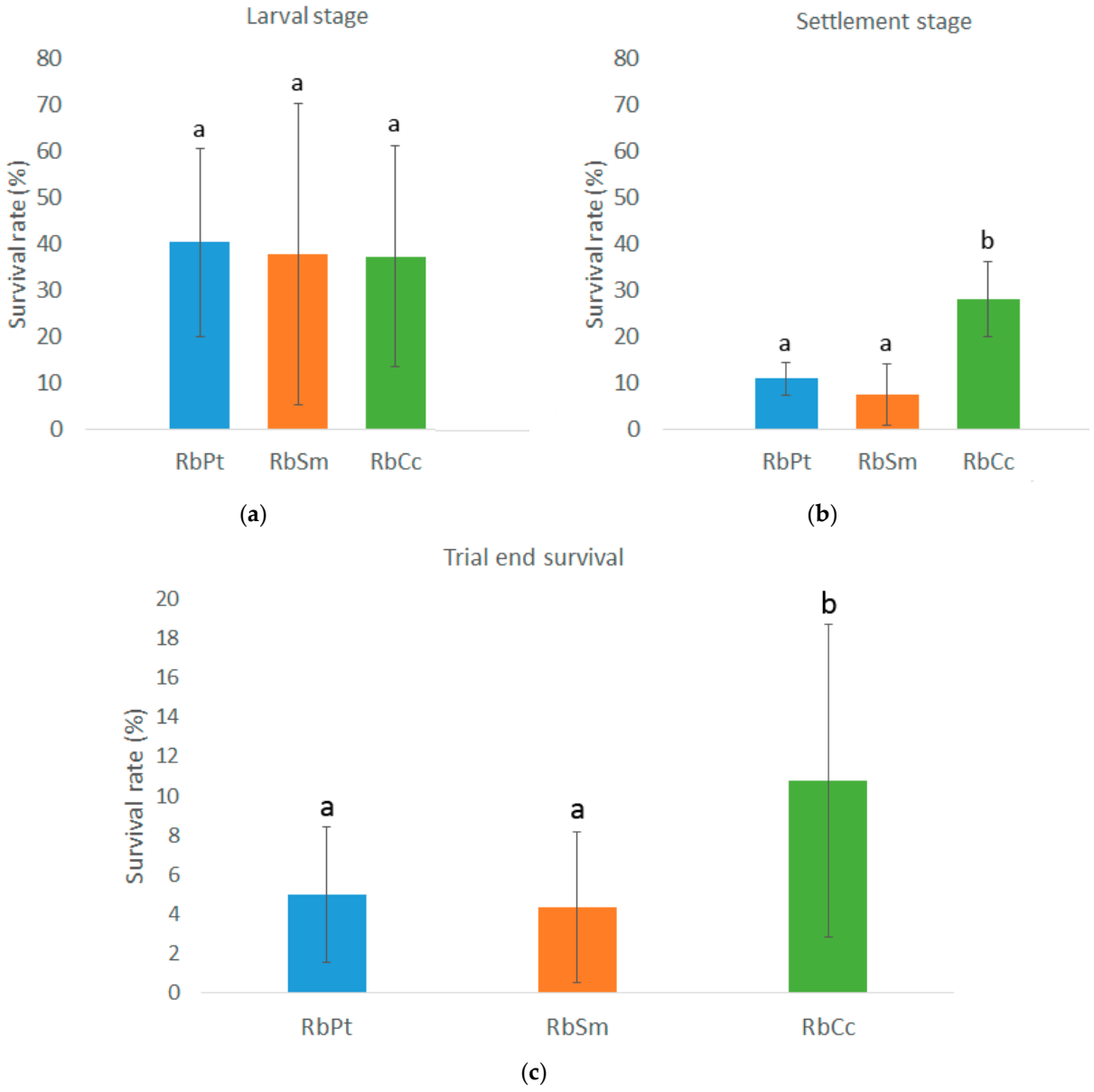


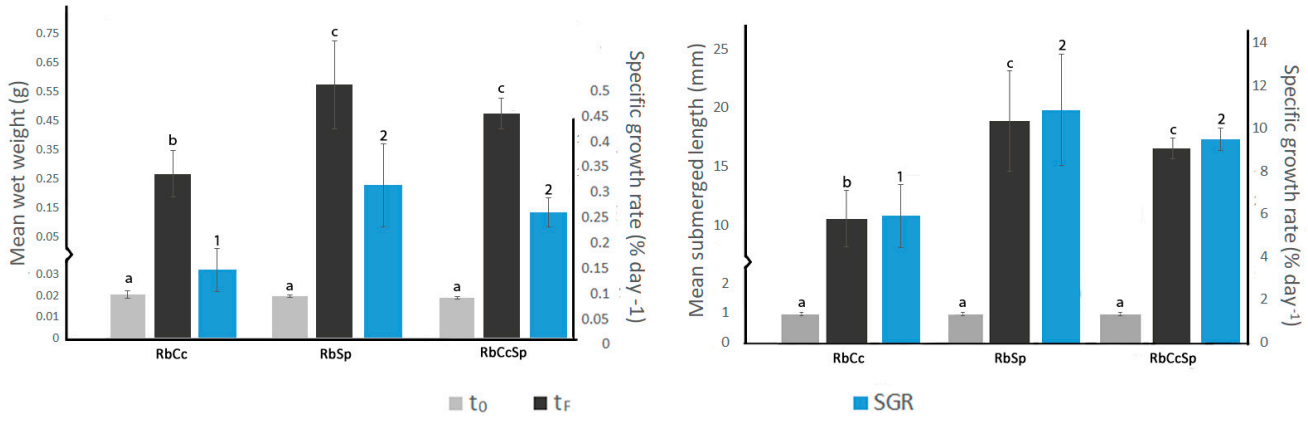
Figure 8. Survival percentage throughout the trial, across all tested surfaces and tank walls, between (**a**) larval stage (3rd to 16th day of trial); (**b**) settlement stage (16th to 21st day of trial) and (**c**) throughout the growth trial (between 3rd and 21st day of trial), for all 3 chosen diets (Diet RbPt = *Rhodomonas baltica* + *Phaeodactylum tricornutum*; Diet RbSm = *Rhodomonas baltica* + *Skeletonema marinoi* and Diet RbCc = *Rhodomonas baltica* + *Chaetoceros calcitrans).* Different letters indicate statistically significant differences of survival ($p < 0.05$) between diets.

Examining the total number of sea cucumbers at the end of the trial, and comparing them to the initial number of early auricularia observed per tank on the 3rd day, holothuroids fed with diet RbCc presented a higher survival percentage (10.79 ± 7.94%) than the other diets (Kruskal–Wallis [$H_{(2)} = 16.984$; $p < 0.001$; Dunn; $p < 0.05$]), nearly twice as high as individuals fed with diet RbPt (4.98 ± 3.41%) and RbSm (4.35 ± 3.82%).

3.3. Juvenile Growth

After a period of 6 months, *H. arguinensis* fed with diet RbSp showed a significantly higher survival percentage (84.26 ± 14.25%) than those fed with diet RbCcSp (40.74 ± 21.03%) (One-Way ANOVA [$F_{(2, 7)}$ = 5.2984; p = 0.0472; Tukey < 0.05]). Individuals fed with diet RbCc showed a Survival percentage of 59.26 ± 12.83% with no significant differences with any of the groups according to the same test.

Regarding the growth of surviving individuals, juveniles fed with diet RbSp (Fresh weight = 0.57 ± 0.15 g) and diet RbCcSp (Fresh weight = 0.48 ± 0.05 g) showed a significantly higher weight than individuals fed with diet RbCc (Fresh weight = 0.27 ± 0.08) according to a One-Way ANOVA [$F_{(2, 196)}$ = 3.579; p = 0.0030; Tukey < 0.05]. This weight gain is also reflected in their mean SGR throughout this period, where individuals fed with diets RbSp (SGR = 0.31 ± 0.08 %·day^{-1}) and RbCcSp (SGR = 0.25 ± 0.03 %·day^{-1}) stand out as having a significantly higher growth than individuals fed with diet RbCc (SGR = 0.14 ± 0.04 %·day^{-1}), according to a One-Way ANOVA [$F_{(2, 7)}$ = 6.9610; p = 0.0273; Tukey < 0.05] (Figure 9a).



(a) **(b)**

Figure 9. Mean fresh weight (g) (**a**) and submerged length (mm) (**b**) (±SD) of juvenile *Holothuria arguinensis* at the trial onset (t_0), end (t_F) and their respective specific growth rate after a period of 6 months (SGR), according to each provided diet (Diet RbCc = *Rhodomonas baltica* + *Chatoceros calcitrans*; Diet RbSp = *Rhodomonas baltica* + *Saccorhiza polyschides* and Diet RbCcSp = *Rhodomonas baltica* + *Chaetoceros calcitrans* + *Saccorhiza polyschides*), n = 3 tanks. Different letters indicate statistically significant differences (p < 0.05) between diets at the trial onset (t_0) and trial end (t_F), while different numbers indicate statistically significant differences regarding specific growth rates between diets.

This positive development was not only observed in the weight of the juveniles but also in their length. Juveniles fed with diet RbSp (Submerged length = 18.9 mm ± 4.3) and RbCcSp (Submerged length = 16.5 ± 0.9 mm) presented a final mean length greater than individuals fed with diet RbCc (Submerged length = 10.5 ± 2.4 mm) according to a One-Way ANOVA [$F_{(2, 196)}$ = 10.530; p < 0.001; Tukey < 0.01]. The same can be observed in their respective SGR, with individuals fed with diets RbSp (SGR = 10.96 ± 2.61 %· day^{-1}) and RbCcSp (GR = 9.53 ± 0.53 %·day^{-1}) presenting significantly higher values than individuals fed with diet RbCc (SGR = 5.85 ± 1.47% day^{-1}), according to a One-Way ANOVA [$F_{(2, 7)}$ = 6.8295; p = 0.0284; Tukey < 0.05]) (Figure 9b).

4. Discussion

4.1. Larval Development

Throughout their development, sea cucumber larvae will undergo several changes in size according to their respective stage, with certain stages early in their development presenting an overall larger size than certain later stages, as detailed in Table 2. On top of

their irregular length increments across stages, larval growth of holothuroids is notoriously asynchronous, with individuals kept under the same conditions displaying discrepancies in their development [46,47]. With that considered, individuals fed with diet RbSm still took more time to overcome certain developmental stages (such as late auricularia and doliolaria) when compared to individuals fed with either of the other two diets. This difference in development could suggest a nutritional deficit related to *S. marionoi*'s relatively low protein and lipid contents [38] when compared to other microalgae used in this trial. Another possible explanation for the lack of success from this microalga may be located in its thicker cell wall when compared to the other microalgae in this trial, which may hinder the larvae's digestive process [48]. This seemingly slower development time is also prevalent when comparing mean larval length and width during the mid-auricularia stage, where individuals fed with diet RbSm presented a significantly smaller size than individuals fed with diet RbCc. Subsequently, individuals fed with diet RbPt and diet RbCc ended up showing a faster development, with the former showing precocious individuals in the doliolaria stage (on day 11) while the latter showed precocious individuals in the pentactula stage (on day 13). Both stages occur in the settlement phase, where growing individuals rely on their lipidic reserves within hyaline spheres as they transition from feeding off suspended microalgae to biofilm on the surface of the substrate. These two more successful diets could result from *C. calcitrans* and *P. tricornutum*'s higher nutritional content [37,49] and, especially, their higher EPA values. Duy et al. [50] had already established the importance of certain fatty acids (such as EPA, DHA and ARA) for the larval development of *Holothuria scabra*, namely their positive effect on the development of hyaline spheres as lipidic reserves. The size of these spheres appears to have a direct relationship with the individual size, but larger spheres are dependent on these fatty acids, thus, in turn, leading to larger individuals. With that in mind, it must be noted that *H. arguinensis* supplied with diet RbPt displayed the largest mean width at the end of the growth trial, most likely due to *P. tricornutum* seemingly presenting the highest lipidic levels of the supplemented microalgae on top of their elevated EPA, DHA, ARA and overall PUFA levels. The nutritional content of diet RbPt, while similar to diet RbCc, seems to also present the highest content of ARA and overall lipids. Following the findings of Bai et al. [51] and Gianasi et al. [52], this higher rate of nutritionally important fatty acids could account for how developing holothuroids fed with this diet displayed the fastest growth. It may also provide an explanation for their largest size at the end of the trial, seeing as all sampled individuals from this diet were at the juvenile stage on the 21st day, as opposed to those fed with diets RbSm and RbCc. However, while not demonstrating a final mean juvenile size as high as those fed with RbPt, results presented herein regarding RbCc seem to be in congruity with published literature, as Domínguez-Godino and González-Wangüemert [17] also demonstrated how a diet comprised of *C. calcitrans* and one other microalga could lead to fast growth during the mid-auricularia to doliolaria stages of development, a crucial tipping point in the development of growing *H. arguinensis* as it encompasses the metamorphosis from pelagic to benthic behaviour. Li and Li [53] also demonstrated an optimal growth and survival percentage with *Apostichopus japonicus* larvae fed with a *C. calcitrans* diet, maintained at a similar temperature of 24 °C and salinity levels close to the ones presented in this study.

4.2. Larval Survival and Settlement

At the start of the trial, there was a significant reduction in the number of observable individuals across diets, as to be expected from the embryonic stage [16,17,21,27,29]. While there were no differences between treatments at this point, this sharp decline in observable individuals is what most likely influenced the low overall survival percentages at the end of the trial. Throughout larval development (between the 3rd and 16th day), where pelagic larvae could still be observed in the water column, there were also no registered differences between survival percentages. It was only after the settlement stage (between the 16th and 21st days of the trial) that differences in survival were registered, as Holothuroids fed RbCc

presented a significantly higher survival (28.83 ± 8.35%) than either RbPt (11.19 ± 3.59%) and RbSm (7.70 ± 6.76%). Looking at the overall survival percentages, between the 3rd and 21st days of trial (RbPt = 4.98 ± 3.41%; RbSm = 4.35 ± 3.82% and RbCc = 10.79 ± 7.84%), only RbCc treatment manages to reach similar survival percentages obtained in previous trials (8.5% with *Holothuria leucospilota* and 11.66% with *Holothuria arguinensis*) [11,21]. As all tanks were maintained under the same conditions, with the same settlement substrata, this marked increase in survival suggests an effect of diet RbCc.

Once larvae settle on the substrate, their diet changes from planktonic microalgae to biofilm on surfaces [54]. The more biofilm adheres to these structures, the more likely it is for developing larvae to settle in search of food, with structures devoid of biofilm being the least enticing for settlement [55]. Biofilm formation is dependent on the biosynthetization of Extracellular Polymeric Substances (EPS), which varies greatly between bacterial and microalgae strains [56]. This biofilm builds up on all hard surfaces, including the tank walls, which presented the highest density of settled individuals for the most successful diet in terms of survival (Diet RbCc), due to their higher surface area when compared to the settling structures (Figure 5).

Examining larval settlement rate in the different substrates supplied, there seems to have been a general preference for substrata at lower depths, with the clear exception of wavy pvc plaques at higher depths. As indicated by the GLM, individuals fed with diet RbPt presented a significantly higher settlement rate for the provided substrata than those fed with diet RbCc and no difference with those fed with diet RbSm. This preference for transparent substrata in a more exposed placement in the water columns seems to go against the previous literature where sea cucumbers, namely their young or smaller individuals, show a preference for dark and sheltered places [42,57]. However, these results are not without precedent in laboratory conditions, as Li et al. [55] presented plastic sheets covered in diatom-based biofilms as the optimal settling structures for the sea cucumber *Apostichopus japonicus* in a trial conducted with smaller 3 L aquaria.

In the previous section, it was shown that individuals fed with diet RbPt, generally presented faster growth, indicating that this diet is just as effective in providing energy to the growing larvae to metamorphize as diet RbCc. However, diet RbCc presented the highest mean survival percentage for the settlement stage, as the tank walls ended up being the most adhered substrate for this diet. Given how all tanks were identical and, therefore, presented the same surface area, the significantly higher number of individuals settled in tank walls where diet RbCc was supplied indicates that *C. calcitrans* may be more suitable microalgae for stimulating settlement than *P. tricornutum* supplied in diet RbPt [58].

While individuals fed with diet RbSm did not present any significant differences in terms of settlement and survival as those fed with diet RbCc, their admittedly slower development time made this diet less suitable to supply. The opposite can be applied to individuals fed with diet RbPt; while they ended up presenting the fastest development time, their significantly smaller settlement and survival percentage make this diet less suitable than diet RbCc, which balanced fast growth with higher survival percentages.

4.3. Juvenile Rearing and Growth

Once growing holothuroids reach the juvenile stage, the scientific consensus points to the introduction of powdered macroalgae into their dietary needs as means to satisfy their nutritional demands [17,59,60]. Considering that *C. calcitrans*, a microalga, was supplied in concentrations of 5.0×10^8 cells mL^{-1} and 6.6×10^8 cells mL^{-1}, the available food ended up dispersed across the entire water column, as opposed to *S. polyschides*, a macroalga, which was supplied in a fine powder that sedimented itself on the sandy substrate. Looking at *S. polyschides* nutritional content, this macroalgae presents a relatively lower protein (7 to 13%) and lipidic (0.7 to 8%) content in terms of dry weight [61,62] when compared to the microalgae *C. calcitrans'* reported nutritional content. However, as the microalgae end up diffused not only throughout the entirety of the sandy substrate, but some also adhered to the tank walls, spreading the supplied food in a wider surface area [58]. Powdered

macroalgae, on the other hand, did not adhere to the tank walls and settled exclusively on the bottom. Being deposit feeders that ingest vast amounts of sediment [63–65], sea cucumbers, in general, are adapted to procuring nutritional content on the surface of the substrate. Considering holothuroid juveniles' low mobility on top of their feeding behavior, the macroalgae's size and weight make it sediment itself on the sandy substrate much more adequately than free-floating microalgae. This points to powdered macroalgae being more suitable to satisfy these echinoderms according to their feeding habits than fresh microalgae, further reflected by the highest SGR (weight and length gain) of the juveniles in this trial.

With no significant differences between juveniles fed with either diet RbSp or RbCcSp in terms of weight and length gain, results seem in accordance with previously published literature. Asha and Muthiah [65] had already demonstrated that the implementation of a powdered macroalgae (*Sargassum* sp), very closely resembling *Sacchoriza polyschides* in terms of nutritional content, had a positive effect in the specific growth rate of *Holothuria spinifera* juveniles.

While the works by Slater et al. [59] presented a mean SGR higher than the results obtained, around 30%, for the juvenile sea cucumber *Australostichopus mollis* using wastewater (water containing suspended organic material, nitrogen and phosphorous content expelled from other organisms, such as fish and other invertebrates, like mussel) instead of macroalgae, these results only extended for the duration of the first 3 months after reaching the juvenile stage. The same can be said regarding the work by Domínguez-Godino et al. [23], using the same sea cucumber species as the one in this trial. Their study also presented a higher SGR for *H. arguinensis* fed with powdered seagrass (*Zostera noltii*). However, much like Slater et al. [61], the trial was concluded after a period of 2 months after reaching the juvenile stage. As such, it becomes hard to properly establish comparisons with the two experiments performed by these authors, as growth rates typically slow down while holothuroids keep growing [66], and the present trial ran for a longer period of time. Additionally, different species may have substantially different growth rates hampering the comparison of growth indices. In conclusion, results lead to the notion that it might be more beneficial in terms of growth, as well as more cost-effective, to introduce some form of macroalgae and/or seagrass into juveniles' diets. However, further differences between macroalgae species still need to be properly assessed.

5. Conclusions

Although a diet composed of *Rhodomonas baltica* and *Phaeodactylum tricornutum* may provide a higher source for lipidic reserves, which can lead to faster development and higher body mass, as seen at the end of the larval growth trial, the number of individuals settling is markedly reduced when compared to a diet with *Rhodomonas baltica* and *Chaetoceros calcitrans*, making this the most suitable microalgae to provide favouring *Holothuria arguinensis'* survival. Mixing these diets across targeted stages of larval development may optimize results in both size and survival; however, future trials must be conducted on this matter.

Regarding settling structures, with not many significant differences noted between substrata, settlement and survival seem more dependent on the biofilm formed than the substrate itself.

Lastly, growing juvenile individuals displayed the best growth performance when fed with powdered macroalgae, whose larger particle size seems to have better enriched the sandy substrate as opposed to microalgae diffused entirely across the tanks' surface area. Another nutritional resource to take into consideration may lie in wastewater from other organisms grown in captivity (such as teleost fish, for example) in an Integrated Multi-Trophic Aquaculture system (IMTA), which could further enrich the sediment the sea cucumbers feed off of.

In conclusion, *H. arguinensis'* larval development is a continuously changing event that requires detailed attention throughout each distinct stage to mitigate mortality rates.

As such, holothuroid rearing techniques can be further developed in each of its main life cycle stages, from dietary optimization during larval development to adequate substrate for settlement and finally back to dietary optimization during juvenile growth.

Author Contributions: P.M.F., A.C.B. and A.P. designed and supervised the study. T.S. and F.A.e.S. conducted the fieldwork in obtaining the wild *Holothuria arguinensis* broodstock, with assistance from J.S., E.V. and J.S. conducted all experiments during the different trials and maintained the microalgae stock for feeding. A.A. and M.L.D.-R. collaborated in microalgae production. J.S., P.M.F. and A.P. conducted all data analyses, and all listed authors contributed to the revision and optimization of the drafted paper. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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