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Development of larviculture protocols for the long-spined sea urchin (*Diadema antillarum*) and enhanced performance with diets containing the cryptophyte *Rhodomonas lens*

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Abstract
Slow or nonexistent natural recovery of the Caribbean long-spined sea urchin (*Diadema antillarum*) following a mass mortality event in 1983–1984 has prompted interest in hatchery-origin production and restocking to aid coral-reef restoration. A critical first step is the ability to propagate *D. antillarum* from gametes, at scale. However, a unique larval biology and difficult and lengthy culture period of ~ 40 days has resulted in inconsistent success over the past 20-plus years. The purpose of this study was to develop protocols for rearing *D. antillarum* within a novel 1800-L recirculating aquaculture system capable of scaled production. Five separate experiments investigated larval development in response to diet quantity, diet composition, and initial stocking density within 40-L replicate culture tanks. The initial experiment was used to develop a microalgae reference diet consisting of *Tisochrysis lutea* and *Chaetoceros* sp. and revealed similar growth and survival between high quantity (40.0×10^3 cells mL^{-1}) and low quantity (10.0×10^3 cells mL^{-1}) treatments at 21 days post-fertilization (DPF). Experiments 2–4 examined diet quality by comparing carbon-equivalent microalgae compositions. Mixed diets containing *Rhodomonas lens* outperformed the reference diet in multiple experiments and a tripartite diet containing all three species resulted in significantly higher survival at 42 DPF. The highest growth overall occurred from a monoalgal *R. lens* diet, which indicated that this species is critically important. Further observations of density-dependent growth dynamics revealed that initial stocking densities > 1 larvae mL^{-1} significantly reduced growth over 28 DPF. Data generated were used to establish fundamental larviculture protocols that have since led to the production of over 1000 juveniles.

Keywords Coral reef · Restoration · RAS · Invertebrate · Microalgae · Sea urchin

Introduction

Aquaculture has been increasingly utilized in recent years to attempt enhancement of populations of organisms for ecological restoration with the goal of recovering and increasing ecosystem services (Alleway et al. 2019; Lorenzen 2014; Patterson 2019).

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Relevant examples of aquaculture-based ecological enhancements include *Crassostrea virginica* (Jaris et al. 2019), *Zostera marina* (Tanner and Parham 2010), *Tripneustes gratilla* (Neilson et al. 2018; Westbrook et al. 2015), and coral (Boström-Einarsson et al. 2020; Leal et al. 2016; Lirman and Schopmeyer 2016) restoration. Unprecedented global declines of coral reefs have resulted in the rapid growth of propagation programs attempting direct enhancement of ecologically important coral species (Barton et al. 2017; Osinga et al. 2011; Rinkevich 1995). Restoring propagated corals alone, however, does not address the stressors that led to reef decline and long-term outplant survival can be low (Ware et al. 2020). Practically, coral enhancement should exist within a larger reef restoration framework that aims to restore structural habitat and biodiversity via a multi-niche ecological approach. Key to this approach in the Caribbean is the re-establishment of lost herbivory via enhancement of long-spined sea urchin, *Diadema antillarum.* Historically, this species was the primary generalist herbivore on Caribbean reefs (Ogden 1977; Sammarco 1982), until a mass mortality event in 1983–1984 reduced populations by 93–100% and pervasively altered reef ecosystem dynamics via reduced herbivory and subsequent declines of hard coral cover, habitat complexity, and biodiversity (Hughes et al. 2010; Lessios 2016).

Despite several established methods to commercially produce echinoderms from gametes (Harris and Eddy 2015; McBride 2005), and implementation of aquaculture-based urchin enhancements on coral reefs in Hawaii (Neilson et al. 2018; Westbrook et al. 2015), a scalable hatchery process for *D. antillarum* has not yet been established. Past attempts to culture this species over the last 20-plus years have been met with varying degrees of success. Eckert (1998) recorded the first instance of complete development, having produced 5 juveniles after a 36-day larviculture period within 1-L plastic beakers with mechanical paddle stirrers. Subsequent failed culture attempts and anecdotal reports of difficulties from other culturists led the same author to conclude that, "[*D. antillarum*] seems very sensitive to poor culturing conditions", and that the larvae could be particularly susceptible to pathogens and/or have specific nutritional requirements leading up to metamorphosis. Water quality is a primary, related concern as abnormal embryo-larval development occurs in response to dissolved metal concentrations as low as 15 and 11 µg/L of nickel and copper, respectively (Bielmyer et al. 2005). A unique larval morphology poses additional challenges. Compared to more commonly cultured species, which exhibit a typical echinopluteus larval form, *D. antillarum* larvae are characterized as echinopluteus transversus, with two distinctly long postoral arms protruding at low angles of elevation from the larval body (Fig. 1b).

This trait is disadvantageous to captive larviculture as relatively turbulent flow dynamics result in mechanical damage to the postoral arms, increased infection potential, and diminished swimming and feeding capacity. Additionally, there are physiological constraints on growth, including reduced feeding, digestive and metabolic efficiency, that are possibly inherent to transversus form larvae (Rendleman and Pace 2018). Cumulatively, these constraints could contribute to the long, 2–8 month (Hernandez et al. 2006) and 28-day plus *D. antillarum* planktotrophic larval duration observed in the wild and in captivity, respectively.

Continued efforts to culture *D. antillarum* led to the creation of a prototype system with the potential for mass culture (Moe 2014). Still, speculative factors including environmental toxins, water quality, and general culture methods precluded reliable development through metamorphosis (Leber et al. 2009). Outstanding restoration goals led to the development of a novel recirculating aquaculture system (RAS) to investigate larviculture bottlenecks and improve the feasibility of scaled production (Pilnick et al. 2021). These culture

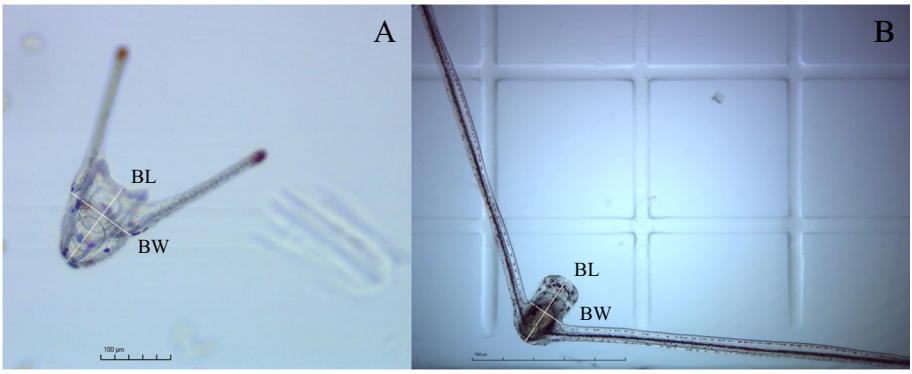

Fig. 1 Larval morphometrics including body width (BW) and body length (BL) used to calculate body size (calculated as area). (**A**) Larvae at 3 DPF and (**B**) larvae at 30 DPF

efforts represented the first attempt to culture *D. antillarum* within a RAS and have to-date produced over 1000 juveniles. As with any novel intensive aquaculture system, an investigation of appropriate culture protocols was required to achieve successful development.

Practically, diet quantity, diet composition, and initial larval stocking density are important considerations for *D. antillarum* larviculture, given that the development of planktotrophic sea urchin larvae is strongly linked to exogenous feeding (Bertram and Strathmann 1998) and that sea urchin larvae are subjected to competition for resources and space within culture tanks (Azad et al. 2012). Different food quantities, feeding frequencies, and species of live microalgae, used for captive larval diets, can directly influence larval growth, survival, and metamorphic success (Carboni et al. 2012; Cárcamo et al. 2005; Kelly et al. 2000; Liu et al. 2007) and appear to be species-specific. The nutritional quality of different microalgae species can vary depending on factors such as cell size, digestibility, and biochemical composition (Guedes and Malcata 2012). While mixed microalgae diets generally perform better (Azad et al. 2011; Carcámo et al. 2005; Gomes et al. 2021), it has been suggested that the cryptophyte *Rhodomonas lens* is an essential dietary component for larval *D. antillarum* (Eckert 1998; Leber et al. 2009). Other microalgae highly regarded for marine larviculture used in prior culture attempts include *Tisochrysis lutea* (formerly *Isochrysis galbana*), *Chaetoceros gracilis*, and *Dunaliella tertiolecta* (Eckert 1998; Leber et al. 2009; Moe 2014). However, a diet protocol for rearing *D. antillarum* within a production-oriented RAS has not yet been developed. Likewise, few studies have investigated the impact of stocking density on sea urchin larviculture within commercial production settings (Azad et al. 2011; Buitrago et al. 2005; Suckling et al. 2018). Apart from a single experiment conducted in small-scale 1.3-L culture vessels that revealed an inverse relationship between larval density and growth (Leber et al. 2009), a determination of appropriate *D. antillarum* stocking density within a production-oriented RAS has also not yet occurred. Larval nutrition and stocking density remain extremely relevant for *D. antillarum* larviculture, especially when considering the feasibility of production for restoration. The current study outlines an experimental process used to determine an appropriate (1) diet quantity, (2) diet composition, and (3) initial larval stocking density for *D. antillarum* larviculture within a novel RAS capable of scaled production for restoration.

Methods

Broodstock maintenance and spawning

Adult *D. antillarum* were collected in March 2018 from reefs at < 8-m depth off Marathon, Florida, by the Florida Fish and Wildlife Research Institute under Florida Keys National Marine Sanctuary permit # FKNMS-2018–023. These broodstock were transported to a land-based restoration aquaculture facility operated by The Florida Aquarium in Apollo Beach, FL. Urchins were quarantined in a RAS within a greenhouse for 45 days. Following quarantine, 14 broodstock were transferred to 450-L fiberglass tanks within a 2400-L seawater RAS. Broodstock husbandry adhered to methods previously described in Pilnick et al. (2021).

Spawning was induced via thermal shock following methods described in Leber et al. (2009) and Moe (2014). Broodstock were transferred to a polyethylene tank filled with 150-L of 1-µm filtered 35 ppt artificial seawater (ASW) prepared by mixing a commercial grade marine salt (Tropic Marin, Wartenburg, Germany) and municipal freshwater purified by reverse osmosis and de-ionization. Water was heated to 5 °C above holding temperature and aeration was provided. Eggs were collected in a 60-mL syringe following release from females and gently rinsed in 2-L egg collectors with 35-µm mesh. Typically, males released gametes first and eggs were fertilized upon collection by residual sperm in the spawning bin. Fecundity and fertilization rates were assessed via 1-mL volumetric subsample counts using a Sedgewick rafter counting slide.

General larval rearing methods

Larval rearing followed the methods established in Pilnick et al. (2021). Fewer than 1 million embryos were incubated at 25–26 °C in semi-circular 40-L culture tanks with pulsed aeration supplied through perforated rigid tubing at intervals of 3–5 s on and 20–30 s off. At 3 days post-fertilization (DPF), pluteus larvae were volumetrically transferred to separate, randomly assigned 40-L replicate culture tanks within a single 1800-L RAS (system picture and schematic supplied in Pilnick et al. 2021). Pulsed aeration within replicate tanks suspended negatively buoyant larvae throughout the culture period. ASW was used for incubation and larval culture throughout. Water quality parameters remained within the range of those described in Pilnick et al. (2021). Feeding with live microalgae was initiated at 3 DPF. Larvae were exposed to food for a 16-h static feeding period during which flow-through from the RAS was turned off. Filtered water from header tanks in-line with the RAS was subsequently sent to the culture tanks at 1–2 LPM for an 8-h flushing period. Effluent wastewater passed through a 5-µm filter sock and protein skimmer (Reef Octopus Regal 200-INT, Honya Co. Ltd, Shenzhen, China), 20-µm cartridge filters, and 50-W ultraviolet filtration to remove uneaten microalgae before water returned to the header tank. Microalgae stock cultures were obtained from the University of Florida Tropical Aquaculture Lab or purchased from a commercial culture facility (AlgaGen LLC, Vero Beach, FL, USA) at regular intervals. Growth of these cultures was extended by the addition of modified F/2 nutrient medium with reduced copper (AlgaGen LLC, Vero Beach, FL, USA) and sterilized ASW.

Experiments

Five separate experiments were conducted to iteratively evaluate the impacts of diet quantity, diet composition, and initial stocking density on larval growth and survival. All experiments began at 3 DPF, except for the fourth experiment which was re-initiated with surplus larvae at 6 DPF following an unexpected culture crash. The duration of each experiment ranged from 21 to 42 DPF. Various microalgae diets included *T. lutea*, *C. gracilis*, *C. muelleri*, and *R. lens* and are described for each experiment below. Diet treatments in experiments 1–4 were standardized based on microalgal carbon content, as described in Strathmann (1967). In mixed diets containing more than one species, each species contributed an equal proportion of carbon (pg) to the total pool. In experiments comparing diet compositions, total carbon content was also equivalent between treatments. The carbon content values for *T. lutea*, *C. gracilis*, and *R. lens* were obtained from Ohs et al. (2010) and equaled 7.0, 15.0, and 40.7 pg cell^{-1}, respectively. Both *C. muelleri* and *C. gracilis* were assigned a carbon content value of 15.0 pg cell^{-1} due to similar values for *C. muelleri* reported by Leonardos and Geider (2004) and were used interchangeably in this study. Microalgae culture densities were enumerated daily with a hemocytometer and used to calculate volumes to feed to each replicate tank. Excluding the first experiment, the total amount of microalgae fed increased equivalently at 14 DPF across all treatments. Table 1 contains information on microalgae diet composition, quantity, and larval stocking density for each treatment level for the following experiments.

Experiment 1, diet quantity

A reference diet consisting of *T. lutea* and *C. muelleri* was used in an initial experiment to determine an appropriate microalgal cell concentration for early larval development. Larval performance on high (40×10^3 cells mL^{-1}) and low (10×10^3 cells mL^{-1}) quantity diets was compared over 21 DPF ($n=5$ replicate tanks/treatment). This duration was chosen due to interest in early larval development. The target initial stocking density for each replicate tank was 4 larvae mL^{-1}.

Experiment 2, diet composition

A diet composition experiment was conducted to compare larval performance among three diet treatments ($n=4$ replicate tanks/treatment) over 21 DPF: (1) *T. lutea* + *C. muelleri* (reference diet), (2) *R. lens* + *T. lutea*, and (3) *R. lens* + *C. muelleri.* This duration was also chosen due to interest in early larval development. The target initial stocking density for each replicate tank was 4 larvae mL^{-1}.

Experiment 3, diet composition

A subsequent diet composition experiment was conducted to compare larval performance among (1) *T. lutea* + *C. gracilis* (reference diet, with substitution of *C. muelleri* with *C. gracilis*), (2) *R. lens* + *C. gracilis* (the numerically best performing diet from experiment 2), and (3) *R. lens* + *T. lutea* + *C. gracilis* (a tripartite diet with higher microalgal diversity) over 42 DPF ($n=4$ replicate tanks/treatment) to capture full larval development. The target initial stocking density for each replicate tank was lowered to 2 larvae mL^{-1}.

Table 1 Microalgae diet quantity and composition and initial larval stocking density information for each experiment and treatment level. Both cell and carbon quantities fed before (initial) and after (final) increasing at 14 DPF are presented. The group *Chaetoceros* sp. includes both *C. muelleri* and *C. gracilis*. Carbon content values used for *T. lutea*, *Chaetoceros* sp., and *R. lens* equaled 7.0, 15.0, and 40.7 pg cell^{-1}, respectively. Asterisks (*) denote reference diet. *SEM*, standard error of the mean

Experiment	Duration (days)	Treatment	*n*	Cells mL^{-1} fed, initial/final			Total cells mL^{-1} fed, initial/final	pg carbon mL^{-1} fed, initial/final	Stocking density (larvae mL^{-1} ± SEM)
				Tisochrysis lutea	*Chaetoceros* sp.	*Rhodomonas lens*			
1: Diet quantity	21	High*	5	27,273/27,273	12,727/12,727	-	40,000/40,000	381,816/381,816	3.78 ± 0.21
		Low*		6818/6818	3182/3182	-	10,000/10,000	95,455/95,455	3.89 ± 0.13
2: Diet composition	21	1*	3	6818/13,636	3182/6364	-	10,000/20,000	95,455/190,909	3.56 ± 0.19
		2		6818/13,636	-	1173/2345	7991/15,982	95,455/190,909	4.14 ± 0.20
		3		-	3182/6364	1173/2345	4355/9709	95,455/190,909	4.06 ± 0.19
3: Diet composition	42	1*	4	10,227/20,455	4773/9545	-	15,000/30,000	143,182/286,364	2.02 ± 0.18
		2		6818/13,636	3182/6364	1173/2345	7991/15,982	143,182/286,364	1.98 ± 0.14
		3		-	4773/9545	1759/3518	6532/13,063	143,182/286,364	2.09 ± 0.16
4: Diet composition	28	1	3	-	10,961/21,921	4040/8079	15,000/30,000	328,815/657,630	2.12 ± 0.13
		2		-	-	8079/16,158	8079/16,158	328,815/657,630	2.39 ± 0.06
5: Stocking density	35	Low	3	-	-	8079/16,158	8079/16,158	328,815/657,630	0.82 ± 0.07
		Medium		-	-	8079/16,158	8079/16,158	328,815/657,630	1.43 ± 0.16
		High		-	-	8079/16158	8079/16,158	328,815/657,630	2.21 ± 0.20

Experiment 4, diet composition

A desire to economize larviculture methods justified an investigation of mixed and monoalgal diets. Larval performance was compared between two diet treatments, (1) *R. lens* + *C. gracilis*, and (2) *R. lens* over 28 DPF ($n = 3$ replicate tanks/treatment), at which point the experiment was concluded to maintain adequate replication. The target initial stocking density for each replicate tank was 2 larvae mL^{-1}.

Experiment 5, initial larval stocking density

Density-dependent growth dynamics observed in experiments 3–4 indicated that reduced larval densities resulted in higher growth. Thus, an experiment was conducted to evaluate performance among three initial target stocking densities, (1) ~ 0.75 larvae mL^{-1}, (2) ~ 1.5 larvae mL^{-1}, and (3) ~ 2.25 larvae mL^{-1} over 35 DPF ($n = 3$ replicate tanks/treatment), at which point the experiment was concluded to maintain adequate replication. All replicate tanks were fed a monoalgal diet of *R. lens* throughout.

Data collection

Larval performance was evaluated by estimating survival and analyzing growth at weekly or biweekly intervals beginning at 3 DPF. Larval density (larvae mL^{-1}) within each replicate tank was estimated from triplicate 10-mL subsample counts performed using a plankton wheel counter. Subsample means were used as the value for each replicate tank. Percent survival was calculated based on the estimated number of larvae remaining at the final day relative to initial stocking. To estimate growth, morphometric data was analyzed from larvae within each replicate tank. A homogenized volume of water containing approximately 30 larvae was concentrated using a 100- or 200-µm mesh sieve and transferred to a Sedgewick rafter counting slide. All larvae were photographed using a compound microscope at 4 × or 10 × magnification depending on body size. A minimum of 12 dorsoventrally oriented individuals were haphazardly selected for measurements. An image analysis software (Motic Images Plus 3.0) was used to measure larval body length (BL) and body width (BW) (Fig. 1). Larval body area was calculated using the formula for the area of an ellipse (Formula 1). Mean larval body area from subsampled larvae was used as the value for each replicate tank. Postoral arm length was not included as a factor of interest due to the tendency for mechanical damage during growth in this species.

$$\textit{Larval body area} = \pi \times \left(\frac{BL}{2}\right) \times \left(\frac{BW}{2}\right) \quad (1)$$

Statistical analysis

Larval growth, as measured by mean body area, was compared between treatments with either an unpaired *t*-test or ANOVA at the final time point. Assumptions of normally distributed data and homogeneity of variances were assessed graphically and confirmed with Shapiro–Wilk and Bartlett's tests, respectively. Following an ANOVA, pairwise post hoc analyses were performed with Tukey's HSD test. Survival data were analyzed statistically by comparing the proportion of estimated number of larvae alive to the estimated

number of larvae not alive (larvae stocked at the initial time point minus larvae alive at the final time point) at the final time point using a logistic regression (glm function in R, family = quasibinomial) followed by a Wald-chi-square test. For trials with greater than 2 treatment groups, pairwise comparisons of survival data were further analyzed with a Tukey HSD post hoc test on the log odds of survival. A p-value of 0.05 was used to assess significance. All analyses were performed with R (R Core Team 2021). All means are reported as mean ± standard error.

Results

Detailed information for each spawn used to initiate an experiment is provided in Table 2.

Experiment 1, diet quantity

An initial experiment was conducted to approximate an appropriate microalgae diet quantity and to establish a reference diet within the novel larval rearing system. No significant differences in body size ($t = 2.28$, $df = 8$, $p = 0.054$) or survival (glm, $p > 0.05$) were detected between the 40.0×10^3 cells mL^{-1} and 10×10^3 cells mL^{-1} diets at 21 DPF (Fig. 2a, b). Survival was 26.02% ± 2.28 and 17.03% ± 3.77 for the high and low quantity diets, respectively. Larval performance overall was similar between diets.

Experiment 2, diet composition

After approximating an appropriate diet quantity, subsequent experiments were conducted to identify high-performing microalgae species and carbon equivalent species combinations. Significantly lower survival (glm, $p < 0.05$) was observed from the reference diet (15.30% ± 5.84) than from *R. lens* + *T. lutea* (41.14% ± 4.43) and *R. lens* + *C. muelleri* (52.33% ± 9.55) at 21 DPF (Fig. 2d). Similarly, both diets containing *R. lens* resulted in significantly greater body size compared to the reference diet ($F = 16.33$, $df = 2$, $p < 0.05$) (Fig. 2c). The incorporation of carbon equivalent amounts of *R. lens* improved larval performance.

Experiment 3, diet composition

A second diet composition experiment was conducted to test larval performance with mixed diets containing *R. lens*, including a novel diet with three algae species, over a longer duration. A significant difference in survival was detected among all three diet treatments at 42 DPF (glm, $p < 0.005$) (Fig. 2f). Survival, in order of decreasing performance, resulted from the tripartite *R. lens* + *T. lutea* + *C. gracilis* sp. diet (26.00% ± 5.23), *R. lens* + *C. gracilis* (12.28% ± 3.88), and the reference diet (1.05% ± 0.49). While further diet diversification improved survival, no significant difference was observed in body size among any of the treatments ($F = 3.38$, $df = 3$, $p = 0.09$) (Fig. 2e).

Table 2 Spawning and gamete collection data for each experiment. *SEM*, standard error of the mean. *CI,* confidence interval

Experiment	Date	Males spawned	Females spawned	Fertilization (% ± SEM)	Millions of eggs collected (95% CI)	Approximate number of embryos used (millions)
1: Diet quantity	Oct. 5, 2018	1	1	98.50 ± 0.20	0.79–2.74	1.53
2: Diet composition	Nov. 7, 2018	4	3	94.87 ± 2.73	4.22–5.17	1.41
3: Diet composition	Feb. 18, 2019	2	2	90.23 ± 1.37	1.76–5.79	0.97
4: Diet composition	May 21, 2019	1	1	99.87 ± 0.13	6.10–12.57	0.54
5: Stocking density	Jan. 29, 2020	3	3	93.33 ± 2.33	4.24–7.29	0.54

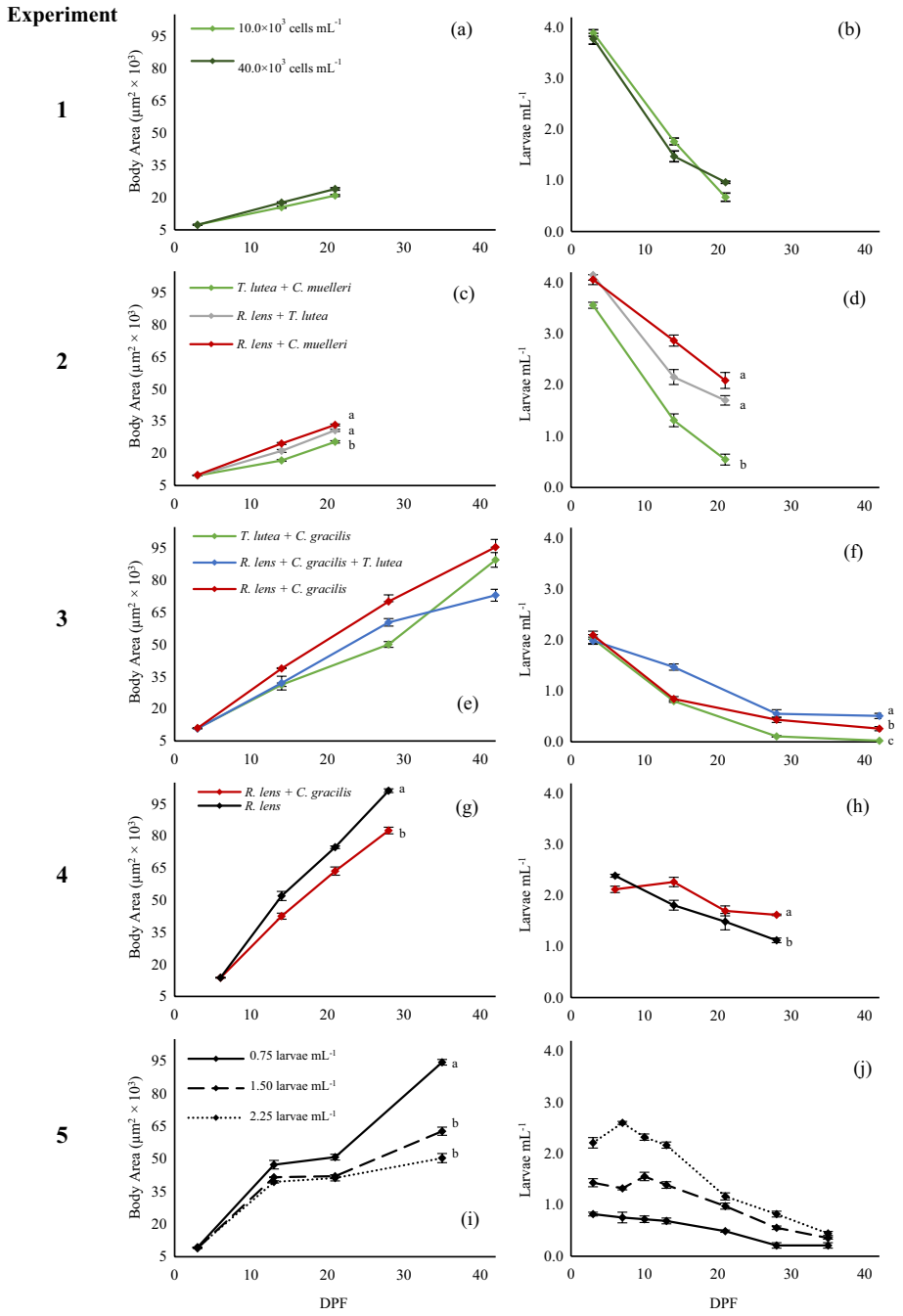

Fig. 2 Larval body size (area; $\mu m^2 \times 10^3$) and density (larvae mL^{-1}) presented for experiment 1 (**a** and **b**), experiment 2 (**c** and **d**), experiment 3 (**e** and **f**), experiment 4 (**g** and **h**), and experiment 5 (**i** and **j**) over time. Lower case alphabetical letters adjacent to size and density curves denote significant differences between treatments. Data points are presented as means ± standard error. Microalgae species included *Tisochrysis lutea*, *Chaetoceros muelleri*, *Chaetoceros gracilis*, and *Rhodomonas lens*

Experiment 4, diet composition

Following the identification of an individually important microalgae species (*R. lens*), experiment 4 was conducted to test the feasibility of culturing *D. antillarum* on a monoalgal diet. Significantly higher survival (glm, $p < 0.005$) resulted from the *R. lens* + *C. gracilis* diet (76.93% ± 4.46) compared to the monoalgal *R. lens* diet (47.15 ± 4.69) at 28 DPF (Fig. 2h). Despite improved survival from the mixed diet, the monoalgal *R. lens* diet resulted in significantly larger body size at 28 DPF ($t = -5.25$, $df = 4$, $p = 0.01$) (Fig. 2g).

Experiment 5, initial larval stocking density

Observations indicating density-dependent growth dynamics led to an investigation of the influence of initial stocking density on larval performance. No significant difference in survival was observed among the 0.75 larvae mL^{-1} (27.63% ± 14.07), 1.50 larvae mL^{-1} (25.31% ± 4.14), and 2.25 larvae mL^{-1} (20.31% ± 3.47) treatments at 35 DPF (glm, $p = 0.82$) (Fig. 2j). While survival was not impacted, body size from the 0.75 larvae mL^{-1} treatment was significantly greater than from the other higher density treatments ($F = 40.36$, $df = 2$, $p < 0.005$) (Fig. 2i).

Discussion

Determining a microalgal cell concentration appropriate for the specific feeding regime within the novel RAS was an essential first step towards developing production-oriented *D. antillarum* culture protocols. Broadly, Hodin et al. (2019) state that most echinoderm larvae grow rapidly when fed between 5.0 and 10.0×10^3 cells mL^{-1} of ~ 10-µm diameter live microalgae every 2–3 days. Optimal cell concentrations, however, can be species-specific and depend on the microalgae type and feeding regime used. Two separate small-scale *D. antillarum* culture attempts reported the most success when larvae were exposed to 5.0–10.0×10^3 cells mL^{-1} mixtures of *R. lens* + *T. lutea* (Eckert 1998), and different 10.0×10^3 cells mL^{-1} combinations of *T. luteau*, *C. gracilis*, and *R. lens* (Leber et al. 2009). These feedings occurred every 3–7 days after 100% water exchange in standalone 1–2.4-L vessels. Larger-scale culture attempts in standalone 50-L vessels were supplied between 30.0 and 50.0×10^3 cells mL^{-1} combinations of *R. lens* + *T. lutea* + *C. gracilis* every 3–4 days after 100% water exchange (Moe 2014). Production-oriented recirculating or flow-through culture systems require more frequent feeding of fewer cells than standalone tanks due to constant or periodic water exchange and active removal of unconsumed food. For example, *Paracentrotus lividus* larvae exhibit greater survival and similar growth when cultured in flow-through systems and fed daily with 1.0–6.0×10^3 cells mL^{-1} compared to standalone tanks fed every 3 days with 3.0–18.0×10^3 cells mL^{-1} (Carboni et al. 2012). In the present study, *D. antillarum* were fed every 24 h after 8 h of flow-through representing at least 100% water exchange. In experiment 1, growth and survival between the 10.0×10^3 and 40.0×10^3 cells mL^{-1} treatment combinations of *T. lutea* + *C. muelleri* were similar. This suggested that larvae were not limited by the lower cell concentration over 21 DPF with this feeding regime and diet composition. An even lower initial concentration of 4.4×10^3 cells mL^{-1} of *R. lens* + *C. muelleri* improved growth and survival compared to 10.0×10^3 cells mL^{-1} of *T. lutea* + *C. muelleri* in experiment 2. Disregarding differences in

diet quality between these treatments, this result suggests that particle encounter rates were likely sufficient down to 4.4–10.0 × 10^3 cells mL^{-1} over a 16-h feeding period. Low initial cell concentrations were preferred to limit the amount of unconsumed food and reduce the risk of fouling and disease. Despite the lack of statistical differences in growth between treatments in experiment 1, numerical divergence between treatments after 14 DPF suggested that moderately increasing the cell concentration at this timepoint could be beneficial. Regardless, similarly poor survival from both treatments averaging 17–26% indicated that some factor(s) other than food quantity, such as food quality and/or larval stocking density, impacted performance.

Comparisons between carbon-equivalent microalgae compositions in experiments 2–4 revealed the importance of diet quality on *D. antillarum* larval development. Larvae were stocked at equivalent densities across treatments within each experiment, unlikely to be limited by algal cell concentrations and had access to the same quantity of dietary carbon. Therefore, differences in larval performance likely resulted from different algal compositions. The reference diet developed in experiment 1 (*T. lutea* + *C. muelleri*) also helped to improve the quality of diets over successive experiments through comparisons to a benchmark (Glencross et al. 2007). The incorporation of *R. lens* improved *D. antillarum* larval performance, corroborating prior recommendations (Eckert 1998; Leber et al. 2009; Moe 2014). Both diets containing *R. lens* in experiment 2 significantly improved growth and survival at 21 DPF compared to the reference diet without *R. lens*. A similar trend extended into late larval development, as both diets containing *R. lens* in experiment 3 significantly improved survival at 42 DPF compared to the reference diet. The apparent dietary benefit of this microalgae is not unique to *D. antillarum. Rhodomonas* spp. have been deemed a high-quality diet for numerous filter-feeding invertebrates including copepods (Dayras et al. 2021; Knuckey et al. 2005; Ohs et al. 2010), artemia (Seixas et al. 2009), rotifers (Coutinho et al. 2020), scallops (Tremblay et al. 2007), oysters (Brown et al. 1998), mussels (Jose Fernández-Reiriz et al. 2015), and sea urchins (Castilla-Gavilán et al. 2018; Gomes et al. 2021; Hinegardner 1969). Nutritional factors, including cell size and morphology, biochemical composition, and/or digestibility, vary by microalgae species (Brown et al. 1997; Guedes and Malcata 2012) and can help to explain improved *D. antillarum* larval performances from diets containing *R. lens*.

Filter feeding invertebrates can ingest a variety of food particles; however, optimum size ranges exist (Fernandez 2001; Lavens and Sorgeloos 1996) and larger, yet still ingestible, microalgae are thought to improve growth (Cárcamo et al. 2005; Fernández-Reiriz et al. 2015; Seixas et al. 2009). Echinoderm larvae cannot actively select food prior to ingestion and instead consume particles that can be captured efficiently and passed through the esophagus into the gut (Strathmann 1971). The ideal particle size range for larval *D. antillarum* is unknown; however, smaller particles are thought to be less readily captured by urchin larvae than larger ingestible particles (Strathmann et al. 1972). Thus, it is possible that fewer *T. lutea* and *Chaetoceros* sp. (3–5-µm and 5–8-µm, respectively [Brown et al. 1997]) cells were captured and ingested when compared to larger *R. lens* cells (8–12-µm [Brown et al. 1997]). Increased cell size and capture efficiency is unlikely to fully explain improved *D. antillarum* larval performance as a similarly sized and commonly used microalgae species, *Dunaliella tertiolecta* (10–12-µm [Brown et al. 1997]), yielded poor results in other studies (Leber et al. 2009; Wijers et al. unpublished data). *Rhodomonas* spp. have favorable biochemical properties for marine aquaculture in general due to desirable fatty acid profiles and relatively high protein and carbohydrate contents (Brown et al. 1997; Castilla-Gavilán et al. 2018; Coutinho et al. 2020; Dunstan et al. 2005; Fernández-Reiriz et al. 2015; Seixas et al. 2009). These microalgae and other cryptophytes can notably

synthesize a diversity of polyunsaturated fatty acids (PUFAs) including eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3) (Peltomaa et al. 2018), which are both essential fatty acids for healthy marine larval development (Sorgeloos et al. 1998). The importance of individual or relative proportions of these long-chain PUFAs for *D. antillarum* development is unknown, as is their ability to elongate and desaturate their precursors, a feature which has already been documented in other urchin species (Liu et al. 2007; Schiopu et al. 2006). Interestingly, *Chaetoceros* spp. and *T. lutea* also contain relatively high amounts of long-chain PUFAs, but neither are rich in both EPA and DHA (Brown et al. 1997). In the present study, these species underperformed when combined in the absence of *R. lens*. Castilla-Gavilán et al. (2018) demonstrated that *Paracentrotus lividus* larvae fed *Rhodomonas* sp. contained higher total lipid content than those fed other microalgae. A similar dynamic could have positive implications for larval *D. antillarum* development.

Dietary protein is an important nutritional source of nitrogen and amino acids and can directly influence growth rates of marine invertebrates (Enright et al. 1986; Kreeger and Langdon 1993). Direct comparisons of protein content between all three microalgae species used in this study are unavailable and can vary depending on culture conditions. Despite this, *R. lens* has been shown to produce and contain higher levels of total protein than *T. lutea* (Fernández-Reiriz et al. 2015; Seixas et al. 2009) and *T. lutea* has also been shown to have higher proportional protein content than *C. gracilis* (Lora-Vilchis et al. 2004). *Rhodomonas* spp. produce a phycobiliprotein pigment called phycoerythrin that can account for up to 12% of total protein content (Seixas et al. 2009) and may have additional undescribed nutritional benefits. Nutrients within poorly digestible cells are unlikely to be assimilated effectively, regardless of cell size or biochemical composition. The digestive capabilities of larval *D. antillarum* are unknown; however, enzymes capable of hydrolyzing carbohydrates, lipids, and proteins have been described from other urchin species (Annunziata et al. 2014; Fenaux 1982; Stumpp et al. 2013) and larvae are thought to prioritize defecating less digestible particles (Strathmann 1971). Diet quality in this study could have been affected by differences in digestibility of microalgae cells and respective degrees of nutrient and energy assimilation. In separate studies, mussel and oyster species exhibited higher absorption rates and efficiencies when fed *Rhodomonas* sp. compared to *T. lutea* (Fernández-Reiriz et al. 2015; González-Araya et al. 2012). Larval sea urchins reportedly do not break down *T. lutea* within the gut as easily as other microalgae and, while diatoms are thought to be digestible (Strathmann 1971), the silica-based frustules that comprise *Chaetoceros* spp. cell walls could be more recalcitrant than *R. lens* membranes.

While *R. lens* appeared to be a crucial dietary component, *D. antillarum* nonetheless benefited from mixed microalgae diets. A comparison of mono-algal *R. lens* and mixed *R. lens* + *C. gracilis* diets in experiment 4 resulted in significantly higher survival from the mixed diet. Similarly, the tripartite diet in experiment 3 resulted in significantly higher survival compared to the *R. lens* + *C. gracilis* diet, which indicated a possible positive correlation between overall diet diversity and performance. This is unsurprising, given that mixed diets are more likely to provide nutritional balance (Brown et al. 1997; Ohs et al. 2010) and have been shown to improve survival of other sea urchin larvae (Gomes et al. 2021). The comparative importance of *T. lutea* or *Chaetoceros* sp. as supplements to *R. lens* is unclear given statistically similar growth and survival between mixed diets in experiment 2. However, *R. lens* + *Chaetoceros* sp. diets performed well and produced the numerically largest larvae in experiments 2 and 3 and the statistically highest survival in experiment 4. Despite improved survival from higher diversity diets and potential benefits from the inclusion of a diatom, the mono-algal *R. lens* diet in

experiment 4 produced significantly larger larvae than the mixed diet and the highest growth overall in this study. This suggests that, as for other sea urchin species (Castilla-Gavilán et al. 2018; Hinegardner 1969), *Rhodomonas* sp. can viably be used as a mono-algal diet for *D. antillarum.*

A density-dependent relationship between larval survival and growth was observed and should be considered concurrently to diet quantity and quality. Higher survival from the mixed-algal diet treatment in experiment 4 corresponded with lower growth, indicating an inverse relationship between larval density and growth. Similarly, the tripartite diet in experiment 3 resulted in the highest survival and lowest growth. Within the same experiment, the production of unexpectedly large larvae from the reference diet treatment may have resulted from a drastic increase in algal cells per larvae due to extremely low survival of ~ 1%. In these instances, reduced survival and lower larval densities potentially lessened competition for physical space and/or resources, including food, leading to higher growth. This dynamic has been observed in the larval culture of other sea urchin species (Azad et al. 2012; Buitrago et al. 2005; Suckling et al. 2018) and warranted investigation in *D. antillarum*. Experiment 5 was conducted to test the hypothesis that, other factors being equal, *D. antillarum* growth and survival improves at lower larval densities. Indeed, a similar trend revealed that the lowest initial stocking density resulted in significantly larger larvae than higher density treatments. While survival was statistically similar between all three larval density treatments, proportionally fewer larvae remained in the higher density cultures. Statistical differences could have resulted from extending the experiment past 35 DPF or from stocking the 1.5 and 2.25 larvae mL^{-1} treatments at higher initial densities. Regardless of initial stocking densities and diet compositions, experiments extending past 30 DPF (experiments 3 and 5) into late larval development concluded at average densities less than 1 larvae mL^{-1}. This observation, in conjunction with the highest growth observed from the 0.75 larvae mL^{-1} treatment in experiment 5, supports culturing *D. antillarum* at initial densities ≤ 1 larvae mL^{-1}.

This study represents a series of investigations intended to develop *D. antillarum* larval culture protocols within a novel RAS capable of scaled production for restoration. Variables of interest included (1) microalgae diet quantity, (2) microalgae diet composition, and (3) initial larval stocking density, which improved outcomes over multiple culture attempts. In summary, daily fed microalgae concentrations down to $4.4–10.0 \times 10^3$ cells mL^{-1} were adequate for rearing *D. antillarum* over 21 DPF with at least 100% daily water exchange. Gradually increasing cell concentrations as larvae grow and presumably increase consumption rates past this point was likely beneficial, but this was not empirically confirmed. The microalgae *R. lens* was a critical dietary component and other *Rhodomonas* species with similar nutritional profiles are likely to perform equally well. Increasingly diverse mixed microalgal diets containing *R. lens* improved larval survival and supplementing with a diatom may have been beneficial. Lastly, density-dependent growth dynamics were observed, whereby reduced larval densities resulted in higher growth. Production-oriented *D. antillarum* larval cultures should be conducted at densities ≤ 1 larvae mL^{-1}. The experimentally derived protocols outlined here eventually resulted in complete development of this species within the novel RAS (Pilnick et al. 2021). Over 1000 juveniles have been produced to-date and have been utilized for pilot-scale restocking studies as well as juvenile behavior and growout research (Hassan et al. unpublished data). Further larval culture optimizations aimed at improving yields are yet necessary. Suggestions for additional research include determining prey capture and consumption rates, digestion efficiencies of different microalgae, and types and ratios of supplementary microalgae to best support *R. lens* diets. Subsequent understanding of how larval nutrition affects settlement and post-settlement success

will further improve production viability. Continued investigations into juvenile growout and restocking methods can further advance *D. antillarum* restoration objectives.

Author contribution Aaron R. Pilnick: conceptualization, formal analysis, investigation, data curation, writing — original draft, visualization. Keri L. O'Neil: conceptualization, resources, writing — review and editing, funding acquisition. Matthew A. DiMaggio: conceptualization, methodology, resources, writing — review and editing, supervision. Joshua T. Patterson: conceptualization, methodology, writing — review and editing, supervision, funding acquisition.

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

Declarations

Competing interests The authors declare no competing interests.

Ethics approval No specific approval of research ethics committees was required to accomplish the goals of this study because experimental work was conducted with an unregulated invertebrate species.

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