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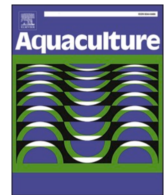

Novel shaker bottle cultivation method for the long spined sea urchin (*Diadema antillarum*; Philippi, 1845) results in high larval survival and settlement rates

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A B S T R A C T

The long spined sea urchin *Diadema antillarum* was an abundant grazer on Caribbean coral reefs, until 1983–1984, when densities were reduced by ~98% during a region wide die-off. Since then, there has been very little natural recovery of the species and interest is growing in applying aquaculture as a tool for population enhancement. In this study we optimized a new shaker bottle cultivation method for *D. antillarum*. The method was tested in a series of experiments by culturing *D. antillarum* from egg to juvenile in the Netherlands as well as the USA. Larvae were cultured in standard 1-L glass reagent bottles, suspended by gentle constant movement on an orbital shaking table and fed with either the microalgae *Rhodomonas lens* or *Rhodomonas salina*. Effects on larval growth and survival were evaluated for different microalgal feeding concentrations, larval densities, and culture temperatures. Larval density and growth were measured twice a week over a period of up to 56 days.

Larvae grew significantly faster on a higher feeding concentration up to 90,000 *Rhodomonas* sp. cells mL^{-1}, twice weekly, compared to 30,000 and 60,000 cells mL^{-1}. A density of 1 larvae mL^{-1} resulted in the highest body size and survival compared to densities of 2 or 4 larvae mL^{-1}. Overall survival from larva to settled juvenile urchin increased from 8 to 10% settlement to 32–33% when the initial density was lowered further from 1.2 to 0.4 larvae mL^{-1}. Growth, survival, competency and settlement did not differ between larval cultures kept at 25 °C or 28 °C.

We believe that this novel method for culturing *D. antillarum* larvae, once scaled-up and validated to pilot scale, could provide juveniles for restocking of urchin-depleted reefs that suffer from algae overgrowth.

1. Introduction

The long spined sea urchin, *Diadema antillarum* (Philippi, 1845) (Echinodermata: Echinoidea: Diadematidae), was historically one of the most important herbivore on Caribbean coral reefs. For at least the last 100,000 years it inhabited Caribbean reefs at high densities, which were possibly enhanced in overfished areas (Lessios et al., 2001). However, between 1983 and 1984, 98% of the population died-off in a mass mortality event (Lessios, 2016), most likely due to a water-borne pathogen that entered through the Panama Canal (Lessios et al., 1984). This event was a major contributor to the phase shift from coral dominated to macroalgae dominated Caribbean reefs, especially in areas where herbivorous fishes were removed (Hughes et al., 1987 & 2003). *D. antillarum* recovery since the mass die-off has been slow or non-existent on most reefs (Lessios, 1995, 2016), probably due to a combination of low larval supply and poor post-settlement survival (Miller et al., 2009; Williams et al., 2011), fertilization limitation (Lee, 2006), lack of suitable settlement substrate (Hylkema et al., 2022b) and a decrease in reef complexity and thereby shelter availability (Lee, 2006; Rogers and Lorenzen, 2016; Tuohy et al., 2020). At reefs where *D. antillarum* populations did recover, algal cover was reduced and coral recruitment and growth was higher compared to zones without urchins

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present (Edmunds and Carpenter, 2001; Idjadi et al., 2010). This demonstrates the viability of contemporary *D. antillarum* population recovery in reversing the previously described phase shift. Such a recovery could possibly be initiated by restocking reefs with cultured *D. antillarum*, while detailed research on the larval to settled juvenile survival phase could potentially answer questions on what is limiting the natural recovery. A consistent and reliable culture method will be vital to these efforts.

Sea urchins have been used for embryological studies as early as 1847, but the groundwork for controlled urchins culture (6 species, *D. antillarum* not included) was only laid in the late 1960s by Hinegardner (1969). Aquaculture of sea urchins has partly been developed because the annual reproductive cycle limits availability for consumption and culture can relieve some of this pressure (McBride, 2005). Large scale sea urchin farming was first demonstrated by Grosjean et al. (1998) for the edible species *Paracentrotus lividus*. In Japan a reported 60 million *Strongylocentrotus intermedius* juveniles are cultured every year in land based systems and are released in the wild to increase standing stock for fisheries (McBride, 2005; Takahashi et al., 2002). Culturing sea urchins for restoration or to control macroalgal growth is relatively new. Hatchery reared *Tripneustes gratilla* can control invasive macroalgae (Westbrook et al., 2015) as demonstrated at scale in Hawaii by releasing ~99,000 cultured juvenile *T. gratilla,* in combination with manual removal of macroalgae (Neilson et al., 2018). This is the first example that large scale sea urchin culture and restocking can help to restore the balance in disturbed reef communities.

Almost 40 years after the mass die-off, several *D. antillarum* culture approaches have produced settled juveniles, but to our knowledge, no reliable culture method yet exists that can consistently produce large numbers of urchin juveniles. *D. antillarum* is particularly difficult to culture due to a relatively long larval phase of 30–55 days (Eckert, 1998), a high sensitivity to metals like nickel and copper (Bielmyer et al., 2005; Moe Jr., 2022), negatively buoyant larvae that must be kept in constant motion during culture, and a generally high sensitivity to water and diet quality (Eckert, 1998; Leber et al., 2009; Nedimyer and Moe, 2006).

Diet type, food concentration, initial larval stocking density and culture temperature can greatly affect survival, and growth of echinoid larvae (Azad et al., 2010). For *D. antillarum* Eckert (1998) was first to report a successful culture that resulted in settled juveniles (5 in total) by culturing larvae in 1 L beakers that contain paddles for water movement at 23 °C, a larval density of ~1 mL^{-1}, fed with the microalgae *Rhodomonas lens* and *Isochrysis galbana* at a concentration of 5000 cells mL^{-1}. Idrisi et al. (2003) cultured at least 200 juvenile *D. antillarum* in 200 and 550 L fiberglass culture tanks, but further culture details such as feeding regime, larval density and temperature were not reported. Leber et al. (2009) cultured over 1200 competent larvae and induced metamorphosis for an undefined number of those in roller bottles, with the most optimal growth, survival and development in 2.4 L bottles at a density of 0.1 larvae mL^{-1}, at 23–26 °C, fed once a week with 10,000 cells of *R. lens* mL^{-1}. Pilnick et al. (2021) were able to produce 1200 competent larvae resulting in 100 juveniles by culturing larvae in custom engineered 40-L acrylic culture tanks, at a density of 1 and 2 larvae mL^{-1}, 25–27 °C, feeding *R. lens* and *Chaetoceros gracilis* at between 5000 and 40,000 cells mL^{-1}. Most recently Pilnick et al. (2022) established an ideal starting density of ≤1 larvae ml^{-1} in the same 40-L tanks and described *R. lens* as critically important for *D. antillarum* larval culture.

In this study we present a novel culture method for *D. antillarum* larvae up to settled juveniles, using 1 L bottles on an orbital shaking table. The aim of the study was to increase the stability and consistency of the shaker bottle culture by providing new knowledge and experience. The method was subsequently applied in labs in the Netherlands as well as the United States to demonstrate consistency and reproducibility even with slight variations in culture conditions. We studied the effects of microalgal diet, initial larval density, and temperature on larval size, density and survival. Larvae of biggest cohort were used for settlement induction trials for which larval competency was scored as well.

With the resulting culture method, that is capable of producing hundreds of settlers, stability, consistency and output can be further optimized in order to realistically produce (hundreds of) thousands of larvae and juveniles year-round for the first time in almost 40 years since the mass die-off.

2. Material and methods

2.1. Brood stock maintenance

2.1.1. Leeuwarden, the Netherlands

At the marine laboratory facilities of University of Applied Sciences Van Hall Larenstein in Leeuwarden (the Netherlands) a brood stock of 10 adult wild caught *D. antillarum,* imported from Cuba, were kept in two groups of 5 animals in recirculation system of 450 L artificial seawater (ASW) (Tropic Marin® REEF-MIX sea salt, Wartenburg, Germany) on full strength (35 g/L) at 25 °C with a light/dark period of 12/12 h with artificial lighting. The animals were fed a commercially available herbivore diet ([31.9% crude protein, 12% crude fat, 4.5% crude fiber], Vitalis Marine Grazer Mini, Thorne, Great Britain) to apparent satiation five days per week.

2.1.2. Apollo Beach, United States

Adult urchins for The Florida Aquarium, Apollo Beach (United States) brood stock were collected in the Florida Keys by Florida Fish and Wildlife Conservation Commission personnel under National Oceanic and Atmospheric Administration permit # FKNMS-0218-023 to FWC. In total, 14 animals were maintained in a greenhouse recirculating system of 2380 L with ASW (Tropic Marin salt REEF-MIX) on full strength (35 g/L) at 25 °C under natural photoperiod. The animals were fed a commercially available herbivore diet ([34% crude protein, 8% crude fat, 8% crude fiber], Algaemax Wafers, New Life Spectrum, Homestead, FL, USA) to apparent satiation five days per week. A more detailed description on the brood stock maintenance in the United States can be found in Pilnick et al. (2021).

2.2. Gamete collection

Spawning was induced by acute transfer of all adult urchins to a tank containing water 5 °C above ambient holding conditions (Leber et al., 2009). Eggs and sperm were collected directly after release of the gametes with a 10 mL pipette. Adults were kept in this warm water for a maximum of 45 min. As soon as both a male and female released their gametes, the collected sperm and eggs were combined in an aerated 1 L borosilicate glass bottles (101 mm diameter x 230 mm height) with screw cap. Two hours after spawning, eggs were checked for fertilization.

The total amount of collected eggs was determined by counting a diluted (1:10) subsamples ($n = 3$) of 1 mL of the 1 L bottle with eggs. After counting, the collected eggs were divided at a concentration of ~100 eggs mL^{-1} over 1 L bottles containing 500 mL ASW. To keep the eggs from sinking to the bottom the bottles were placed on an orbital shaking table (Innova'40 in Leeuwarden and Heathrow Scientific 120,460 in Apollo Beach) and were constantly shaken at an RPM of 135 at 25 °C with a light/dark period of 12/12 h (natural photoperiod in Apollo Beach).

2.3. Larval culture

Larvae at 3–6 days post fertilization (DPF) were enumerated by counting 10 mL subsamples (n = 3) using an acrylic Ward zooplankton counting wheel. Larvae were diluted into replicate 1 L bottles, containing 500 mL filtered ASW into a density of 0.3–4 larvae mL^{-1} depending on the experimental plan (Table 1). Following volumetric

Table 1
Overview of performed culture experiments to optimize larvae rearing from 3 to 6 days post fertilization (DPF) till the end of the experiment. Shown is the focus of the study, culture location, number of bottles per treatment, larval density, temperature and feeding regime for each experiment.

Focus of experiment	Culture location	Replicate bottles per treatment	Larval density (larvae mL^{-1})	Temperature (in °C)	Feeding regime (1000 x cell mL^{-1})
1: Feeding concentration 1	Leeuwarden (NL)	n = 3	4.0 ± 0.2	28.2 ± 0.3	30 *R. salina* 3–42 DPF 60 *R. salina* 3–42 DPF 90 *R. salina* 3–42 DPF
2: Larval density	Leeuwarden (NL)	n = 3	3.9 ± 0.2 2.0 ± 0.3 1.0 ± 0.2	26.0 ± 0.2	60 *R. salina* 3–16 DPF
3: Feeding concentration 2	Apollo Beach (USA)	n = 3	1.2 ± 0.2	25.7 ± 0.4	3.3 (low) 10 (high) *R. lens* 6–12 DPF 10 (low) 30 (high) *R. lens* 13–32 DPF 30 (low) 90 (high) *R. lens* 33–44 DPF
4: Temperature	Leeuwarden (NL)	n = 8	0.3 ± 0.1	24.9 ± 0.2 28.2 ± 0.1	15 *R. salina* 3–14 DPF 30 *R. salina* 14–28 DPF 60 *R. salina* 28–56 DPF

distribution, each replicate bottle was counted again (n = 3) to verify the initial larval density. Bottles were placed on a shaking table as described in 2.2 to keep the larvae in suspension during culture, at temperatures ranging between 25 and 28 °C. Bottles were cleaned twice a week by first removing any floating debris with a pipette. Water quality parameters were within acceptable ranges for this species as reported in Pilnick et al. (2021). Remaining ASW with the larvae was gently poured onto a 50–250 μm (depending on the size of the larvae) mesh filter to retain the larvae. Larvae were then gently washed from the filter with ASW in a clean 1 L bottle with a final volume of 500 mL and fed at an initial concentration between 3300 and 90,000 cells mL^{-1} with microalgae *Rhodomonas salina* in the Netherlands and *Rhodomonas lens* in the United States. Microalgae were cultured on adjusted f/2 medium (Guillard, 1975) without the addition of zinc and copper, at 4× the feeding concentration. Experiments were run until all larvae had settled, died, or did not show any rudiment formation after 40–42 days.

2.4. Larval measurements

2.4.1. Size

After cleaning the bottles, but before feeding, each larval culture was measured for size and survival. For size, a picture of a subsample of 5 randomly selected larvae from each replicate bottle for each treatment was taken. In Leeuwarden, pictures were taken with an Olympus Stereomicroscope SZ61 using cellSense Image software, in Apollo Beach with a Moticam 10+ camera with Motic Images Plus 2.0 software. Body length and body width were determined for each larvae (Fig. 1A) and were used to calculate size (as body surface area in μm^2) using the formula for an ellipse shape:

$$Size = \pi \times \left(\frac{Body\ length\ in\ \mu m}{2}\right) \times \left(\frac{Body\ width\ in\ \mu m}{2}\right) \quad (1)$$

2.4.2. Density

To determine larval density, three subsamples of 10 mL were counted using an acrylic Ward zooplankton counting wheel from each replicate bottle for each treatment. After each counting, larvae were rinsed back in the bottles for further culture.

2.4.3. Competency and settlement

For the temperature experiment, 10 randomly collected larvae were weekly checked for competency starting at 35 DPF for each replicate bottle and both temperatures. Larvae were regarded as being competent if they had a clearly developed external (Fig. 1B) or internal rudiment (Fig. 1C). Settlement was defined as larvae that were attached to the surface of the petri dish with their rudiment.

2.5. Experiment 1: Feeding concentration 1

To test the effect of algal density on survival and growth of *D. antillarum*, larvae were fed with 30,000, 60,000 or 90,000 cells mL^{-1} of *R. salina* twice a week with 3 replicate bottles for each treatment (*n* = 3). The experiment was conducted in Leeuwarden, the Netherlands, from July to September 2020. Starting density for each of the treatments was 4.0 ± 0.2 larvae mL^{-1} (average ± SD, *n* = 9) with 1983 ± 104 larvae (average ± SD, n = 9) in each replicate bottle. Culture temperatures were maintained at 28.2 ± 0.3 °C (average ± SD, *n* = 36), except between 17 and 21 DPF, when a local heat wave and lack of temperature control increased culture temperature to up to 31 °C.

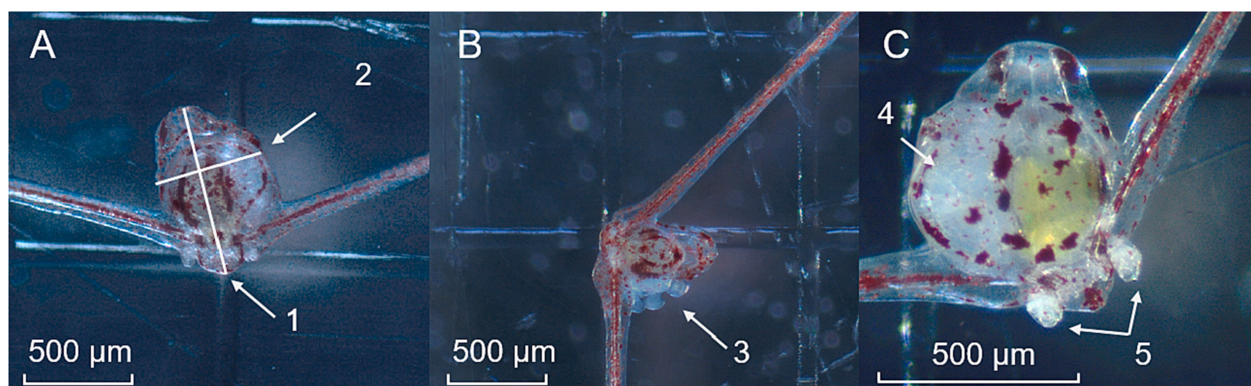

Fig. 1. Dorsal view of *D. antillarum* larvae showing (**A**) body length (1) and body width measurement (2), (**B**) a larvae with developing external rudiment (3), not yet fully developed and (**C**) a competent larvae with internal rudiment (4) and pedicellariae, which are not always present but seem to be a good indication of competency (5). The yellow circular shape, most noticeable on picture **C**, is the stomach of the larvae. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

2.6. Experiment 2: Larval density

The effect of a starting density of 3.9 ± 0.2, 2.0 ± 0.3 and 1.0 ± 0.2 larvae mL^{-1} (average ± SD, n = 3), with 1956 ± 108, 1000 ± 174 and 511 ± 79 larvae (average ± SD, n = 3) in each replicate bottle respectively, on growth and survival for *D. antillarum* was tested with 3 replicate bottles for each treatment (n = 3) in Leeuwarden, the Netherlands, from September to October 2020. Culture temperature was maintained at 26.0 ± 0.2 °C (average ± SD, n = 36). All treatments were fed 60,000 cells of *R. salina* mL^{-1} twice a week until 17 DPF. At 17 DPF the *R. salina* culture failed and the experiment had to be ended before any larvae developed competency.

2.7. Experiment 3: Feeding concentration 2 (including settlement)

2.7.1. Larval culture

The effect of feeding concentration on survival, growth and settlement of larvae was tested for a low or high feeding concentration with 3 replicate bottles for each treatment (n = 3) with *R. lens* twice a week in Apollo Beach, United States, from September to October 2020. Larvae starting density was 1.2 ± 0.2 larvae mL^{-1} (average ± SD, *n* = 6) with 619 ± 80 larvae (average ± SD, n = 6) in each replicate bottle and culture temperature was maintained at 25.7 ± 0.1 °C (average ± SD, *n* = 35). The low feed treatment received an with larval development (DPF) increasing amount of 3300 (3–14 DPF), 10,000 (14–28 DPF) and 30,000 (28–44 DPF) cells mL^{-1} and in the high feed treatment an increasing amount of 10,000 (3–14 DPF), 30,000 (14–28 DPF) and 90,000 (28–44 DPF) cells mL^{-1}. Previous experiments showed microalgae remain un-eaten when feeding at a concentration 60,000–90,000 cells mL^{-1} in earlier culture (3–28 DPF), visible as a pink hue in culture bottles. Therefore, the amount of feed was increased in three steps depending on DPF.

2.7.2. Settlement

A small scale settlement test was initiated at 42 DPF. Five randomly selected larvae from each bottle (n = 6) were moved into 12 mL covered culture well plates. In total, 90 larvae from both the low feed and high feed treatment were divided over 36 culture wells. Each well contained a conditioned ceramic tile (15 × 15 × 5 mm) and 10 mL of filtered ASW, 50% of the water was changed daily. Ceramic tiles were conditioned for a period of 3 months within brood stock holding tanks. Culture well plates were observed daily for 6 days and the total number of settled urchins was quantified for both treatments.

2.8. Experiment 4: Temperature

To test the effect of temperature on growth, density, competency and settlement, larvae were cultured at 24.9 ± 0.2 °C and 28.2 ± 0.1 °C (average ± SD, *n* = 36), the range of the previous three culture experiments, with 8 replicate bottles for each treatment (*n* = 8). The experiment was conducted in Leeuwarden, the Netherlands, from October to December 2020. All larvae were fed 15,000 (3–14 DPF), 30,000 (14–28 DPF) and 60,000 (28–56 DPF) cells of *R. salina* mL^{-1} twice a week. A starting density of 0.3 ± 0.1 larvae mL^{-1} (average ± SD, *n* = 18) with 167 ± 47 larvae (average ± SD, n = 18) in each replicate bottle was used to minimize any possible negative effect of density or food availability or physical interaction of larvae. Larvae were used for the settlement experiment described under 2.9 from 35 DPF until 56 DPF, when the experiment was ended.

2.9. Experiment 5: Competency and settlement

2.9.1. Petri dish settlers

After the first settlement occurred in the bottles during culture at 34 DPF of the *Temperature* experiment (2.8), larvae were used to quantify *competency* and evaluate *settlement* at 35, 42, 49 and 56 DPF. Ten larvae were randomly collected from each bottle, visually checked for competency as described in 2.4, and transferred into one petri dish (89 mm diameter) per replicate bottle (n = 8). Larvae that were cultured at 25 °C were also settled at 25.2 ± 0.1 °C (average ± SD, *n* = 4), similarly the 28 °C cultured larvae were left to settle in an environment at 28.3 ± 0.2 °C (average ± SD, n = 4). Prior to the settlement test, each petri dish was conditioned in a brood stock tank for one week, to facilitate biofilm formation to provide settlement cues, and filled with 25 mL autoclaved ASW. The diameter of each settler was quantified under the same stereoscope used to measure the larvae (2.4). Settlement was recorded after 48 h incubation time in the petri dish.

2.9.2. Bottle settlers

All larvae that settled during culture in the culture bottles were recorded and removed twice a week (during water cleaning and feeding).

2.9.3. End settlers

All remaining larvae that were not used for weekly petri dish testing and that did not settle in the bottles at 56 DPF were transferred into a petri dish (89 mm diameter) for final settlement. Each petri dish was conditioned in a brood stock tank for four weeks, filled with 25 mL autoclaved ASW, the temperature was kept at 25 °C or 28 °C depending on the treatment and settlement was recorded after 48 h.

2.10. Statistical analyses

Statistical analyses were performed with R (R Core Team, 2021) using R studio version 4.0.4. Linear Mixed Models (LMM, lmer function in the R package "lme4") (Bates et al., 2015) were used to model competency, settlement, size (formula 1) and larval density for all culture experiments except the larval density experiment. To be able to analyse the data for the larval density experiment with the same statistical model, survival was calculated at each sampling point (formula 2), as larval densities were different from the start per design.

$$Survival = \left(\frac{Measured\ larval\ density}{Initial\ larval\ density}\right) \times 100\% \qquad (2)$$

For all models, treatment and DPF were considered as fixed factors, while the culture bottle ID was included as random factor to control for the multiple observations per bottle. Model selection and validation was performed according to Zuur et al. (2009) and the Akaike Information Criterion (AIC) was used to select the best fitting models. The larval density models of the *Feeding concentration 1* and *Feeding concentration 2* experiments had the lowest AIC when only DPF was included as fixed factor, while the larval density models of the *Larval density survival* and the *Temperature* experiments, all body surface models and the competency and settlement models had the lowest AIC when both treatment and DPF were included. Residuals of the larval density models of *Feeding concentration 1*, all body surface models and *Settlement* model indicated heteroscedasticity, which was solved after the data were cube-root transformed. For statistical inference, F-tests with Kenward-Roger's approximation to degrees of freedom were performed using the Anova function of the "car" package (Fox and Weisberg, 2019). If treatment consisted of more than two levels, pairwise comparisons, corrected for multiple comparisons, were performed using estimated marginal means (EMM) from the package "Emmeans" (Lenth et al., 2018). Data is expressed as "average ± SE".

3. Results

Cultured animals developed well from fertilized egg to larvae and finally juvenile within a span of 35–56 days, fed with either *R. salina* or *R. lens* (Fig. 2). Two of four culture experiments resulted in juvenile *D. antillarum*, respectively 16 and 874 in total. Pedicellaria, a claw

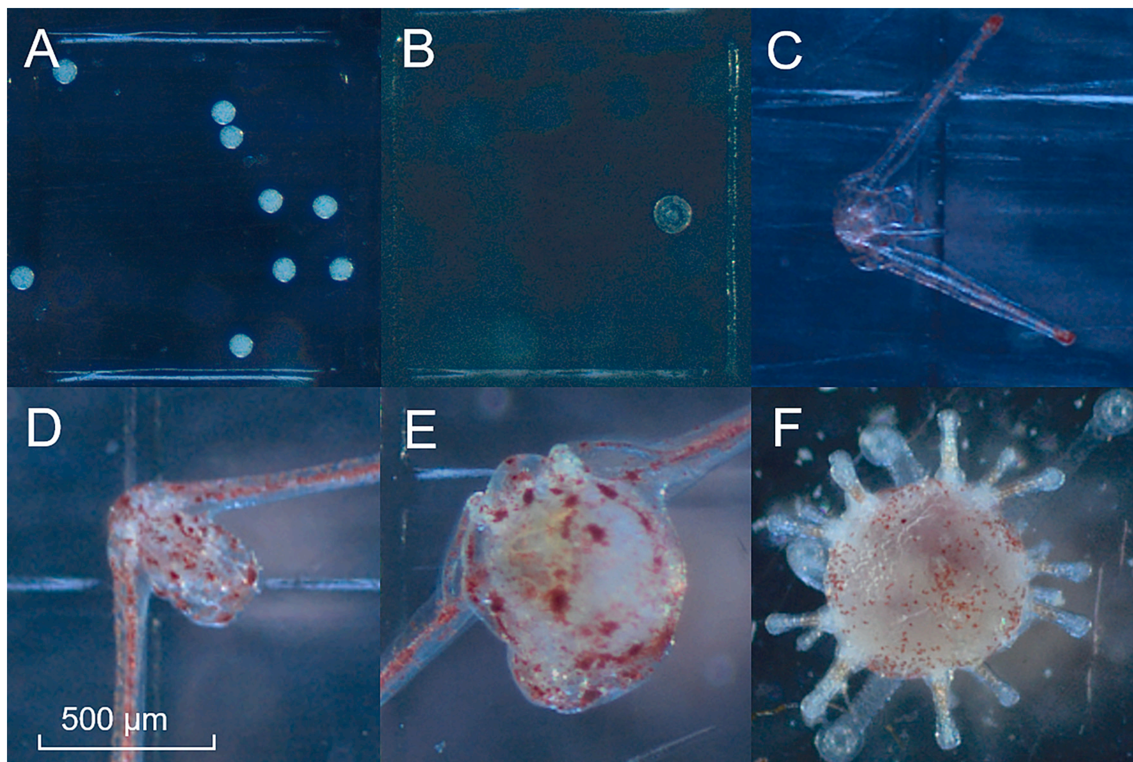

Fig. 2. Larval development from gamete to settled urchin showing, (**A**) fertilized eggs two hours after spawning, (**B**) one day old blastula, (**C**) 7 day old early larvae, (**D**) 21 days old larvae with elongated arms but no formation of rudiment yet, (**E**) 31 day old competent larvae with large internal rudiment and pedicellariae and (**F**) two day old settled and metamorphosed juvenile.

shaped appendage (Fig. 1C), were clearly visible in many of the larvae in the experiment producing the most juveniles and seem to be a good indicator for a healthy larvae and competency (personal observations). Using this novel method, juveniles were cultured in both the Netherlands and the United States. Larvae settled in the culture bottles as early as 35 DPF, but most juveniles settled in the settlement experiments using biofilm (1 and 4 weeks old) as a settlement cue with larvae between 35 and 56 DPF.

3.1. Induced spawning and gamete collection

Induced heat shock resulted in 36–100% of brood stock spawning, with on average 350,000 (100,000-500,000) fertilized eggs collected per female (Table 2). This was more than enough for the experiments presented here and not seems likely to be a bottle neck when upscaling culture. Every attempted spawn was successful with at least one male and one female that spawned. Fertilization was not determined but is generally high for *D. antillarum* without any observations of polyspermy (Pilnick et al., 2021). No mortality or other observed stress due to spawning was recorded with the brood stock animals.

3.2. Experiment 1: Feeding concentration 1

DPF was a significant (F = 395.39, df = 314, $P \leq 0.001$) predictor for the larvae density, but feeding density was not included in the best fitting statistical model. Under all feeding densities, larval density declined drastically during the first 10 days of culture and slowly kept decreasing (Fig. 3A). Each treatment with an initial 4 larvae mL^{-1} ended with a density below 1 mL^{-1} at 42 DPF and the experiment was ended as no competency was observed.

Feeding 90,000 cells of *R. salina* mL^{-1} resulted in the fastest growing larvae (Fig. 3B). Both feeding density (F = 69.71, df = 6, $P \leq 0.001$) and DPF (F = 811.95, df = 530, $P \leq 0.001$) were significant predictors for body surface. Post-hoc testing revealed that differences between treatments 30,000–60,000 (P = 0.0036), 30,000–90,000 (P = 0.0001) and 60,000–90,000 (P = 0.0019) were all significant.

3.3. Experiment 2: Larval density

The initial larval stocking density significantly affected survival of the larvae. Where a density of 1 larvae mL^{-1} showed almost no mortality during 17 days of culture, an initial stocking density of 4 larvae mL^{-1} quickly dropped and ended below 2 larvae mL^{-1} (Fig. 4A). Larval density (F = 13.61, df = 6, P = 0.006) and DPF (F = 31.31, df = 125, $P \leq$ 0.001) were significant predictors for survival. Survival was lower in 4 compared to 1 (P = 0.006) and 2 (P = 0.0201) larvae mL^{-1} but 1 and 2 larvae mL^{-1} were not significantly different from each other (P = 0.499).

Larvae with an initial stocking density of 1 larvae mL^{-1} grew significantly bigger compared to 4 but not 2 mL^{-1} initial stocking

Table 2
Gamete collection summary where for each larval culture experiment a different batch of gametes was collected with the data shown here.

Focus of experiment	Date	# Urchins (# spawned)	Spawn %	Male	Female	# Fertilized eggs (1000 x)
1: Feeding density 1	July 2020	6 (4)	67	2	2	450
2: Larval density	Sept. 2020	5 (2)	40	1	1	400
3: Feeding density 2	Nov. 2020	14 (5)	36	2	3	1,500
4: Temperature	Oct. 2020	3 (3)	100	2	1	100

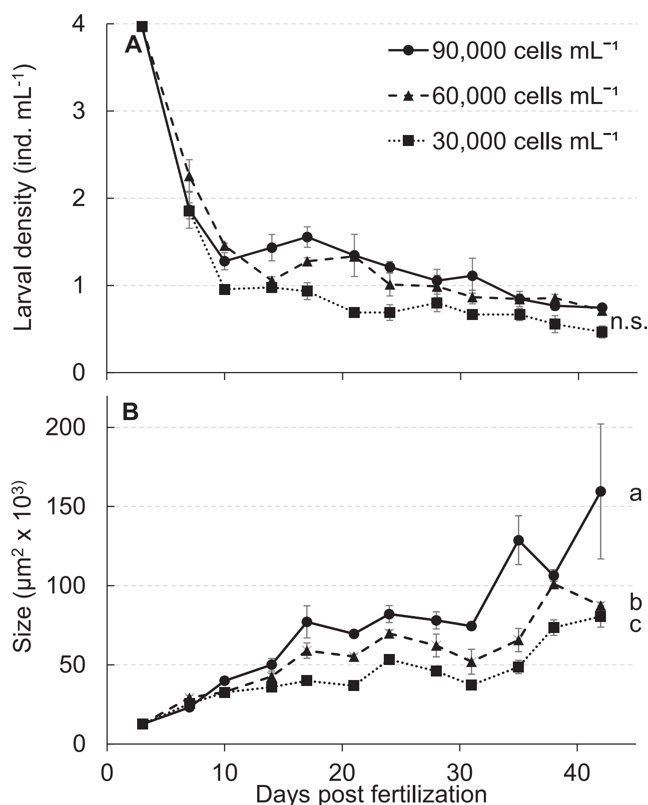

Fig. 3. *Experiment 1:* The growth and density of *D. antillarum* larvae from day 3 to 42 days post fertilization fed with 30,000 (line), 60,000 (dotted line) and 90,000 (dashed line) cells of *R. salina* mL^{-1} twice a week, cultured at 28.2 ± 0.3 °C (average ± SD, n = 36) showing (**A**) larval density described as number of individuals mL^{-1} where DPF was a significant (F = 395.39, df = 314, $P \leq$ 0.001) predictor for the larvae density, but feeding density was not and was not included in the final statistical model and (**B**) body size (μm^2 x 10^3) where both feeding density (F = 69.71, df = 6, $P \leq$ 0.001) and DPF (F = 811.95, df = 530, $P \leq$ 0.001) were significant predictors for body surface. Post-hoc testing revealed that all differences between treatments were significant, 30,000–60,000 (P = 0.0036), 30,000–90,000 (P = 0.0001) and 60,000–90,000 (P = 0.0019). Treatments with different letters indicate a significant difference (n = 3).

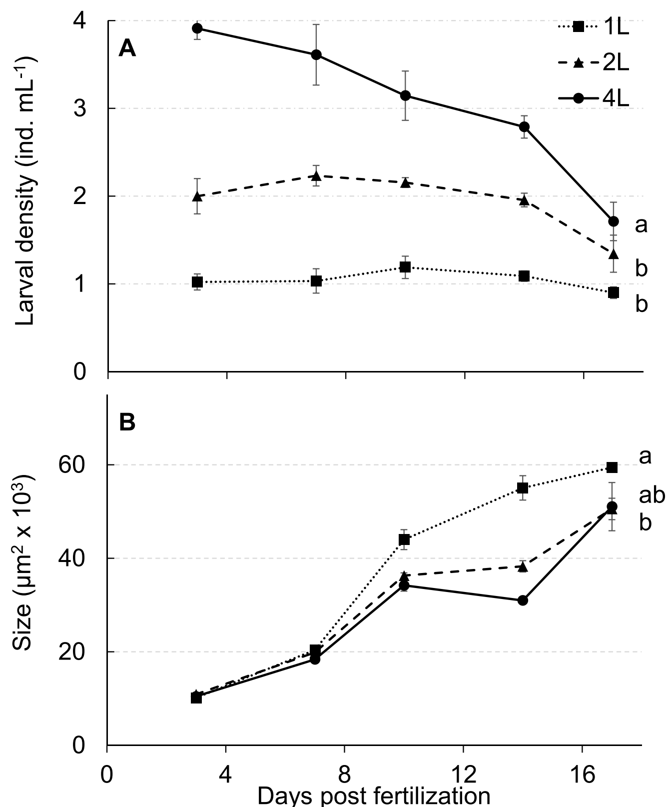

Fig. 4. *Experiment 2 (Larval density):* The size and survival of *D. antillarum* larvae from 3 to 17 DPF with 1 (*1 L* = line), 2 (*2 L* = dotted line) and 4 (*4 L* = dashed line) larvae at start mL^{-1} fed with 60,000 cells of *R. salina* mL^{-1} cultured at 26.0 ± 0.2 °C (average ± SD, n = 36) showing (**A**) larval density described as number of individuals mL^{-1} where larval density (F = 13.61, df = 6, P = 0.006) and DPF (F = 31.31, df = 125, $P \leq$ 0.001) were significant predictors for survival. Survival was lower in 4 compared to 1 (P = 0.006) and 2 (P = 0.0201) larvae mL^{-1} but 1 and 2 larvae mL^{-1} were not significantly different from each other (P = 0.499) and (**B**) body size (μm^2 x 10^3) where both larval density (F = 6.94, df = 6, P = 0.028) and DPF (F = 415.96, df = 215, $P \leq$ 0.001) were significant predictors where 1 was significantly different than 4 larvae mL^{-1} (P = 0.023) but 2 was not significantly different from 1 (P = 0.281) and 4 (P = 0.187) larvae mL^{-1}. Treatments with different letters indicate a significant difference (n = 3).

density (Fig. 4B). Both larval density (F = 6.94, df = 6, P = 0.028) and DPF (F = 415.96, df = 215, $P \leq 0.001$) were significant predictors of larval density, where 1 was significantly different than 4 larvae mL^{-1} (P = 0.023). An initial density of 2 was not significantly different from 1 (P = 0.281) and 4 (P = 0.187) larvae mL^{-1}.

Due to failure in the algal cultures the experiment was ended prematurely at 17 DPF without any observations of rudiment formation or competency.

3.4. Experiment 3: Feeding concentration 2

Mortality was high and gradual throughout culture in both high and low feeding regimes, with only ~20% survival after 42 days for both treatments (Fig. 5A). DPF (F = 108.14, df = 56, $P \leq 0.001$) was a significant predictor for larval density but feeding density was not.

The high feeding regime resulted in significantly bigger larvae (F = 21.08, df = 4, P = 0.010) and both treatments resulted in juveniles (Fig. 5B). DPF was also (F = 581.02, df = 466.24, P ≤ 0.001) a significant predictor for size.

During the settlement trial a total of 16 larvae settled and transformed into juveniles, 7 in the low feed treatment and 9 in the high feed treatment, respectively 8% and 10% of the total larvae.

3.5. Experiment 4: Temperature

Larval survival throughout the experiment was high (83–116%) for both temperature treatments (Fig. 6A). Inherent error in measuring larvae at low densities, especially underestimating numbers in early culture, appeared to contribute to high survival rates (e.g. >100%). However, survival was good overall. Larvae kept growing throughout the experiment, only showing signs of flattening at the last day of measurements, reaching the size of almost 300,000 μm^2 (Fig. 6B). For larval density, both DPF (F = 2.25, df = 463, P = 0.135) and temperature (F = 4.06, df = 14, P = 0.064) were no significant predictors. For body size, DPF (F = 4026.94, df = 1267.47, $P \leq$ 0.001) was a significant predictor but temperature was not (F = 24.86, df = 1.13, P = 0.297).

3.6. Experiment 5: Settlement

Competency for both temperature treatments gradually increased over time reaching around 75% of the larvae being scored as competent at 56 DPF (Fig. 7A). Settlement also gradually increased over time reaching around 50% settlement between 49 and 56 DPF for both treatments (Fig. 7B). Overall, settlement was not as high as the percentage of competent larvae, meaning that not all larvae that were

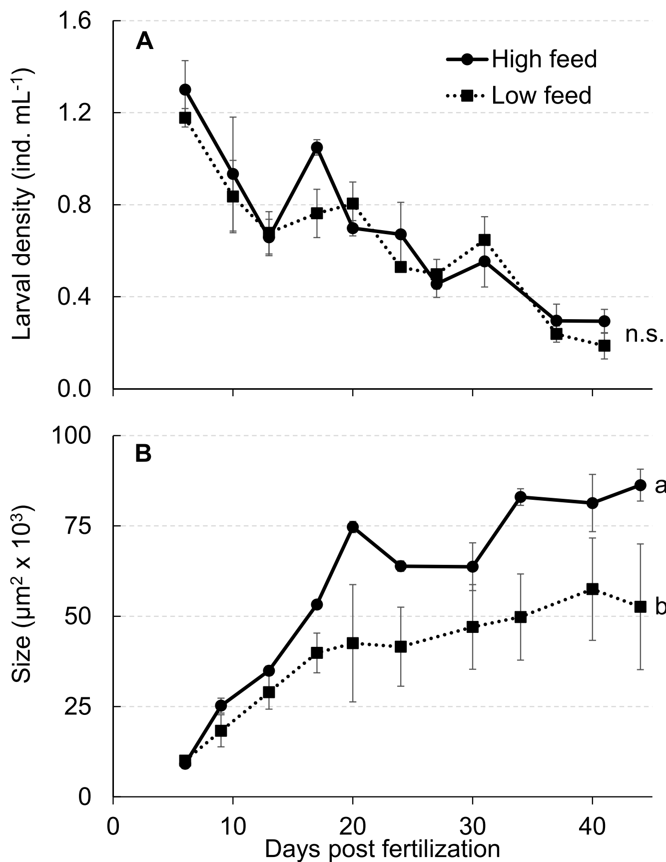

Fig. 5. *Experiment 3 (Feeding density 2):* The growth from 6 to 41 DPF and survival of *D. antillarum* larvae from 6 to 44 DPF fed incrementally depending on DPF with 10,000, 30,000 and 90,000 (*High feed* = line) and 3300, 10,000 and 30,000 (*Low feed* = dotted line) cells of *R. lens* mL^{-1} cultured at 25.7 ± 0.1 °C (average ± SD, *n* = 35) showing (**A**) larval density described as number of individuals mL^{-1} where DPF (F = 108.14, df = 56, *P* ≤ 0.001) was a significant predictor, but feeding density was not included in the final statistical model and (**B**) body size (µm^2 x 10^3) where both feeding density (F = 21.08, df = 4, P = 0.010) and DPF (F = 581.02, df = 466.24, P ≤ 0.001) were significant predictors. Treatments with different letters indicate a significant difference, n. s. indicates there was no significant difference (n = 3).

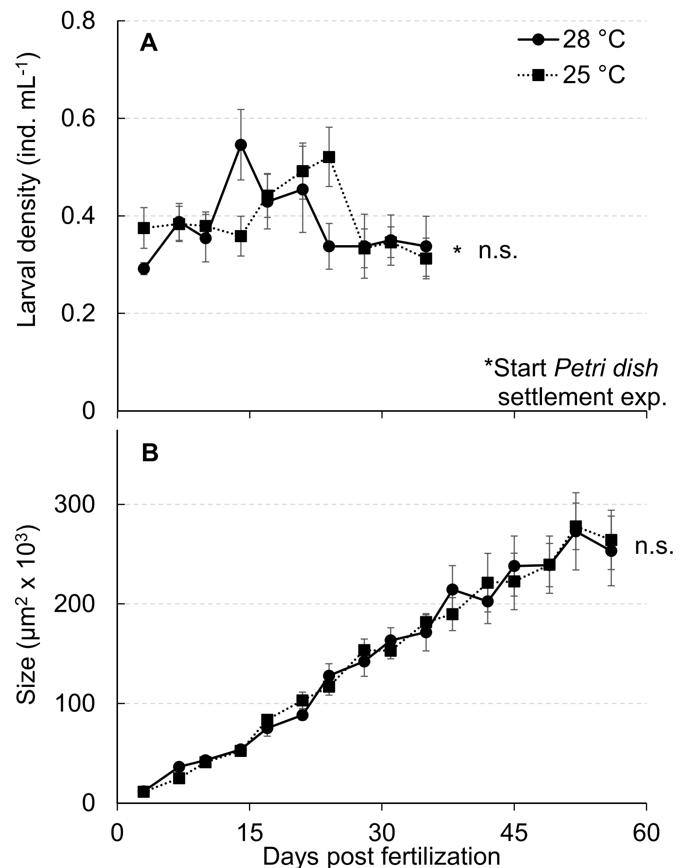

Fig. 6. *Experiment 4 (Temperature):* The growth and survival of *D. antillarum* larvae from 3 to 56 DPF fed incrementally depending on DPF with 15,000, 30,000 and 60,000 cells of *R. salina* mL^{-1} reared at 28 °C (line) and 25 °C (dotted line), measured data 28.2 ± 0.1 °C and 24.9 ± 0.2 °C (average ± SD, *n* = 36), showing (**A**) larval density described as number of individuals mL^{-1} (starting at day 35 larvae were used for petri dish settlement tests, artificially lowering larval density and therefore no longer shown) where both DPF (F = 2.25, df = 463, *P* = 0.135) and temperature (F = 4.06, df = 14, *P* = 0.064) were no significant predictors and (**B**) body size (µm^2 x 10^3) where DPF (F = 4026.94, df = 1267.47, *P* ≤ 0.001) was a significant predictor but temperature was not (F = 24.86, df = 1.13, *P* = 0.297). N.s. indicates there was no significant difference (*n* = 8).

scored as competent also settled in the petri dishes with a one week old biofilm. In total 189 larvae settled combining both treatments (see Table 3) with an average diameter size of 651 ± 103 µm (mean ± SD). DPF was a significant predictor for both competency (F = 73.55, df = 47, *P* ≤ 0.001) and settlement (F = 26.43, df = 47, *P* ≤ 0.001) and temperature was not for both competency (F = 0.058, df = 14, *P* = 0.813) and settlement (F = 1.60, df = 17, *P* = 0.227).

The first settlers in the culture bottles were observed at 34 DPF during cleaning and a total of 84 settlers have been collected from the culture bottles throughout the culture experiment, ending at 56 DPF (see Table 3).

4. Discussion

The novel *D. antillarum* culture method presented here is a bench top method that could be replicated anywhere where there are microalgae to feed, relatively small volumes of saltwater, and urchins to spawn. The use of relatively small, static culture units (1-L media bottles) facilitates replicated, hypothesis-testing studies without the need for a recirculating system or significant infrastructure. The method resulted in competent larvae and juveniles both in the Netherlands and the USA, suggesting a general ease of replication and potential for reproducibility that could facilitate aquaculture production at scale. The fact that half of the culture attempts reported here resulted in juveniles is good basis for potential and further technological development. The method has also been employed on Saba (Dutch Caribbean) in 2021 and 2022, resulting in over six hundred settlers from four culture runs (Tom Wijers, unpublished data).

1. All spawn attempts in the current study were successful, confirming the speculation by Pilnick et al. (2021) that holding the brood stock at a more constant temperature can increase spawn success. Our results also confirm that gametes can be collected year round, not being dependent on natural reproduction cycles that peak in spring and early summer (Hylkema et al., 2022a; Vermeij et al., 2010; Williams and Yoshioka, 2010).
2. Feeding concentration, combined with larval density determines food availability and influences growth and survival. For echinoid larvae in general the optimal feeding concentration is between 3000 and 9000 algal cells mL^{-1} with an optimal larval density of 1–2 larvae mL^{-1} (Azad et al., 2010). *D. antillarum* larvae have previously been cultured with microalgal concentrations between 5000 and 40,000 cells mL^{-1} (Eckert, 1998; Leber et al., 2009; Pilnick et al., 2021) but never as high as the 90,000 cells mL^{-1} tested here. However, previous feeding regimes added algae more frequently,

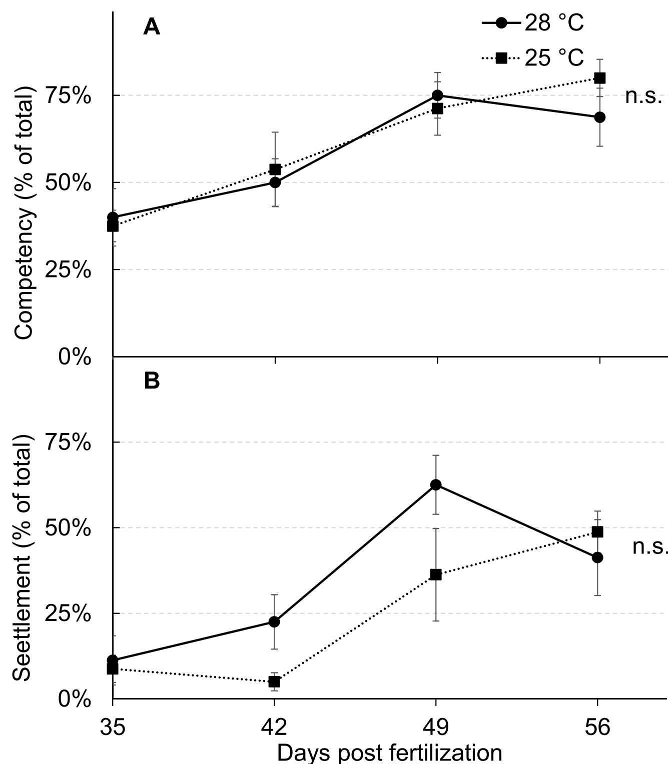

Fig. 7. *Experiment 5 (settlement):* Larvae from the temperature experiment (*Experiment 4*) cultured at 28 °C (line) and 25 °C (dotted line) were selected at random and scored for (**A**) competency, as a percentage of total, where DPF ($F = 73.55$, $df = 47$, $P \leq 0.001$) was a significant predictor and temperature ($F = 0.058$, $df = 14$, $P = 0.813$) was not and (**B**) settlement as a percentage of total, where DPF ($F = 26.43$, $df = 47$, $P \leq 0.001$) was a significant predictor and temperature ($F = 1.60$, $df = 17$, $P = 0.227$) was not. Per time point 10 larvae from each temperature treatment and bottle were scored for competency, these larvae were transferred to a petri dish with a one week old biofilm for each individual bottle and settlement was scored after 48 h, expressed as a percentage settled of the total. N.s. indicates there was no significant difference ($n = 8$).

Table 3
The total number of larvae for both 25 °C and 28 °C and total of the combined at the start of the temperature culture experiment (at 3 PDF) and the total number of settlers in the weekly petri dish experiment between 35 and 56 DPF (*Petri dish*), settlers in the bottles during culture between 34 and 56 DPF (*Bottle*) and total number of settlers at the end after 56 DPF (*End*). Between brackets is the percentage of settlers of the number of larvae at start in the described treatment and settlement method.

Treatment	Larvae at start	Number of settlers (and % of total larvae)			Total settlers
		Petri dish	*Bottle*	*End*	
25 °C	1500	79 (5.3%)	65 (4.3%)	356 (23.7%)	500 (33.3%)
28 °C	1167	110 (9.4%)	19 (1.6%)	245 (20.1%)	374 (32.0%)
Total	2667	189 (7.1%)	84 (3.5%)	601 (22.5%)	874 (32.8%)

increasing the overall feed input over time. This suggests some flexibility in potential feeding schedules for *D. antillarum* larvae, which could be important given inherent difficulties in live microalgae production. Larvae fed with 90,000 cells mL^{-1} were bigger compared to lower feeding concentrations, but with lower initial larval densities in later experiments, incrementally increasing feeding concentrations to a maximum of 60,000 cells mL^{-1} and feeding twice a week gave the best results. Leber et al. (2009) found an optimal larval density of 0.1 mL^{-1} for *D. antillarum* and our results confirm that lower larval densities result in higher survival and growth.

3. Experimental results have been successfully attained. However, if the objective is production of significant amounts of juveniles for repopulation activities, validation and scaling up of these results, is essential. Increasing and standardizing larval settlement is the next step that needs to be investigated before large scale culture is possible. Preliminary settlement tests with *D. antillarum* without any cues did not result in settlement, highlighting the importance of cues in triggering settlement and offering a simple negative control for future experimentation. Biofilm contains (bio)chemical cues that triggered larvae to settle, and our results corroborate previous studies indicating that the age and complexity of the biofilm is important (Dworjanyn and Pirozzi, 2008; Hadfield, 2011; Nelson et al., 2020). In the settlement experiment presented here, settlement was substantially lower than the amount of larvae that were scored as being competent, indicating that the one-week-old biofilm did not contain all cues needed to induce optimal settlement in all of the competent larvae. Determining the optimal age of biofilm and investigating the effect of other (natural) substances on the settlement of *D. antillarum* larvae can further improve culture output. Histamine (Sutherby et al., 2012; Swanson, 2006; Swanson et al., 2012), gamma-aminobutyric acid (Pearce and Scheibling, 1990; Rahmani and Ueharai, 2001) and potassium chloride (Carpizo-Ituarte et al., 2002) are substances that are known to induce settlement in other echinoids and should be tested with *D. antillarum*. Other natural cues, including various macroalgae, have been shown to induce higher settlement rates in some urchin species than biofilms and/or histamine (Mos et al., 2011; Swanson et al., 2012) and also warrant examination in *D. antillarum*. Mos et al. (2011) also indicate that some cues can induce settlement before larvae are fully competent, which stresses the importance to evaluate post-settlement survival in future studies.
4. With the shaker bottle method hundreds of juveniles were produced in a period of under two months. The method can be scaled up by increasing the number of shaker tables and potentially the size of individual culture vessels. For restocking purposes, culturing thousands or even millions of juveniles will be needed to have an observable impact on reef herbivory. For example, in Puerto Rico Williams (2022) found that stocked *D. antillarum* at 2 individuals m^{-2} resulted in a significant decrease in macroalgal cover while Olmeda-Saldaña et al. (2021) showed no effect at 1 individual m^{-2}. Given a required density in this range and the vast expanse of Caribbean reef that would be viable for *D. antillarum* restocking, achieving culture at scale is clearly an imperative. In addition to scaling up the shaker bottle method, culture in larger vessels such as the specialized 40-L tanks described by Pilnick et al. (2021) and the 300-L cylindro-conical tanks mentioned by Mos et al. (2020) to rear a Pacific diadematid (*Centrostephanus rodgersii*), could help increase overall efficiency and production. Rearing methods with high growth and survival need to be established or adapted from existing echinoid culture facilities in order to facilitate this large scale production.
5. The method presented here is reproducible and consistent as long as vital culture conditions are met: a constant temperature between 25 °C and 28 °C, a stable supply of *R. lens* or *R. salina* and a constant gentile shaking motion to keep the larvae suspended in 1 L bottles. The method has a high larval survival and settlement rate and our study presents the first consistent method for the culture of *D. antillarum*, resulting in hundreds of juveniles from just two successful culture runs. The results presented here suggest a good level of transferability for this method; brood stocks were of different geographic origins, two different species of *Rhodomonas* were fed, and personnel were completely different between successful cultures

in the Netherlands and USA. We were able to settle a third of the cultured larvae from the best performing cohort. The reproducibility of this work needs further confirmation, as two of the four cultures were not successful. Reproducibility is extremely important for scaling up to reef-scale relevance in terms of *D. antillarum* stocking. Nevertheless, the shaker bottle method is easy to reproduce, requires little specialized equipment and can be used to further optimize the culture of *D. antillarum* and to culture juveniles year round for restocking purposes. Hassan et al. (2022) reports an initial investigation into juvenile grow out of hatchery propagated urchins. The next steps will be scaling up larviculture research to develop a novel pilot technology, technically and economically viable large scale *D. antillarum* rearing in a hatchery setting. A feasible tool for aquaculture mass production of million juveniles could enhance restocking activities that may help address declined wild populations and some lost ecosystem function of *D. antillarum* in the Caribbean.

CRediT authorship contribution statement

Tom Wijers: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Alwin Hylkema:** Conceptualization, Formal analysis, Investigation, Supervision, Funding acquisition. **Aaron R. Pilnick:** Conceptualization, Formal analysis, Investigation, Data curation. **Albertinka J. Murk:** Conceptualization, Writing – review & editing, Supervision. **Joshua T. Patterson:** Conceptualization, Writing – review & editing, Supervision.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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