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Effect of Microalgal Diets on Sunray Venus Clam (*Macrocallista nimbosa*) Production and Fatty Acid Profile

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Abstract: The sunray venus (sunray) clam, *Macrocallista nimbosa*, is an alternative clam species reared in hard clam hatcheries in Florida. Current feeding practices follow those used for hard clam culture. This study aimed to identify whether a hard clam bi-algal *Tisochrysis lutea* and *Chaetoceros neogracile* diet was an optimal diet for post-set sunray clams or whether other microalgal dietary combinations could improve production. Six dietary bi-, tri-, or tetra-algal combinations consisting of four microalgae species (*Tisochrysis lutea*, *Diacronema lutheri*, *Chaetoceros neogracile*, and *Thalassiosira weissflogii*) were fed for 6 weeks; the growth, survival, and fatty acid profiles of post-set clams were evaluated. Clams fed equal proportions of *T. lutea*, *D. lutheri*, *C. neogracile*, and *T. weissflogii* had higher growth, while those fed equal proportions of *T. lutea* and *C. neogracile* had higher survival. The poorest-performing diet consisted solely of diatoms. A contrasting polyunsaturated fatty acid (PUFA) profile was found in post-set clams fed flagellate- or diatom-only diets. Clams fed the bi-algal flagellate diet had a higher percentage of docosahexaenoic acid (DHA) but a lower percentage of (n-6) PUFA, whereas those fed the bi-algal diatom diet had a higher percentage of arachidonic acid (ARA) and eicosapentaenoic acid (EPA) but a lower percentage of DHA. The percentages were similar and neither very high nor very low in clams fed the remaining dietary treatments. The results of this study show that sunray venus post-set clams can be successfully produced when fed a typical hard clam bi-algal flagellate and diatom diet, but they indicate that growth may be accelerated by the addition of other microalgae species.

Keywords: sunray venus clam; microalgae; fatty acids

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1. Introduction

Shellfish aquaculture in Florida is an established and economically viable industry driven mainly by the hard clam market. The northern quahog (*Mercenaria mercenaria*) represents 92% of total shellfish sales in Florida [1]. Industry diversification to increase economic stability has been noted, and feasibility studies have been conducted with alternative bivalve species, such as the angel wing clam (*Cyrtopleura costata*), bay scallop (*Argopecten irradians*), the blood ark (*Anadara ovalis*), the ponderous ark (*Noetia ponderosa*), and the sunray venus clam (*Macrocallista nimbosa*) [2–7].

The sunray venus (sunray) clam, *Macrocallista nimbosa* (Lightfoot, 1786), an attractive venerid clam that gets its name from the markings on its shell, is native to Atlantic waters ranging from North Carolina to Florida and the Gulf of Mexico [8]. Research conducted with this clam determined that it could be cultured using techniques similar to those used for hard clam culture [6,9]; however, little research has been conducted toward optimizing sunray culture.

Clam hatcheries are dependent on cultured microalgae as a food source for larval and post-set production [10–13]. Success at these early life stages is dependent on high-quality microalgae. Nutritional profile is the primary criteria for algal species selection although algal cell size, cell mobility and form, toxicity, and ease of culture may also be considered when choosing a microalgae species [10,11,14]. During early stages (first 7–10 days), larval clams are typically fed small flagellates, such as *Isochrysis* sp. Diatoms, such as *Chaetoceros* sp. are introduced during settlement, and a mixed diet of flagellates and diatoms is fed post settlement [13]. Due to the ease of culture, availability, and culture requirements, only a few species of microalgae are widely used in aquaculture. Species cultured in bivalve hatcheries include flagellates, such as *Tisochrysis lutea*, previously known as *Isochrysis* aff. *galbana* or T. Iso [15], *Diacronema lutheri*, *Tetraselmis suecica*, and diatoms, such as *C. neogracile*, *C. muelleri*, and *Thalassiosira* sp. [16], with most hatcheries culturing only one or two predominant flagellate and diatom species.

The nutritional value of algal species Is determined by biochemical composition, i.e., lipids, carbohydrates, and proteins [14,17]. Lipids provide the principal source of energy for bivalves, followed by protein; little energy comes from carbohydrates [18–22]. Growth and survival of bivalve larvae are particularly impacted by the fatty acid (FA) profile of the diet. Species rich in n-3 polyunsaturated fatty acids (PUFA), such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), result in increased growth and survival of early-stage bivalves [23,24]. The n-6 PUFAs linoleic acid (LA, 18:2n-6) and arachidonic acid (ARA, 20:4n-6) are precursors of EPA and DHA synthesis in bivalves, and their ratios influence the quality of bivalve microalgal diets [25]. Bivalve shellfish must obtain these fAs from their diet as they have limited ability to synthesize essential fatty acids required for growth [26,27]. Mixed microalgae diets offer complementary nutritional benefits needed for bivalve physiological processes over that of mono-species diets and have been shown to enhance production [11,28]. The combination of flagellates and diatoms in the diet nutritionally is complementary, resulting in potentially enhanced larval development and growth [28]. Although FA profiles of algae species have been reported in the literature, they have been shown to vary on the basis of culture conditions, such as light intensity, temperature, and culture medium [29–31].

The cost of algal production in bivalve hatcheries is high, with the production of live algae comprising 30% of the operating costs [32–34]. As the Florida shellfish industry continues to grow and diversify, it is essential to determine whether the nutritional needs of these alternative bivalve species are similar to those of the hard clam. A better understanding of the nutritional profile of algal species cultured and their effects on clam production and nutritional profile may assist hatcheries in determining feed management strategies that result in increased seed production and increase their economic bottom line. To our knowledge, no research has been conducted to establish an optimal diet for the sunray clam. The objective of this study was to determine an effective live microalgal diet to maximize growth and survival for post-set sunray clams using bi-, tri-, and tetra-algal dietary combinations of *Tisochrysis lutea*, *Diacronema lutheri*, *Chaetoceros neogracile*, and *Thalassosioria weissflogii*. These species were chosen on the basis of their use in aquaculture operations, ease of culture, and the known differing FA profiles of diatoms and flagellates.

2. Methods

2.1. Spawning and Larval Production

This study was conducted at the Florida Atlantic University Harbor Branch Oceanographic Institute (FAU-HBOI) aquaculture facility. Post-set clams used in this study were obtained from sunray broodstock conditioned for 8 weeks at 21 °C and fed a mixed (50:50) diet of flagellates (*T. lutea*) and diatoms (*C. neogracile*) to satiation prior to spawning. Fertilized eggs were stocked in 700 L conical bottom larval rearing tanks filled with UV-treated 1 µm filtered seawater and maintained at 28 °C with light aeration. Larvae were fed *T. lutea* 25,000 algal cells/mL/day starting on day 1, with a gradual increase to 50,000 cells by day 7. Upon reaching the pediveliger stage (7 days) clams were transferred to floating

setting containers (downwellers) (55 cm wide by 76 cm tall, 120 µm screen) immersed in shallow tanks. Clams were maintained on the downwellers for 2 weeks and fed a mixed diet of *T. lutea* and *C. neogracile*. Clams were then transferred to the experimental system.

2.2. Experimental System

The experimental setup consisted of 60 L round tanks each containing three mini-downwellers with airlifts (three replicate tanks per treatment). Thin-walled PVC pipes, 10 cm in diameter, were cut to 8 cm in length and placed horizontally at the bottom of the tank to support a plastic grid (5 × 5 cm wide cells) to act as a tank bottom. Downwellers were placed on top of the grid, providing a 10 cm space between the bottom of the downweller and the bottom of the tank. Downwellers were created using 15 cm wide thin-walled schedule 20 sewer-grade PVC pipes cut to 23 cm in length with a 150 µm screen secured to the bottom with a 15 cm thin-walled slip coupler (Figure 1). Each downweller was outfitted with an airlift made from 15 mm thin-walled PVC, a small air stone, and air tubing connected to an airline manifold. Airlifts were cut to 30 cm in length extended below the plastic grid bottom to ensure proper mixing and eliminate the possibility of dead zones with regard to water movement and algae settlement.

Following initial measurement, 0.5 mL of post-set clams (1074 ± 39 individuals) were stocked into each downweller (n = 36). Water quality was measured to maintain optimal growing parameters for post-set clam culture (pH 7.5–8.5, DO > 5 mg/L:, temperature 27–29 °C, salinity 26–30 ppt, and total ammonia nitrogen < 0.25). Tanks were cleaned on alternate days, and 100% of the water was changed.

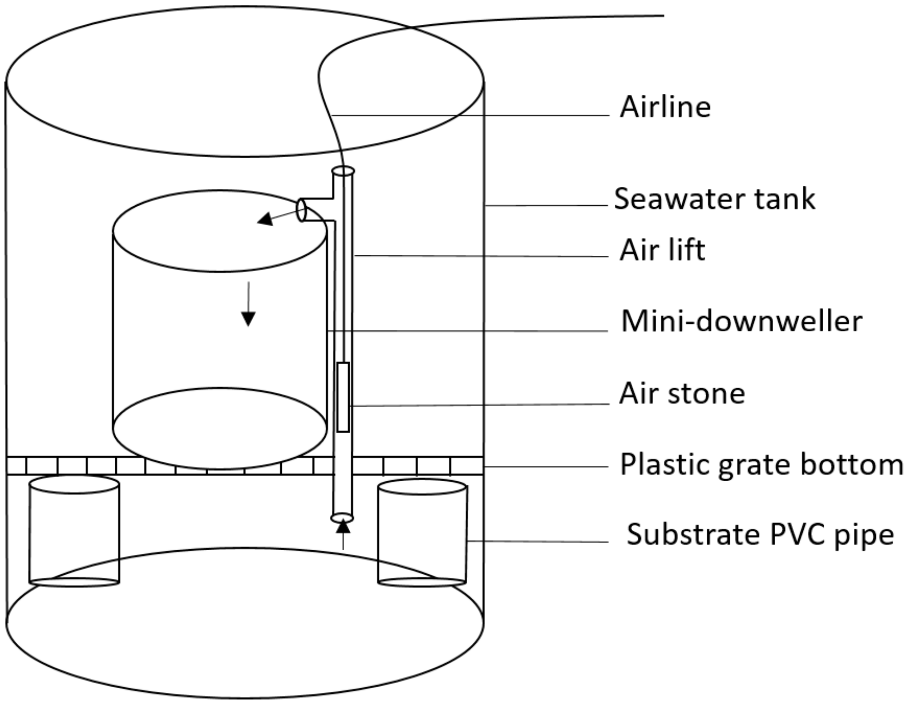

Figure 1. Diagram of experimental post-set rearing system used in both feed studies. Arrows indicate the direction of seawater flow through airlift and downweller (figure reproduced from [35]).

2.3. Algae Culture and Standardization

The four species of microalgae (*T. lutea* (TL), *Diacronema lutheri* (DL), *C. neogracile* (CN), and *Thalassiosira weissflogii* (TW)) used in this study were produced in batch culture (flasks, 18 L carboys, 200 L fiberglass suntubes) in the indoor microalgae culture facility at FAU-HBOI using UV-treated, 1 µm filtered salt well water. Temperature was maintained at 20 °C, along with salinity at 27–30 ppt and pH at 8.0–8.2, through CO_2 injection. Microalgae were cultured with f/2 nutrient media [36], with metasilicates added to diatom cultures.

Size variations of microalgae species were standardized according to dry weight [37]. A total of 100 mL of each species was filtered using pre-weighted 1.2 µm GF filters in triplicate and washed with 50 mL of 0.5 M ammonium formate to remove salt residue [38]. The filters were then dried in an oven at 70 °C for 18 h. Microalgae cell concentrations were determined using a hemocytometer, and the dry weight of one algal cell was calculated on the basis of algae concentration and total dry weight of the filtered algae. The algae feed ration was calculated as a function of the clam biomass and standardized on the basis of the dry weight of each species according to the following formula: $F = (S \times R)/7$, where F is the algae dry weight per day (mg), R is the ration as dry weight of algae per mg clam per week, and S is the weight of algae at the start of each week. As is standard practice when calculating a ration for bivalve diets incorporating two or more species, the representation of each species in the ration was calculated on a cell volume equivalency basis [26]. The dry weights (picogram/cell) of microalgae used were as follows: *T. lutea*, 13.1 pg; *D. lutheri*, 13.4 pg; *C. neogracile*, 24.6 pg; *T. weissflogii*, 75 pg.

2.4. Experimental Design

The study consisted of feeding six dietary treatments (bi-, tri-, and tetra-algal species combinations), with three replicates per treatment for 43 days (Table 1). Juveniles were starved for 24 h prior to the start of the experiment. Each tank was fed at a rate of 1,000,000 algal cells of *T. lutea* per clam (or equivalent cell number based on dry weight of other microalgae species), and feed rates were increased by 20% weekly.

Table 1. Microalgae dietary treatments fed to post-set sunray venus clams (*Macrocallista nimbosa*).

Treatment	Live Microalgae Diet (% Inclusion)
T1 (TL + DL)	*Tisochrysis lutea* (50%) + *Diacronema lutheri* (50%)
T2 (CN + TW)	*Chaetoceros neogracile* (50%) + *Thalassiosira weissflogii* (50%)
T3 (TL + CN)	*T. lutea* (50%) + *C. neogracile* (50%)
T4 (TL + DL + CN + TW)	*T. lutea* (25%) + *D. lutheri* (25%) + *C. neogracile* (25%) + *T. weissflogii* (25%)
T5 (TL + DL + CN)	*T. lutea* (33.3%) + *D. lutheri* (33.3%) + *C. neogracile* (33.3%)
T6 (TL + CN + TW)	*T. lutea* (33.3%) + *C. neogracile* (33.3%) + *T. weissflogii* (33.3%)

2.5. Clam Growth and Survival

Shell lengths of post-set clams were measured prior to stocking using an Olympus S2X7 microscope (Olympus Corporation, Tokyo, Japan) outfitted with an Olympus DP71 camera and Olympus cellSens Standard imaging software (Olympus cellSens Standard, version 1.11). The initial average shell length was 0.913 ± 0.242 mm. At 2, 4, and 6 weeks, shell length measurements were taken from 20 randomly selected clams per replicate (three replicates per treatment) using a vernier caliper. At experimental termination (week 6), all live clams were counted to calculate the total percentage survival (final number of clams/Initial number of clams $\times$ 100).

2.6. Clam and Microalgae Fatty Acid Profile

At experimental termination, clams and microalgae were freeze-dried for 48 h and ground to a fine powder with a mortar and pestle. Microalgae were first filtered on a 2 µm GF filter and collected filtrate placed into 15 mL falcon tubes. Processed clams (50 to 70 mg, triplicate samples) and microalgae (20 mg, duplicate samples) were sent to Microbial ID Inc. (Newark, DE) for fatty acid analysis. The relative fatty acid content was determined using fatty acid methyl ester (FAME) analysis (MIDI technical note #101, Sasser). FAMEs were separated by gas chromatography (GC) using a hydrogen carrier gas and a nitrogen makeup gas. The electrical signal from the GC detector was compared to a stored database of the Sherlock pattern recognition software. Individual fatty acids from microalgae and post-set sunray clams are presented as the percentage of total fatty acids. The FA profile of microalgae is listed below (Table 2).

Table 2. Fatty acid profile (as a percentage of total fatty acid) of microalgae species used to feed post-set sunray venus clams (*Macrocallista nimbosa*). ND: not detected. Data are presented as the means ± SD (n = 2).

Fatty Acid	*Tisochrysis lutea*	*Diacronema lutheri*	*Chaetoceros neogracile*	*Thalassiosira weissflogii*
12:0	0.1 ± 0.0	0.1 ± 0.0	0.14 ± 0.0	ND
14:0	25.4 ± 0.2	12.3 ± 0.1	20.9 ± 0.7	17.4 ± 0.2
15:0	0.7 ± 0.0	1.0 ± 0.1	1.0 ± 0.1	1.4 ± 0.1
16:0	10.6 ± 1.3	21.3 ± 1.0	18.3 ± 0.4	13.4 ± 0.1
17:0	2.3 ± 0.0	3.3 ± 0.5	3.4 ± 1.6	4.5 ± 0.3
20:0	0.2 ± 0.0	1.8 ± 0.6	0.9 ± 0.0	2.8 ± 0.1
Total SFA	39.2 ± 1.1	39.7 ± 0.1	44.6 ± 0.4	39.6 ± 0.2
14:1n-5	0.4 ± 0.0	0.1 ± 0.0	0.4 ± 0.0	0.3 ± 0.0
14:1n-7	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	ND
15:1n-6	ND	ND	0.2 ± 0.0	0.3 ± 0.0
16:1n-5	0.1 ± 0.0	0.2 ± 0.0	0.6 ± 0.1	0.9 ± 0.0
16:1n-6	1.0 ± 0.0	0.7 ± 0.0	3.2 ± 0.1	7.4 ± 0.1
16:1n-7	6.3 ± 0.1	20.9 ± 0.0	37.7 ± 0.3	21.9 ± 0.9
17:1n-8	0.5 ± 0.0	ND	0.1 ± 0.0	0.2 ± 0.1
18:1n-7	1.4 ± 0.0	8.2 ± 0.5	2.4 ± 0.0	3.4 ± 0.3
18:1n-8	9.7 ± 0.3	ND	ND	ND
18:1n-9	12.6 ± 1.1	ND	0.8 ± 0.0	0.5 ± 0.0
Total MUFA	32.2 ± 1.0	30.1 ± 0.4	45.6 ± 0.5	34.9 ± 1.2
18:2n-6	4.7 ± 0.1	0.5 ± 0.0	0.9 ± 0.0	1.6 ± 0.0
18:3n-6	0.3 ± 0.0	0.2 ± 0.0	0.9 ± 0.1	ND
20:3n-6	ND	0.04 ± 0.0	0.1 ± 0.1	0.2 ± 0.0
20:4n-6 ARA	0.2 ± 0.1	0.2 ± 0.0	0.7 ± 0.00	0.9 ± 0.0
22:5n-6	0.8 ± 0.0	0.3 ± 0.0	ND	ND
Total PUFA (n-6)	6.1 ± 0.0	1.2 ± 0.0	2.6 ± 0.2	2.8 ± 0.1
18:4n-3	13.4 ± 0.8	5.2 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
20:5n-3 EPA	0.7 ± 0.0	17.8 ± 0.2	5.5 ± 0.2	13.9 ± 0.0
22:6n-3 DHA	6.8 ± 0.2	5.1 ± 0.1	0.2 ± 0.0	2.5 ± 0.0
Total PUFA (n-3)	21.0 ± 1.0	28.2 ± 0.3	6.1 ± 0.2	16.9 ± 0.1
Total Combined PUFA (n-3, n-6)	26.3 ± 1.0	29.4 ± 0.4	8.7 ± 0.4	19.7 ± 0.1

2.7. Statistical Analysis

All data were tested for assumptions of normality and homogeneity of variance using Shapiro–Wilk and Levene's tests. Survival data and fatty acid content, which are expressed as proportions, were arcsine square-root transformed prior to analysis. One-way analysis of variance (ANOVA) was used to evaluate differences among the dietary treatments. Tukey's honestly significant difference test and a test of simple effects were used when significant differences were found among response variables. Differences among means were considered significant at $p < 0.05$. All data were analyzed using SPSS v. 27 (IBM, Armonk, NY, USA).

3. Results

3.1. Growth

Significant differences ($p < 0.05$) in growth were seen among treatments at 2, 4, and 6 weeks (Table 3). The final size of post-set clams fed various microalgae diets was significantly different ($p = 0.002$). The largest clams were those fed T4 (TL + DL + CN + TW), while the smallest clams were those fed T2 (CN + TW), i.e., the diatom-only diet. Tetra- and tri-algal species diets outperformed bi-algal species diets regardless of whether they were diatom- or flagellate-dominant. Although the average size was the smallest in clams fed T2, final size variations were also higher in T2 than in other treatments.

Table 3. Size (mm, mean length ± SD) of post-set sunray venus clams (*Macrocallista nimbosa*) fed live microalgae diets. Different letters in the same column indicate significant differences among the treatments (one-way ANOVA, $\alpha = 0.05$, a > b > c). DPF: days post fertilization.

Diet Treatment	Duration of Feeding		
	2 Weeks (Age: 44 DPF)	**4 Weeks (Age: 58 DPF)**	**6 Weeks (Age: 72 DPF)**
T1(TL + DL)	2.57 ± 0.68^{a}	3.53 ± 0.75^{a}	3.8 ± 0.91^{b}
T2 (CN + TW)	2.12 ± 0.67^{c}	3.22 ± 0.86^{ab}	3.38 ± 1.46^{bc}
T3 (TL + CN)	2.36 ± 0.64^{ab}	2.84 ± 0.50^{bc}	3.59 ± 0.72^{bc}
T4 (TL + DL + CN + TW)	2.30 ± 0.68^{abc}	3.41 ± 0.70^{ab}	4.37 ± 0.97^{a}
T5 (TL + DL + CN)	2.44 ± 0.74^{a}	3.29 ± 0.73^{ab}	4.0 ± 0.95^{ab}
T6 (TL + CN + TW)	2.28 ± 0.85^{bc}	2.98 ± 0.87^{b}	3.9 ± 0.79^{b}

There was a significant difference in daily growth rate among treatment groups ($p = 0.006$) (Figure 2). Sunray venus clams fed T4 (TL + DL + CN + TW) had the highest average daily growth (80.4 µm ± 5.8 µm), while the lowest daily growth rate was seen in T2 (CN + TW) (57.4 µm ± 10.0 µm). There was no significant difference ($p < 0.05$) among the three bi-algal dietary treatments or between the two tri-algal dietary treatments.

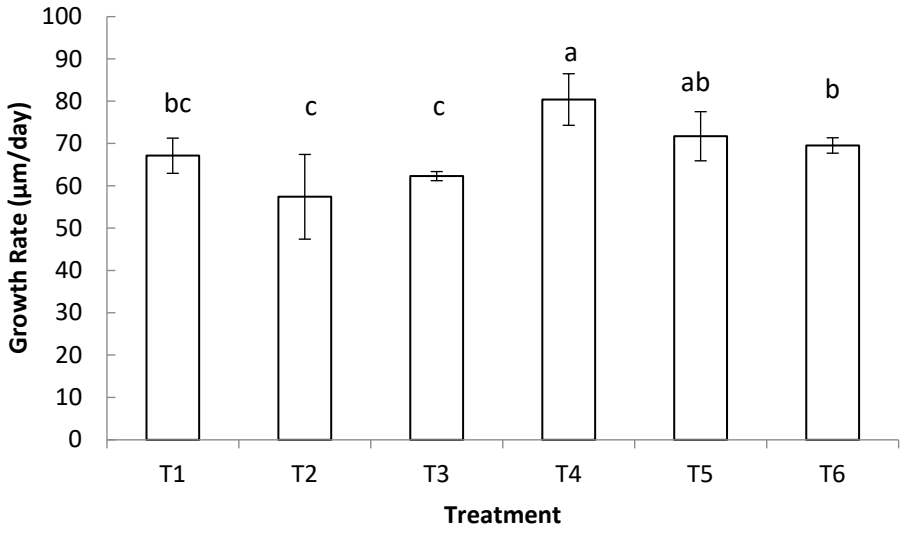

Figure 2. Daily growth rate of post-set sunray venus clams (*Macrocallista nimbosa*) fed live microalgae diets. Different letters indicate significant differences among the treatments (one-way ANOVA, $\alpha = 0.05$, a > b > c). Each bar represents the mean ± SD of three replicates.

3.2. Survival

The survival of post-set sunray venus clams was significantly affected by dietary treatment ($p = 0.0008$) (Figure 3). Clams fed T3 (TL + CN) had the highest survival (88.1% ± 6.3%), whereas the lowest survival (61.9% ± 6.7%) was seen in clams fed T5 (TL + DL + CN). There was no statistical difference in the survival of post-set clams fed T2 and T5 or T1, T4, and T6.

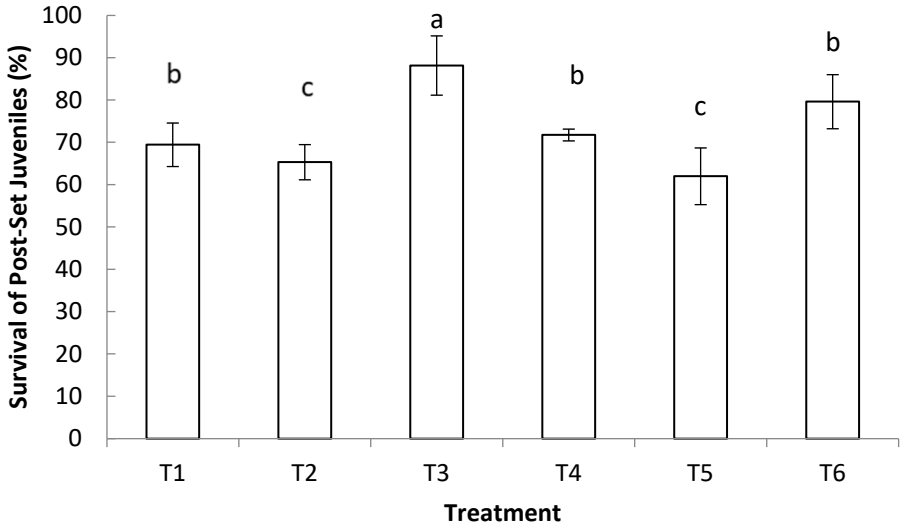

Figure 3. Survival of post-set sunray venus clams (*Macrocallista nimbosa*) fed various live microalgal species combinations. Different letters indicate significant differences among the treatments (one-way ANOVA, $\alpha = 0.05$, a > b > c). Each bar represents the mean ± SD of three replicates.

3.3. Clam Fatty Acid Profile

The FA profiles of post-set sunrays fed the various dietary treatments are presented in Table 4. Significant differences were seen in percentages of saturated fatty acids (SFAs) ($p = 0.019$), monounsaturated fatty acids (MUFAs) ($p < 0.0001$), and polyunsaturated fatty acids (PUFAs), n-3 PUFAs ($p < 0.0001$) and n-6 PUFAs ($p < 0.0001$). Regardless of diet, sunrays had higher total PUFAs or SFAs compared to MUFAs. PUFAs were the most abundant FA group in clams fed diets T2 and T4, SFAs were the most abundant FA group in clams fed T1, and proportions were similar in clams fed T3 and T5. The dominant FA in all sunrays was 16:0, followed by either 20:5n-3 eicosapentaenoic acid (EPA) (T2) or 22:6n-3 docosahexaenoic acid (DHA) (T1, T3–T6). Other FAs found in high percentages in sunray clams included 17:0, 16:1n-7, 18:1n-9, and 20:2n-6. Clams fed the flagellate-only T1 (TL + DL) diet had the highest DHA and lowest arachidonic acid (ARA), (20:4n-6) percentages, while clams fed the diatom-only T2 (CN + TW) diet had the lowest DHA and highest ARA percentages. Clams fed diets T3 and T5 had similar DHA, EPA, and ARA percentages; likewise, clams fed diets T4 and T6 had similar percentages of the same three highly unsaturated fatty acids (HUFAs).

Table 4. Proportions of fatty acids of post-set sunray venus clams (*Macrocallista nimbosa*) fed different diet combinations of live microalgae.

	Treatment Group					
Fatty Acid	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
14:0	5.9 ± 0.2^{a}	2.0 ± 0.1^{d}	4.1 ± 0.2^{b}	3.0 ± 0.1^{c}	3.8 ± 0.2^{b}	3.3 ± 0.1^{c}
15:0	0.4 ± 0.01^{d}	0.6 ± 0.03^{ab}	0.5 ± 0.01^{cd}	0.5 ± 0.03^{bc}	0.4 ± 0.02^{d}	0.6 ± 0.01^{a}
16:0	25.2 ± 0.2^{ab}	23.6 ± 0.7^{c}	24.7 ± 0.5^{abc}	25.7 ± 0.2^{a}	25.9 ± 0.7^{a}	24.2 ± 0.3^{bc}
17:0	6.3 ± 0.3	7.0 ± 3.0	6.8 ± 0.5	8.1 ± 0.4	7.4 ± 0.1	9.0 ± 0.1
20:0	0.9 ± 0.1^{b}	2.3 ± 0.1^{a}	1.1 ± 0.03^{b}	1.2 ± 0.4^{b}	1.4 ± 0.4^{b}	1.6 ± 0.1^{b}
21:0	0.7 ± 0.2^{a}	0.1 ± 0.1^{b}	0.8 ± 0.2^{a}	0.7 ± 0.05^{a}	0.8 ± 0.1^{a}	0.6 ± 0.1^{a}
22:0	0.1 ± 0.1^{c}	0.8 ± 0.3^{a}	0.3 ± 0.02^{ab}	0.4 ± 0.02^{ab}	0.3 ± 0.04^{ab}	0.4 ± 0.04^{b}
Total SFA	39.5 ± 0.3^{ab}	36.4 ± 2.6^{b}	38.2 ± 0.8^{ab}	39.6 ± 0.1^{a}	40.0 ± 0.5^{a}	39.6 ± 0.5^{a}
16:1n-6	0.2 ± 0.04	0.2 ± 0.2	0.3 ± 0.02	0.2 ± 0.05	0.2 ± 0.04	0.3 ± 0.02

Table 4. *Cont.*

	Treatment Group					
Fatty Acid	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
16:1n-7	3.8 ± 0.3^{d}	9.3 ± 0.7^{a}	4.8 ± 0.2^{bc}	4.8 ± 0.1^{bc}	4.4 ± 0.4^{cd}	5.5 ± 0.1^{b}
17:1n-7	0.3 ± 0.02^{a}	0.0 ± 0.0^{c}	0.04 ± 0.07^{c}	0.0 ± 0.0^{c}	0.2 ± 0.02^{b}	0.0 ± 0.0^{c}
18:1n-6	0.1 ± 0.1^{c}	0.4 ± 0.1^{a}	0.3 ± 0.03^{abc}	0.3 ± 0.03^{abc}	0.2 ± 0.1^{bc}	0.3 ± 0.04^{ab}
18:1n-7	5.7 ± 0.4^{a}	4.0 ± 0.3^{bc}	3.7 ± 0.1^{c}	4.4 ± 0.02^{b}	4.5 ± 0.3^{b}	3.6 ± 0.1^{c}
18:1n-9	9.9 ± 0.5^{a}	3.5 ± 0.2^{d}	9.1 ± 0.2^{b}	7.4 ± 0.2^{c}	8.6 ± 0.2^{b}	7.1 ± 0.1^{c}
20:1n-6	1.1 ± 0.1^{e}	3.0 ± 0.04^{a}	1.3 ± 0.1^{d}	2.1 ± 0.06^{b}	1.6 ± 0.02^{c}	1.9 ± 0.1^{b}
20:1n-9	3.4 ± 0.4^{a}	0.0 ± 0.0^{b}	3.6 ± 0.1^{a}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.2 ± 0.02^{b}
Total MUFA	24.5 ± 1.9^{a}	20.4 ± 1.5^{b}	23.1 ± 0.9^{a}	19.2 ± 0.4^{ab}	19.5 ± 1.2^{bc}	18.9 ± 0.4^{c}
18:2n-6	1.9 ± 0.4^{a}	1.1 ± 0.1^{bc}	1.5 ± 0.04^{ab}	1.0 ± 0.06^{c}	1.1 ± 0.1^{c}	1.0 ± 0.02^{c}
18:3n-6	0.03 ± 0.1^{e}	0.5 ± 0.1^{a}	0.2 ± 0.02^{cd}	0.2 ± 0.02^{bc}	0.1 ± 0.1^{de}	0.3 ± 0.02^{b}
20:2n-6	5.9 ± 0.2^{d}	7.2 ± 0.2^{c}	7.3 ± 0.2^{c}	10.0 ± 0.06^{a}	10.4 ± 0.6^{a}	9.1 ± 0.3^{b}
20:3n-6	0.5 ± 0.0^{d}	1.7 ± 0.1^{a}	0.6 ± 0.01^{d}	0.8 ± 0.02^{c}	0.5 ± 0.1^{d}	0.9 ± 0.1^{b}
20:4n-6 ARA	2.8 ± 0.2^{d}	6.0 ± 0.1^{a}	4.3 ± 0.2^{bc}	4.4 ± 0.02^{b}	4.0 ± 0.1^{c}	4.4 ± 0.1^{bc}
22:4n-6	0.4 ± 0.1^{d}	2.3 ± 0.1^{a}	1.0 ± 0.1^{b}	1.0 ± 0.06^{b}	0.7 ± 0.1^{c}	1.0 ± 0.03^{b}
22:5n-6	3.9 ± 0.1^{a}	0.5 ± 0.02^{e}	3.4 ± 0.2^{b}	3.0 ± 0.1^{c}	3.7 ± 0.2^{ab}	2.2 ± 0.1^{d}
24:4n-6	0.3 ± 0.04	0.1 ± 0.1	0.2 ± 0.2	0.3 ± 0.04	0.3 ± 0.03	0.1 ± 0.1
Total PUFA (n-6)	15.7 ± 1.1^{c}	19.4 ± 1.0^{a}	18.5 ± 0.9^{b}	20.7 ± 0.7^{a}	20.8 ± 1.3^{a}	19.0 ± 0.7^{a}
15:4n-3	0.6 ± 0.02^{d}	2.5 ± 0.1^{a}	0.8 ± 0.02^{c}	1.4 ± 0.1^{b}	1.0 ± 0.06^{c}	1.5 ± 0.02^{b}
18:4n-3	2.1 ± 0.1^{a}	0.0 ± 0.0^{d}	1.8 ± 0.1^{ab}	1.4 ± 0.3^{bc}	1.5 ± 0.2^{bc}	1.3 ± 0.03^{c}
20:5n-3 EPA	3.6 ± 0.04^{c}	10.0 ± 0.2^{a}	4.8 ± 0.2^{c}	6.0 ± 0.01^{b}	4.8 ± 0.2^{c}	6.4 ± 0.1^{b}
22:1n-3	0.3 ± 0.02^{a}	0.1 ± 0.1^{b}	0.3 ± 0.02^{a}	0.3 ± 0.02^{a}	0.4 ± 0.03^{a}	0.3 ± 0.01^{a}
22:5n-3	0.4 ± 0.1^{c}	3.4 ± 0.1^{a}	0.9 ± 0.2^{b}	0.9 ± 0.1^{b}	0.7 ± 0.2^{b}	0.9 ± 0.1^{b}
22:6n-3 DHA	12.0 ± 0.3^{a}	6.3 ± 0.1^{d}	10.7 ± 0.6^{b}	10.3 ± 0.1^{bc}	10.8 ± 0.6^{b}	9.4 ± 0.04^{c}
Total PUFA (n-3)	19.1 ± 0.5^{b}	22.2 ± 0.6^{a}	19.3 ± 1.1^{b}	20.3 ± 0.6^{b}	19.1 ± 1.1^{b}	19.7 ± 0.2^{b}
Total Combined PUFA (n-3, n-6)	34.8 ± 0.3^{c}	41.6 ± 0.8^{a}	37.8 ± 1.4^{b}	41.0 ± 0.2^{a}	39.9 ± 0.4^{b}	38.7 ± 0.3^{b}
EPA/ARA	1.3 ± 0.1^{cd}	1.7 ± 0.02^{a}	1.1 ± 0.01^{e}	1.4 ± 0.01^{bc}	1.2 ± 0.04^{de}	1.5 ± 0.05^{b}
EPA/DHA	0.3 ± 0.01^{d}	1.6 ± 0.04^{a}	0.4 ± 0.01^{c}	0.6 ± 0.01^{b}	0.4 ± 0.04^{c}	0.3 ± 0.02^{d}

Values are given as a percentage of total fatty acids. Data are presented as means ± SD ($n = 3$). Different superscript letters indicate significant differences among groups. Means were compared using one-way analysis of variance (ANOVA), with $p < 0.05$ considered significant.

4. Discussion

The choice of microalgal combinations affects growth, survival, and fatty acid (FA) composition [39–41]. As such, bivalve nutritional research has focused on determining optimal live algal combinations [11,42,43].

This is the first known study to explore various live algae diets to enhance the production of post-set sunray clams, an alternative clam species reared in hard clam hatcheries in FL. Post-set sunray clams achieved the highest growth when fed a tetra-algal dietary combination consisting of two flagellates (*T. lutea* and *D. lutheri*) and two diatoms (*C. neogracile* and *T. weissflogii*), while survival was highest in the bi-algal *T. lutea* and *C. neogracile* treatment.

It is generally agreed that mixed microalgae bivalve diets result in increased production compared to mono-algal diets due to a more nutritionally complete dietary profile [11,39,41,44,45], yet this is not always the case. For the saltwater clam (*Meretrix meretrix*), a mono-species diet of *I. galbana* resulted in better performance than a mixed-species diet [46]. In contrast, mixed diets outperformed mono-species diets in blue mussel (*Mytilus edulis*) and northern quahog (hard clam) (*M. mercenaria*) larvae and post-set clams [35,45,47].

Bivalve hatcheries typically switch from a mono (flagellate) to a bi-algal diet (diatom and flagellate) once clams reach the post-set stage. A standard post-set hard clam diet consists of feeding equal proportions of *C. neogracile* and *T. lutea*, although proportions are not strictly adhered to and are often based on the condition of available algae. Algal species suitable for hatchery culture, such as *I. galbana*, *D. lutheri*, *Tetraselmis suecica*, *T. pseudonana*,

and *C. neogracile*, have been shown to have enhanced nutritional quality compared to other species [33,48].

The growth and survival of sunrays was improved when fed a mixed species diet consisting of equal proportions of flagellates and diatoms, likely due to the complementary nutrition benefits offered. The reported survival seen with post-set sunray clams in this 6 week study is higher than but comparable to the mean survival of 50% seen in post-set sunray clams reared to 1 mm seed at the FAU-HBOI bivalve hatchery (Laramore, unpublished data). Higher growth and survival were reported in hard clam larvae fed a bi-algal diet over a flagellate-based diet; however, the species of diatom fed was important [35]. Increased survival in blue mussel (*M. galloprovincialis*) larvae was reported when diatoms were added to a flagellate-based diet in various proportions [43]. Both the proportion and the species of diatom added affected survival but not growth.

The sunray clams in the present study showed increased growth when diatoms were included in the diet; however, growth was lowest when fed a diatom-only diet. Other studies have shown similar results. Inclusion of diatoms in bivalve diets significantly promoted growth rates in oysters (*Pinctada margaritifera* L.), scallops (*Nodipecten subnodosus*), hard clams (*M. mercenaria*), and geoduck clams (*Panopea generosa*) [24,28,35,47,49], whereas diatom-only or diatom-dominant diets have been shown to result in decreased growth in oysters (*Crassostrea gigas*) and hard clams (*M. mercenaria*) [47,50]. Lower survival was likewise reported in juvenile hard clams fed a diatom-only or a diatom-dominant diet [47].

Polyunsaturated fatty acids (PUFAs) play important roles in bivalve growth and development [31,51]. DHA has been shown to be integral in maintaining the membrane structure and functional integrity of tissues, and EPA has been shown to play a vital role in growth [52,53]; more importantly, as bivalves have limited ability to synthesize these FAs, they must be provided through their diet [25,54,55]. For this reason, nutritional research in bivalves has focused on n-3 PUFAs, although feed studies have shown that ARA (20:4 n-6) plays a key role in fish larval development, as well as immune response stress response in fish and oysters [56–60].

Differing profiles for diatoms and flagellates have been reported in the literature [61,62]. The most common algal combination fed to post-set clams consists of a mixture of *Isochrysis* sp. and *Chaetoceros* sp. as flagellates and diatoms, which complement each other nutritionally [33]. However, variability is sometimes seen within these categories, which is why a mixed-species diet is recommended. The FA profile of microalgae fed was primarily reflected in the FA profile of the clam tissue, as flagellates and diatoms also generally differ with respect to nutritional profile. Of the four microalgae fed in the present study *T. lutea* and *D. lutheri* were high in DHA. Although *C. neogracile* contained high levels of EPA compared to *T. lutea*, concentrations in *D. lutheri* and *T. weissflogii* were higher. The greater amounts of DHA present in the two flagellate species are reflected in the clam tissues, in that the most DHA was found in the flagellate-only diet, and the least amount of DHA was found in the diatom-only diet. This was not the case with EPA. Highest levels of DHA were found in the bi-algal diatom diet, with the lowest levels in the bi-algal flagellate diet. In both instances, diets containing a mix of diatoms and flagellates had moderate and similar amounts of both EPA and DHA.

The best-performing diet in terms of both growth and survival for sunrays in the present study was the tetra-algal diet containing equal proportions of diatoms and flagellates. Sunrays fed the two tri-algal diets showed a differential response in terms of production even though clam FA profiles were similar, with clams fed the tri-algal diatom-dominant diet exhibiting increased survival, and those fed the tri-algal flagellate-dominant diet exhibiting increased growth. Sunrays fed the bi-algal diatom diet performed poorly in terms of both growth and survival, and they differed greatly in terms of FA profile compared to clams in other treatments, having significantly higher concentrations of EPA, and significantly lower concentrations of DHA. Clams fed the bi-flagellate diet performed better in terms of survival than in growth, suggesting perhaps that DHA is more important for survival and EPA is more important for growth. Survival of mussel larvae was enhanced

by increasing levels of DHA, even when EPA levels were similar [43]. High levels of EPA, found in *C. neogracile*, were found to be correlated with inconsistent mussel performance, while moderate EPA levels found in the bi-algal *T. lutea* and *C. neogracile* diet resulted in high performance [50].

Arachidonic acid (ARA) plays a role in the formation of eicosanoids, and it is crucial to various physiological and pathological processes in marine bivalves [59,60]. ARA was shown to be important in maturation and immune response of the mangrove oyster (*Crassostrea corteziensis*) but had no effect on the growth [60].

The ARA concentration was low (<1%) in all four microalgae species fed. However, higher levels of ARA were seen in both species of diatoms, which is reflected in that the bi-algal diatom diet had the highest and the bi-algal flagellate diet had the lowest ARA levels. In contrast, post-set clam tissues had high (2.8–6%) ARA concentrations. This has likewise been reported in the common cockle (*Cerastoderma edule*) and the northern hard clam [23,47].

Some species (i.e., *M. mactroides*) are thought to be able to convert linolenic acid (18:2 n-6) into ARA [63]; however, whether this occurs in venerid clams is unclear. *T. lutea* contained the highest levels of linolenic acid of the microalgae fed in the present study, and, although clams fed the bi-algal flagellate diet had the highest amount of linolenic acid in their tissues, they had the lowest levels of ARA. Neither a high nor a low ARA concentration enhanced growth or survival. The sunrays fed the bi-algal diatom treatment had a significantly larger proportion of ARA, while sunrays fed the bi-algal flagellate treatment had a significantly lower proportion of ARA in their tissues.

Long-chain PUFA ratios may be more important than absolute levels [64,65]. An unbalanced EPA/ARA ratio is thought to lead to harmful effects, as EPA can inhibit the formation of eicosanoids from 20:4n-6 [66]. Development of sea urchin larvae (*Paracentrotus lividus*) was enhanced when the dietary EPA/DHA ratio was lower, and the EPA/ARA ratio was higher [65,67]. Clams fed the poorest-performing diet in this study, the bi-algal diatom diet, had high EPA/DHA and EPA/ARA ratios, while the converse was seen in all other treatments. Clam tissue FA profiles reflected microalgae species. This was evident in the EPA/DHA, as DHA concentrations were higher in both flagellate species despite there being differences in EPA concentrations. Conversely, this was not seen with the EPA/ARA ratio, likely due to the ARA microalgae concentrations being low in all species, while EPA levels were quite variable, leading to an extremely high EPA/ARA ratio of 89 in *D. lutheri*, compared to 3.5 for *T. lutea*. Only one other known study detailed the FA content of sunrays [68]. Although not a dietary study, the post-set sunrays were fed a *T. lutea* and *C. neogracile* bi-algal diet. The clam total PUFA n-3 and n-6 values were lower in that study than in the present study; however, EPA/DHA and EPA/ARA ratios were similar.

Nutritional components other than FA, such as lipids, proteins, carbohydrates, minerals, and vitamins, present in the diets might also have affected sunray post-set production. High carbohydrate levels were shown to increase growth in juvenile oysters (*Ostrea edulis*) and scallops (*Pecten maximus*) [69,70], while higher protein levels increased growth in juvenile mussels (*Mytilus trossulus*) [71].

Due to the amount of clam tissue required for the determination of proximate and mineral analysis and the size and number of clams available at experimental termination, this analysis was unfortunately not conducted. Proximate and mineral analysis of algae was not performed for a similar reason. However, the literature concerning clam nutritional profiles other than FAs indicates that clams are high in protein, low in fat, and high in minerals. Proximate analysis of assorted clam species such the striped venus clam, *Chamelea gallina*, *Anadora semillis*, the blood ark, *Anadora ovalis*, the ponderous ark, *Noetia ponderosa*, and the hard clam *Mercenaria mercenaria* reported protein levels of 7–16 g/100 g wet weight and lipid levels of 0.8–1.5 g/100 g wet weight dependent on species and season [72–74]. These studies showed clams to be a good source of minerals such as sodium, calcium, magnesium, phosphorus, iron, and zinc, which also varied with season. As seasonal variation is often associated with a change in the availability of various phytoplankton species, it is likely

that clams fed different combinations of algae would also show differences in some of these nutritional components.

Microalgae species used in the present study were previously analyzed for these nutritional components, and the values are reported in the literature. The microalga *D. lutheri* has been shown to contain high levels of protein, carbohydrates, and lipids compared to the other three species of microalgae used in this study [12]. Both diatom species were found to have similarly higher protein and similarly lower carbohydrate and lipid concentrations compared to *T lutea* [12]. The enhanced performance in growth seen with the tetra-algal species diet that included *D. lutheri* over that of the bi-species *T. lutea* and *C. neogracile* diet may have been due to increased concentrations of protein, carbohydrates, and/or lipids, as well as differing FA profiles. Survival, however, was not enhanced in diets containing *D. lutheri*, suggesting that these nutritional components may have less of an effect on survival compared to growth.

5. Conclusions

A typical hard clam hatchery diet consists of feeding a microalgae diet consisting of *T. lutea* and *C. neogracile*. Few studies have been conducted concerning an optimal hard clam diet, and none have been conducted with sunrays. A recent study aimed at determining whether other mixed algae dietary combinations would increase hard clam production over the standard hard clam hatchery diet concluded that a tetra-algal species combination of *T. lutea*, *D. lutheri*, *C. neogracile*, and *C. nana* enhanced both growth and survival. While sunrays fed a tetra-gal species combination of *T. lutea*, *D. lutheri*, *C. neogracile* and *T. weissflogii* had higher growth, those fed the standard hard clam bi-algal diet showed the highest survival. This poses a conundrum for clam hatcheries considering sunray culture as the cost of production of multiple microalgae species is likely to be higher. Individual hatchery operators will need to conduct a cost–benefit analysis to determine whether any economic benefits are to be gained by producing multiple algal species to increase growth of post-set sunrays, as no survival benefit was achieved.

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