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Ingestion and Utilization of Microalgae with Different Characteristics by Pearl Oyster *Pinctada fucata* Larvae

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ABSTRACT

Nannochloropsis oculata, *Isochrysis galbana* and *Chaetoceros muelleri* are tropical microalgae that have different shapes, particle sizes, surface structure, and biochemical compositions. As these species are easy to cultivate, can endure high temperatures, and are rich in unsaturated fatty acids, they are widely used in tropical and subtropical regions for invertebrate larval rearing. These three microalgae species were tested for ingestion, digestion, growth and survival of *Pinctada fucata* larvae, using an optical microscope, in order to identify an appropriate diet for *P. fucata*. An experiment was conducted using larvae at 2 dph (days post hatching) (shell length: 79.01 ± 2.00 µm), 8 dph (90.81± 4.61 µm), 14 dph (108.81 ± 6.58 µm), and 20 dph (146.83 ± 8.92 µm). Larvae were stocked in flasks and fed 10^5 cells/mL of each species of microalgae individually. Larvae were fed for one hour and then observed under the microscope to detect ingestion. Larvae were then sieved and placed in flasks containing filtered seawater to measure the area of microalgae in the stomach, and to analyze the digestion of the microalgae ingested every two hours. Of the three species, *N. oculata* and *I. galbana* were ingested at any stage, while *C. muelleri* were not ingested at all, and the ingestion rate of *N. oculata* was higher than that of *I. galbana*. In addition, *I. galbana* were easily digested, and *N. oculata* were difficult to digest. Subsequently, growth and survival rates were determined by feeding larvae *N. oculata* and *I. galbana*. Better growth and survival rates were observed in the larvae fed with *I. galbana*. The un-ingestible algae could not be used by the larvae, and high concentrations of algae were detrimental to larval growth and survival. These findings suggest that the microalgae used as staple foods in the larval culture practice of *P. fucata* should consist of small particle size, spherical shape, smooth surface, and should be easily digested. In addition, algae should be fed to larvae at appropriate concentrations and times.

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Authors' Contribution
ZHD conceived and designed the project, collected the samples and carried out analysis. MQC and YW cultured the microalgae. WZ cultured the parent *P. fucata*. ZHD and MQC cultured the *P. fucata* larvae. ZHD and WZ wrote the manuscript. All listed authors have read, edited, and approved the final manuscript.

Key words
Digestion, Ingestion, Larvae, Microalgae, *Pinctada fucata*

INTRODUCTION

The pearl oyster *Pinctada fucata* is one of the most important bivalves used for the cultivation of seawater pearls worldwide (Guan *et al*., 2017). Since the larval rearing of the pearl oyster *P. fucata* was first successfully carried out in 1965 in China, seawater pearling has been developing rapidly. Currently, it is one of the main industries in some coastal regions of Guangdong, Guangxi, and Hainan provinces in China (Meng *et al*., 2017; Li *et al*., 2017). Although the artificial seed-breeding of *P. fucata* can be successfully achieved, the larvae have a high mortality rate in their pelagic phase, and seed breeding is still difficult (Wang *et al*., 2012). Cultivation of floating larvae is key to the seed cultivation of bivalve mollusks, and in particular the selection of proper feeds and feeding strategies are crucial for larval cultivation (Deng *et al*., 2016). In the rearing of other marine bivalve mollusks, some studies have shown that larval growth rate and survival rate could be increased by adjusting larval cultivation density (Taylor *et al*., 1997; Doroudi and Southgate, 2000; Deng *et al*., 2013), and by adjusting the concentration, types and ratios of microalgae species (Doroudi *et al*., 1999; Doroudi and Southgate, 2000;

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Pettersen *et al*., 2010; Fernández-Pardo *et al*., 2016), based on analysis of nutritional values of microalgae species, and the nutritional needs of larvae (Renaud *et al*., 1999; Martínez-Fernández *et al*., 2006, Martínez-Fernández and Southgate, 2007; Aranda-Burgos *et al*., 2014; Liu *et al*., 2016). Microalgae species are the main type of feed used for the cultivation of bivalve mollusk larvae, and the utilization of the nutrition provided by microalgae directly affects larval growth and survival rates (Brown *et al*., 1997). The nutritional values of microalgae determined experimentally in growth trials not only reflect the nutrient compositions of microalgae, but also the ability of larvae to digest them, and the efficiency with which their nutrients are assimilated (Carboni *et al*., 2012; Kaspar *et al*., 2014; Duy *et al*., 2015). Factors, such as the particle size, degree of digestion difficulty, and biochemical compositions of microalgae species, determine whether microalgae species may be ingested and digested by larvae to meet larval nutritional needs for their growth and development. Therefore, it is important for people to select and apply an appropriate feed type and feeding strategy for larval cultivation with microalgae species (Marshall *et al*., 2010). However, not all microalgae species can be utilized by larvae during their cultivation. For example, Lora-Vilchis *et al*. (1997) fed Pacific calico scallop, *Argopecten ventricosus,* larvae with 10 microalgae species, and found that only seven species could be ingested, and only three could be digested. Similarly, Martínez-Fernández *et al*. (2004) fed winged pearl oyster, *Pteria sterna*, larvae with 10 microalgae species, and found that only five species could be ingested, and only two could be digested. To date, studies on the ingestion and utilization of microalgae species by *P. fucata* have primarily focused on the adult mollusks. Deng *et al*. (2016) found that adult *P. fucata* had a higher ingestion rate of large-sized microalgae species than of small-sized microalgae species, while over-feeding with microalgae species reduced feed digestibility, so that the feeds could not be fully utilized. However, the ingestion and utilization of different microalgae species by *P. fucata* larvae, and the optimal feeding standards and feeding strategies for larval cultivation remain unknown.

Nannochloropsis oculata, *Isochrysis galbana*, and *Chaetoceros muelleri* are three tropical microalgae species, which, due to their richness in many kinds of unsaturated fatty acids, their ability to resist high temperatures, and the fact that they are easily cultivated at large scales under natural conditions, are widely used as feeds in the cultivation of marine invertebrate larvae in tropical and subtropical regions (Martínez-Fernández *et al*., 2004; Martínez-Fernández and Southgate, 2007; Duy *et al*., 2015; Liu *et al*., 2016). The three microalgae species belong to the divisions of Chlorophyta, Chrysophyta and Bacillariophyta, respectively, and have different particle sizes, morphology and surface structures. In this experiment, these microalgae species were fed to larvae of different day-ages, and the larval ingestion and digestion efficiency of different microalgae species were monitored. Using the three microalgae species as feeds, we observed their effects on larval growth and survival. We expect that our results will provide a reference for selecting proper feeds and feeding strategies that are suitable for the successful cultivation of *P. fucata* larvae.

MATERIALS AND METHODS

Experimental materials

The parent *P. fucata* (shell length: 57.36 ± 5.69 mm, age: 20 months, $n = 30$) used in this study were cultivated by our research group in Lingshui New Village Harbor (18°25′N, 109°59′E), Hainan Province, China. All the *N. oculata*, *I. galbana* and *C. muelleri* (Fig. 1) used in this study were from our research group, and their particle size and main characteristics are listed in Table I. The experimental site was in Sanya, Hainan Province.

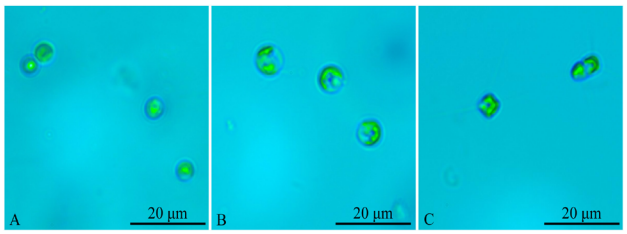

Fig. 1. Three microalgae species. (A) *N. oculata*; (B) *I. galbana*; (C) *C. muelleri.*

Microalgae cultures and larval rearing

The *N. oculata*, *I. galbana* and *C. muelleri* were cultivated by adding F/2 nutrient solution (Guillard, 1975) in glass-fiber barrels (top diameter of 90 cm, bottom diameter of

Table I. Size and characteristics of three microalgae species.

Species	Division	Size/μm	Characteristics
Nannochloropsis oculata	Chlorophyta	3.22 ± 0.45	Very small. Fibrous glycoprotein cell wall
Isochrysis galbana	Haptophyta	5.47 ± 0.67	Two flagella, round–oval shaped
Chaetoceros muelleri	Bacillariophyta	(7.82±0.87) × (5.86±0.76)	Rigid cell wall, large spines

80 cm, and height of 74 cm) under natural light conditions, with an illumination intensity of approximately 0~5000 lx inside the barrels. When fed to the larvae, the three microalgae species were in a logarithmic growth stage and their cell concentrations exceeded 10^6 cells/mL.

After the surface adherents of the parent *P. fucata* were removed, they were rinsed until clean, and then placed in a cool shaded area for a 3-h period of stimulation. When the parent *P. fucata* opened their two shells, individual *P. fucata* with full gonads were selected and transferred to a cement pool, where they were stimulated with flowing water so that they underwent natural ovulation and fertilization. When the fertilized eggs developed to D-shaped larvae 24 h after hatching, a 47 μm mesh sieve was used to collect the floating larvae in the upper layer, and the collected larvae were transferred to 10 square cement pools (each with a size of 1 m ×1 m ×1 m), where they were cultivated at a density of approximately 5 cells/mL. Of the 10 cultivation pools, four used *I. galbana* as the feed, three used *N. oculata* as the feed, and three used *C. muelleri* as the feed (the larvae should be fed with *I. galbana* before ingesting *C. muelleri*). The feeds were added three times a day, at 8:00, 16:00 and 24:00. Feeds were always supplied at a quantity of 1×10^3~5×10^3 cells/mL, and the water was refreshed every two days, so that the volume of refreshed water accounted for 30%~50% of the original water volume. During larval cultivation, water temperature was kept at 26.73 ± 0.58□, salinity was maintained at 28.35 ± 0.78, and pH was 8.05 ± 0.02.

Ingestion and digestion trials

At the day-age of 2 dph (days post hatching), 8 dph, 14 dph, and 20 dph, bolting-silk bags with pore size of 47 μm were used to sample some larvae from a cultivation pool to which *I. galbana* was added as the feed. The collected larvae were subjected to starvation for 12 h in clear seawater that was pre-filtered through filter bags with pore size of 1 μm. The starved larvae were placed (at a density of 5 cells/mL) into three 500-mL flasks, each filled with one of the three microalgae species at a density of 10^5 cells/mL. The larvae were allowed to feed in the flasks for 1 h, and then portions of the larvae in each flask were euthanized with $NaClO_2$ (content 10%). The euthanized larvae were placed under a phenix-ph 50 microscope in order to observe whether the larvae had ingested the microalgae species. Those larvae that had ingested the microalgae species were filter-rinsed with bolting silk and then placed in clear filtered seawater. The above sampling procedure was repeated every 2 h to observe the statuses of microalgae species digestion by the larvae. The microscope was connected to a camera with 8-million pixels. Software Toup View 3.7 was used to measure the area (μm^2) of microalgae species in the larval stomachs, with 30 larvae being measured each time in each group.

Measurement of larval growth rate and survival rate

At a given day-age (i.e., 2 dph, 8 dph, 14 dph, 20 dph and 25 dph), a 1 L water sampler was used to collect 1 L of water five times, once from the center, and four times from the four corners of the cultivation pools, followed by water mixing. For each pool, a volume of water was sampled from the mixed water until the sampled volume contained more than 500 larvae, and 2 drops of $NaClO_2$ were then added to make the larvae precipitate, followed by collection of the settled larvae. Settled larvae were then placed in a 1 mL plankton counting chamber and counted under the microscope, so that the larval survival rate could be calculated. The larval shell length was also measured (n = 30).

Image processing and date analysis

The experimental data are expressed as mean ± standard deviation ($\bar{x}$± SD), and one-way ANOVA was conducted in statistical software SPSS 21. The significance ($P < 0.05$) of inter-group differences was evaluated with the least significant difference (LSD) test. The images were processed with Photoshop CS5, and plots were generated with origin 9.1.

RESULTS

Ingestion and digestion of microalgae species

The statuses of larvae of *P. fucata* after being fed with *N. oculata*, *I. galbana* or *C. muelleri* for 1 h, observed under the microscope, are shown in Figure 2A, B, C. As shown in the figures, the larvae had ingested *N. oculata* and *I. galbana,* but not *C. muelleri*. After the larvae of different day-ages were fed with one of the three microalgae species at a density of 10^5 cells/mL for 1 h, the quantity of ingested microalgae species in the larvae stomachs was expressed as the area of the ingested microalgae species, as shown in Table II. The uptake of *N. oculata* by the larvae varied with larval age, increasing first and then gradually decreasing, with the maximum uptake occurring at 8 dph. However, the uptake of *I. galbana* by the larvae increased with larval age, and the uptake at 2 dph and 8 dph was lower than that for *N. oculata*. The digestion statuses of microalgae species in the stomachs of the larvae that had been transferred to clear filtered seawater are shown in Figure 2D. Figure 2D shows that the digested microalgae species in the stomachs were in a diffuse state, and that the digestive diverticulum after absorbing nutrients showed a color change, from pale under the starvation condition, to the same color as the digested microalgae species. The larval

statuses just after microalgae species were fully digested and excreted from the stomachs are shown in Figure 2E, which indicates that the larval stomachs were empty and the digestive diverticulum still had the same color as the digested microalgae species.

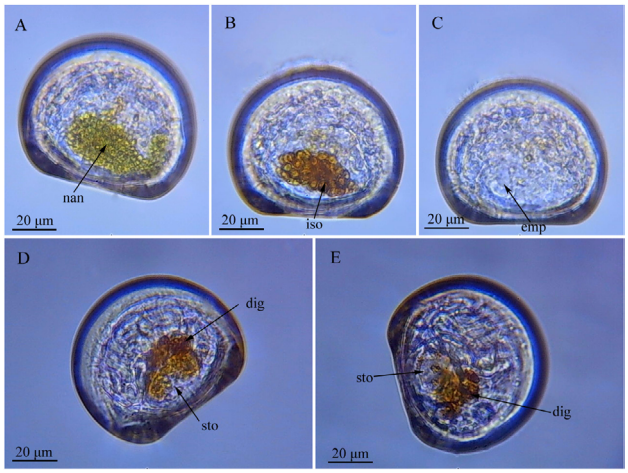

Fig. 2. The starved larvae which ingested three microalgae species for 1 h at 10^5 cells/mL, and then digested these in the filtered fresh seawater. (A) starved larvae ingested *N. oculata*; (B) starved larvae ingested *I. galbana*; (C) starved larvae did not ingest *C. muelleri*; (D) microalgae in the stomach were digested; (E) larvae were had finished digestion; nan, *N. oculata*; iso. *I, galbana*; emp, empty stomach; sto, stomach; dig, digestive diverticula.

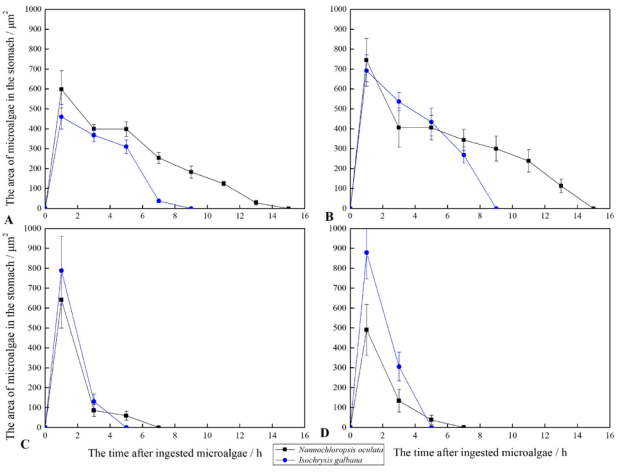

Fig. 3. Changes of microalgae area in the stomachs of larvae of different dph. A, 2 dph; B, 8 dph; C, 14 dph; D, 20 dph.

After the larvae fed on *N. oculata* and *I. galbana* for 1 h, they were filter-rinsed and transferred to clear seawater. The digestion statuses of microalgae species in the larval stomachs were observed every 2 h, as shown in Table III, and the variation of microalgae species area in the stomachs is shown in Figure 3. The results indicate that it was difficult for the larvae to digest *N. oculata,* while it was easy for them to digest *I. galbana*. Regardless of whether the microalgae species were digested in the stomachs, the quality of those microalgae species kept reducing; as larvae age increased, the larvae showed an increasingly enhanced capability for digesting and excreting the microalgae species.

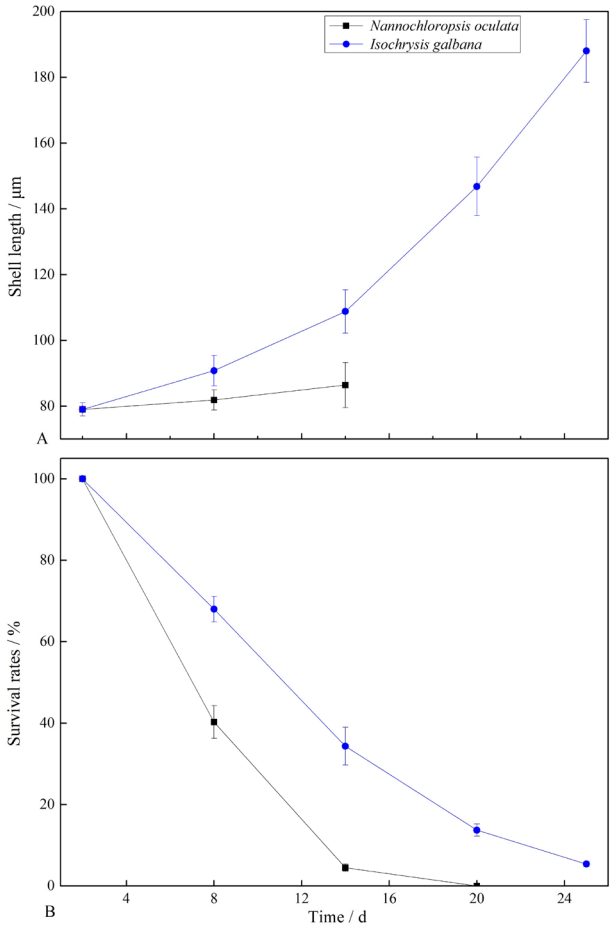

Fig. 4. The effects of growth and survival rates of larvae when fed *N. oculata* and *I. galbana,* respectively. (A) The effect of growth when fed *N. oculata* and *I. galbana,* respectively; (B) The effect of survival rates when fed *N. oculata* and *I. galbana*, respectively.

Different microalgae species were fed to the larvae during their cultivation, and the larval shell length and survival rate were monitored every six days. Larval shell lengths are shown in Figure 4A, and the larval survival

Table II. The three microalgae species area in larval stomachs after larvae fed for one hour, by larvae of different dph (μm²).

Microalgae species	2 dph	8 dph	14 dph	20 dph
Nannochloropsis oculata	597.92 ± 93.34^{b}	745.50 ± 108.67^{c}	641.19 ± 111.91^{b}	490.57 ± 127.64^{a}
Isochrysis galbana	460.41 ± 62.06^{a}	692.66 ± 79.17^{b}	788.27 ± 101.48^{c}	878.81 ± 132.86^{d}
Chaetoceros muelleri	0^{a}	0^{a}	0^{a}	0^{a}

Means in the same rows with different superscript are significantly different ($P < 0.05$).

Table III. Stages of ingestion and digestion of three microalgae species by larvae of different dph.

Microalgae species	2 dph							8 dph							14 dph			20 dph		
	1	3	5	7	9	11	13	1	3	5	7	9	11	13	1	3	5	1	3	5
Nannochloropsis oculata	I	I	I	I	I	II	III	I	I	I	I	I	I III	III	IIII	IIII	III	IIII	IIII	III
Isochrysis galbana	I	II	II	IIIII	III	--	--	III	II	II	IIIII	III	--	--	II	IIIII	III	II	IIIII	III
Chaetoceros muelleri	NO							NO							NO			NO		

I, Whole algal cells well defined in the stomach; II, Whole and lysed algal cells mixed in the stomach or no whole cells present (lysed algae only); III, larvae had finished digestion; --, means no observed; NO, means no ingested.

rates are shown in Figure 4B. During the entire experimental period, the larvae did not feed on *C. muelleri*, and therefore Figure 4 does not illustrate the effect of *C. muelleri* on the larval growth and survival. When fed with *N. oculata*, the larvae at 2 dph had a shell length of 79.01 ± 2.00 μm, while the length became 81.89 ± 3.06 μm and 86.42 ± 4.86 μm at 8 dph and 14 dph, respectively. The larval survival rate decreased with the larval growth, with the rate becoming 0 at 20 dph. When fed with *I. galbana*, the larval growth rate and survival rate were both higher than in larvae fed with *N. oculata*. In particular, at 25 dph the larval shell length of larvae fed *I. galbana* was 183.03 ± 6.54 μm, and some of the larvae started to undergo settlement and metamorphosis, with a larval survival rate of 5.4 ± 0.65 %.

DISCUSSION

All the *P. fucata* larvae of different day-ages could ingest *N. oculata* and *I. galbana,* but not *C. muelleri*. At 8 dph, the larvae had a greater ingestion rate for *N. oculata* than for *I. galbana*. Based on comparative analysis of the shape and particle size of the three microalgae species, we believe that the larvae were more likely to ingest the microalgae species that were small in size, and had a spherical shape and a smooth cell surface, which is in agreement with the reported ingestion behaviors of catarina scallop, *A. ventricosus-circularis* larvae (Lora-Vilchis *et al*., 1997), winged pearl oyster, *P. sterna* larvae (Martínez-Fernández *et al*., 2004), *Paphia schnelliana* (Deng *et al*., 2021a), *Antigona lamellaris* (Deng *et al*., 2021b) and *Trachycardium flavum* larvae (Deng *et al*., 2016) for microalgae species. Algae in the genus *Chaetoceros* are rich in the essential fatty acids that are necessary for normal growth and survival of marine invertebrate larvae (Reitan, 2011; Carboni *et al*., 2012; Scholtz *et al*., 2013), and therefore have been used as a primary feed or supplementary feed for the cultivation of many marine bivalve mollusk and echinoderm larvae (Doroudi *et al*., 2003; Knauer, 2011). However, none of the larvae of different day-ages successfully ingested *C. muelleri*. Observations about the ingestion processes of *T. flavum* larvae (Deng *et al*., 2016), geoduck clam, *Panopea zelandica* larvae (Le *et al*., 2017) and oyster, *Crassostrea angulate* larvae (Qiu *et al*., 2015) in water, suggest that the ingestion of food by bivalve mollusk larvae is achieved by swinging the peripheral cilia of the face plate to form water flow. This moves microalgae species toward the oral groove of the larvae, where they are captured by the swinging cilia of the oral groove, and enter the oral groove, and subsequently the stomach, which completes the ingestion of microalgae species (Deng *et al*., 2021a, b). Constrained by the size of the larval oral groove, microalgae species usually have long, rigid antennal hairs, which can easily prevent microalgae species from entering the oral groove of the larvae, thereby making it impossible for the larvae to successfully ingest the algae (Patinosuarez *et al*., 2004). In this study, until 8 dph, larvae had a greater ingestion rate for *N. oculata* than for *I. galbana,* which suggests that the larvae had a greater ingestion rate for small-sized microalgae species than for large-sized counterparts. This is in contrast to the microalgae species ingestion behaviors of adult *P. fucata* (Deng *et al*., 2016), young Atlantic bay scallops, *Argopecten irradians* and Pacific oysters,

Crassostrea gigas (Wang *et al*., 2000). The ingestion by spats and adult mollusks is conducted through filter-feeding on the gill. It is commonly accepted that with the increase of microalgae species size, the possibility for the gill to miss the capture of microalgae species would decrease, and therefore, given the same concentration of microalgae species, the ingestion rate for large-sized microalgae species is higher than that for small-sized microalgae species (Deng *et al*., 2016). *N. oculata* have a diameter of only about 3 μm while *I. galbana* have a diameter of 5~6 μm. Both species have almost the same likelihood of moving with water flow to reach the oral groove of the larvae, but some *I. galbana*, due to their larger particle size, may touch the cilia, or the oral groove, so that they are easily bounced off, which causes them to be ingested at a lower rate than larger *N. oculata*, and thereby making the larvae have higher ingestion rates for *N. oculata* than for *I. galbana*. After 14 dph, the larval rate of ingestion for *N. oculata* decreased, which may be associated with ingestion behaviors of larvae. The observations made by Qiu *et al*. (2015) on the movement and ingestion behaviors of oyster larvae suggested that larvae in later stages had intermittent bivalve closure, which shortened the effective ingestion time, and thereby decreased the ingestion rate for *N. oculata*. However, the larvae in this study showed an increased ingestion rate for *I. galbana*, which may be attributed to the fact that, with increased larval age and growth, their ingestion capability gradually increased, and therefore the larvae had an enhanced effective ingestion rate for *I. galbana*, thereby offsetting the decrease in effective ingestion time.

It was difficult for the larvae to digest *N. oculata,* while it was easy to digest *I. galbana*. This finding is in agreement with the digestion behaviors of catarina scallop, *A. ventricosus-circularis*, larvae (Lora-Vilchis *et al*., 1997), winged pearl oyster, *P. sterna*, larvae (Martínez-Fernández *et al*., 2004), *T. flavum* larvae (Deng *et al*., 2016) and sandfish, *Holothuria scabra*, larvae (Duy *et al*., 2015) for these two microalgae species. The degree of difficulty associated with microalgae digestion is mainly due to their structure. *N. oculata* is in the division Chlorophyta, and its surface is coated with a layer of cellulose cell wall, which makes it more difficult to degrade and digest *N. oculata* than *I. galbana* in larval stomachs. The observation made by Deng *et al*. (2016) about the digestion and excretion of microalgae by *T. flavum* larvae indicated that microalgae in the larval stomachs kept swinging via microvilli, so that microalgae could rapidly turn over and undergo digestive cleavage due to digestive enzymes. When broken down through digestion to a certain particle size, microalgae that are subjected to the swinging of stomach villi and stomach contractions are discharged from the stomach to the intestine and to the anus, from which they are excreted out of the body to complete a normal digestion and excretion process for microalgae. When the larval stomach is subjected to excessive one-time ingestion, the rapid stirring of microvilli, and the contractions of the stomach would allow for undigested microalgae in the stomach to be squeezed into the intestine, from which they are rapidly excreted out of the body. This makes the larvae unable to fully digest and utilize *I. galbana,* despite the fact that this microalgae is easy to ingest and cleave digestively. This is the main cause for the gradual decrease of larval growth rates and survival rates with increasing concentrations of microalgae beyond a certain threshold value, a phenomenon also observed by Fernández-Pardo *et al*. (2016), who used different concentrations of microalgae to feed *Venerupis corrugata* larvae. Given the same concentration of *N. oculata* and *I. galbana* added to the larval cultivation system, the latter algae led to a greater larval growth and survival rate than the former algae. This is consistent with the observation by Tang *et al*. (2006), who reported that the growth rate and survival rate of Asiatic hard clam, *Meretrix meretrix,* larvae fed with *I. galbana* were higher than those fed with other microalgae species that were more difficult to digest. This means that it is important to consider the degree of digestion difficulty for microalgae in the selection of feeds. Therefore, when cultivating *P. fucata* larvae, we should choose to use those microalgae species that are easily cultivated on a large scale, and are easily ingested and digested by the larvae. Furthermore, implemented feeding regimes should consist of appropriate feeding quantities, and feeding frequencies, based on the ingestion rate and digestion rate of microalgae species by the larvae under specific conditions.

CONCLUSIONS

In the larval cultivation of *P. fucata*, the microalgae should be with small cell size, spherical shape and easy to digest, and the principle of small amount and many times should be followed.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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