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Title: Effect of Diet on Growth and Larval Development of the Sea Cucumber Holothuria nobilis.

Approved: Robert H. Richmond
Robert H. Richmond, Chair Thesis Committee

The effects of diet on larval growth, development, and survival of the economically valuable sea cucumber *Holothuria nobilis* (Selenka) was studied under laboratory conditions. Adult sea cucumbers were collected from the reefs of Guam and induced to spawn in the laboratory. Two sets of experiments were conducted at different times, each with a duration of 30 days. Larvae were raised on the following diets: 0.45 µm filtered seawater (unfed), natural seawater, and cultured algae including Tahitian (T-) *Isochrysis, Pavlova salina,* and a mixed culture of T- *Isochrysis* and *P. salina*. Five 1-liter replicate glass bottles were used for each diet, and larvae were raised at a larval density of 1000 bottle^{-1} . Larval length at the secondary auricularia stage was largest for larvae that were raised on the T- *Isochrysis* and natural seawater diets, and significantly smaller for the unfed larvae. Larval development was initially fastest for larvae raised on natural seawater, with 78 % of the individuals reaching the secondary auricularia stage within one week. However, from week two until the end of week four, development was slower for larvae raised on the natural seawater and T- *Isochrysis* diets compared to those on the unfed treatment. Unfed larvae had faster development than the larvae of any of the other treatments; with 37 % larvae reaching

the doliolaria stage by week four. Larval survival was very low on the mixed diet treatment with a mean survival of 1.4 % compared to 52 % and 26 % for T-*Isochrysis* fed and unfed larvae. Results indicate that diet has a major influence on growth, development, and survival of *H. nobilis* larvae, but other factors may also affect the ability of larvae to complete metamorphosis.

TO THE OFFICE OF GRADUATE SCHOOL AND RESEARCH

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EFFECT OF DIET ON GROWTH AND LARVAL DEVELOPMENT OF THE SEA CUCUMBER, HOLOTHURIA NOBILIS

BY

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INTRODUCTION

Tropical holothurians are ecologically and economically valuable organisms. They are ecologically important as components of reef communities for their role as deposit feeders and recyclers of nutrients and organic matter (Trefz, 1958; Bakus, 1973; McEuen and Chia, 1985; Birkeland, 1988). They are also economically valuable as a food source, sea cucumbers are regarded as a delicacy in many Asian countries and Pacific islands, where they have been collected for centuries (Gentle, 1979; Conand, 1981; Shelley, 1985; Philipson, 1990; Hopper and Richmond, 1988; Yamaguchi, 1990). In addition to their importance as a food source, valuable natural products such as collagen and saponins can be extracted from the body wall or the cuverian tubules of the sea cucumbers and used for the production of glues, gelatins, coatings, and other products of potential importance for the pharmaceutical industry (Bakus, 1973; Foscarini, 1989).

At present, sea cucumber fisheries represent an important source of income for many small fishing communities in the Tropical Indo-West Pacific, with small-scale fisheries being developed throughout this region (Gentle, 1979; Trinidad-Roa, 1987). For example in 1988, Fiji exported about 1000 tons of dried sea cucumber, an equivalent of about 10,000 tons of fresh sea cucumber (Adams, 1992). The development of additional sea cucumber fisheries based on natural stocks alone is a matter of concern since sea cucumbers can be easily overharvested. This has generated interest in the development of techniques for sea cucumber larval rearing, to

manage the resource through stock enhancement and to increase the yields of some of the commercially valuable species.

Sea cucumbers exhibit two types of larval development, lecithotrophic and planktotrophic (McEuen, 1987). Lecithotrophic larvae do not feed but complete development on stored energy, while planktotrophic larvae must feed in order to develop and undergo metamorphosis to the juvenile stage. The eggs of species with nonfeeding larvae are generally much larger than the eggs of the species with feeding larvae (Strathmann, 1985).

Planktotrophic holothurian larvae develop from smaller eggs and go through more developmental stages than lecithotrophic larvae. The first stage is the auricularia, with a looped ciliary band (McEuen, 1987). The time of development to this larval stage varies among species and climatic zones, but auricularia usually appear within three days of fertilization (Barnes, 1987). During subsequent growth, lobes or projections encircled by a continuous ciliary band proliferate on the auricularia (McEuen 1987). After this late auricularia stage, the larvae begin to shrink to approximately half their former body size. They then become barrel-shaped, and the ciliary band breaks up to form several separate hoops that encircle the larvae. This "doliolaria" larva does not feed but remains planktonic, and if sufficient energy reserves were accumulated in the feeding auricularia stage and an appropriate substratum is present, it may settle and complete its metamorphosis to the benthic pentacula stage.

The length of time the larvae remain pelagic as well as the time spent in the different stages varies among species. For *Parastichopus californicus*, a temperate species, the feeding auricularia stage can last from 35 to 52 days prior to metamorphosis to the doliolaria stage (McEuen, 1987). Another temperate holothurian with planktotrophic larvae, *Stichopus japonicus,* lasted 20 days in the pelagic stage before settlement (Uehara, 1970). Larvae of *Actinopyga mauritiana*, a tropical species, remained in the planktonic state from 6 to 20 days before settlement (Hopper, 1989).

To date, studies of holothurian larval biology have focused mostly on temperate species (Strathmann, 1971, 1978; McEuen and Chia, 1985, 1991; Smiley, 1984b, 1986b; Young and Chia, 1982; McEuen, 1987), although some work on tropical species has been done. Mortensen (1937), raised several species of tropical sea cucumbers and partially described their larval development. Chen and Chian (1990) studied the larval development of *Actinopyga echinites* from fertilization to the juvenile stage in Taiwan. Hopper (1990) described the larval development of *Actinopyga mauritiana* larvae from fertilization to the doliolaria stage. Ishida (1978) raised *Stichopus japonicus* to the juvenile stage with a survival rate of 15%. Richmond and his students at the University of Guam Marine laboratory have raised the larvae of three species of tropical sea cucumber (*Actinopyga mauritiana, Holothuria nobilis*, and *Thelenota ananas*) part way through their development cycle (Preston, 1990).

The present study focuses on the tropical species *Holothuria (Microthele) nobilis* (Selenka), commonly known as the black teatfish. This is one of the most

highly valued of the edible species because of its large size (30 - 40 cm length) and thick body wall. Dry specimens of *Holothuria nobilis* collected in areas such as Papua New Guinea, Fiji, Palau, Sri Lanka or India, sell 22pc/Kg at a wholesale price of $67.00 in markets of Singapore (Infofish, 1992), and at a retail price of $19/lb in Hawaii (Richmond pers. comn. 1994).

Holothuria nobilis is distributed throughout the tropical Indo-West Pacific (Rowe and Doty, 1977). This species occurs in the subtidal zone at depths between 2 and 25 meters and is usually covered with a thin coating of sand (Gentle, 1979). Individuals are very dispersed and found in habitats around coral slopes (Conand, 1981). In New Caledonia *H. nobilis* has an annual reproductive cycle with a long spawning period, followed by a short period of gonadal resting and maturation (Conand, 1981). On Guam, *H. nobilis* has several spawnings during the year (Richmond, 1990).

The present study examines the development and growth of the planktotrophic larvae of *Holothuria nobilis* in relation to their diet. I used a series of replicated experiments to determine the effects of diet (cultured and natural phytoplankton) on the growth, development, and survival of larval *H. nobilis*. The objectives of this research were to determine: 1) the importance of feeding during larval development ; and 2) the effects of different diet treatments on growth, development and survival of the larvae.

MATERIAL AND METHODS

Collection of Broodstock

Twenty individuals of *Holothuria nobilis* were collected in June and July 1993 from Gun Beach and Tumon Bay, Guam. The sea cucumbers were placed in large coolers filled with seawater and transported to the running seawater tanks at the University of Guam Marine Laboratory. Due to the absence of sexual dimorphism and an approximate sex ratio of 8:2 (male:female) at these sites, it was necessary to collect a minimum of 20 individuals to assure the presence of both sexes. All of the sea cucumbers collected for the experiment were returned to their appropriate habitats after spawning.

Spawning and Collection of Gametes

Keeping stressed echinoderms in standing water helps to induce spawning (Strathmann, 1992). Immediately after their arrival from the field, all the collected broodstock were placed in a 1-m^3 tank with no exchange of seawater. Male sea cucumbers usually released sperm soon after being placed in the tanks, while females took an additional ten minutes to one hour to spawn. Once spawning began, the gametes were collected with a Pasteur pipette directly from the gonopore, approximately 75 ml of gamete solution was diluted to 150 ml 0.45 µm Millipore filtered, UV-treated seawater (henceforth referred to as UV-filtered seawater) in 250-ml Erlenmeyer flasks, and the flasks covered with sterile aluminum foil. Sperm

and eggs were collected separately to avoid premature fertilization. The collected sperm were kept in the flasks in an air conditioned room at 25^0 C until fertilizations were performed. For each experiment, the eggs used were collected from only one female, while sperm were obtained from two or three males.

Fertilization

Approximately five drops of the diluted sperm solution were added to each 150 ml egg suspension. The mixture was carefully stirred by mild agitation of the flask. After 10 - 15 minutes, the eggs were checked for fertilization under a compound microscope. Fertilized eggs were washed several times with filtered seawater on a 60-µm nylon mesh to remove excess sperm and avoid polyspermy.

Rearing Conditions

The fertilized eggs from two 150 ml eggs suspension were evenly distributed among four 1-liter glass bottles filled with filtered seawater. The bottles were placed on their sides on a roller-type culture device with a rotation of approximately 0.3 RPM and exposed to continuous fluorescent light (40 watts). The temperature of the cultures was maintained at 26 ± 1° C, which is the approximate ambient ocean temperature at 10 - 12 meters depth, where this species is found.

Experimental Design

There were two sets of experiments, each with a duration of 30 days and conducted at different times, to investigate the effects of diet on the growth and development of the *Holothuria nobilis* larvae. Both experiments followed the same basic procedures, number of replicates and sample size, the only differences being the diets and the spawns.

Five replicate bottles of larvae from the same spawn were used for each treatment. Three days after fertilization, approximately 1000 early auricularia larvae were placed in each of 15 1-liter glass bottles. The larval density of each stock bottle was calculated by extrapolating the number of larvae collected in a 40 ml beaker to the volume of the bottle.

Diet

The larvae were fed every other day, starting the third day after fertilization. At this stage, the larvae had already developed mouths and alimentary canals, which allowed them to begin feeding. The diet consisted of the flagellates *Isochrysis* sp. (clone T.ISO; obtained from the Kewalo Marine Laboratory, Hawaii), *Pavlova salina* (from CSIRO, Phytoplankton Culture Collection, Tasmania, Australia), and natural unfiltered seawater. The selection of these microalgae species was based on several factors including their tolerance of tropical culture conditions; their appropriate size for feeding sea cucumber larvae (T-*Isochrysis* is 3-5 µm and *P. salina* is 4 - 6 µm); and their nutritional value. Both contain high amounts of two essential polyunsaturated

fatty acids [20:5ω3 and 22:6ω3] (Jeffrey et al., 1990), necessary for the development and growth of the larvae. These algal cultures have been widely used as diets for feeding larvae of a variety of marine invertebrates (Giese, 1977; Strathmann, 1977). The algal cultures were maintained in Guillard's f/2 medium. Algal cells were collected for feeding the larvae at times of logarithmic growth, when the cells have the best nutritional value (Fulks and Main, 1991). The algal cell density of each culture was determined by counting the cells on a clinical hemacytometer. Prior to feeding the sea cucumber larvae, the algal culture medium was removed by centrifugation and replaced with filtered seawater to avoid the accumulated toxic by-products of the algal cells. Larvae were fed at an algal concentration of 10,000 cells ml^{-1} .

For those cultures raised on natural seawater, fresh seawater was collected on the reef flat at Pago Bay (behind the University of Guam Marine Laboratory) immediately before feeding time. The seawater was strained through 80-μm nylon mesh to remove larger plankton.

The diets used in the first experiment included:

1) an unfed diet treatment of 0.45-μm membrane filtered seawater,

2) natural seawater, and

3) T-*Isochrysis* at a concentration of 1 x 10^4 cells ml^{-1}

The second experiment compared the effects of monocultures versus a polyculture, and the diets included:

1) T-*Isochrysis* at a concentration of 1 x 10^4 cells ml^{-1}

2) *Pavlova salina* at a concentration of 1 x 10^4 cells ml^{-1} and

3) a mixed culture of T-*Isochrysis* at a concentration of 0.5 x 10^4 cells ml^{-1} and *Pavlova salina* at 0.5 x 10^4 cells ml^{-1}

Water Changes

Every four days 75 % of the water in each experimental bottle was gently siphoned off through a 60-µm nylon mesh. While the water level was lowered, the larvae remained in the bottle. The bottles were subsequently refilled with freshly-filtered seawater and cultured algae were added as appropiate.

Observations and Measurements of Larvae

A sample of fifteen larvae were taken from each bottle, beginning on day 4 after fertilization (day 0) and continuing on alternate days until day 28. The larvae were observed under a compound microscope equipped with an ocular micrometer. The following data were recorded for each larva: 1) larval length, 2) presence of lipid spheres, and 3) stage of development. To facilitate the recording of data, and due to the various morphogenic changes exhibited by the auricularia larvae during development, I subdivided this stage into three sub-stages:

First auricularia: when the larva is very transparent and is lightly pigmented along the margins. The body is rather broad and no lateral extensions are present.

Secondary auricularia: when the larva displays a more complex shape, with distinctive extensions along the body margin.

Shrinking auricularia: when the larva exhibits a dense and thick appearance of the epidermis and there are no lateral extensions. At this stage the esophagus and intestines are laterally compressed.

All measured larvae were discarded since they normally degenerate and die after manipulation and prolonged exposure to the microscope light. At the end of each experiment, which corresponded to day 30, all remaining larvae were counted to determine survival within treatments.

Data Analysis

To determine the effects of diet on larval growth, means of total length of the larvae were compared among treatments at four days (first auricularia stage); twelve days (secondary auricularia stage, when most larvae exhibited the maximum size); and at twenty eight days (doliolaria stage). Mean larval lengths among groups were compared with a mixed-model analysis of variance (ANOVA). Diet treatment was considered as a fixed factor; bottle effect was considered as a random factor nested in treatments, and individual measurements were nested within bottles. The data analysis were performed with the BMDP statistical program 3V (BMDP Statistical Software, Inc; Los Angeles, California). Prior statistical comparisons with ANOVA, the data were checked for homogeneity of the variances with Hartley's F_{max}-test (Sokal and Rohlf, 1981). Comparisons of larval development among treatments of experiment 1, at the end of weeks one and four, were made with a one-way ANOVA. The percentage of larvae were arcsine transformed prior to analysis to normalize the data

for ANOVA requirements. The transformed data was the dependent variable. Survival and mean percentages were compared with one-way ANOVA. A Tukey's Test was used to examine differences among treatments. The one-way ANOVA and Tukey's tests were analyzed with the statistical program Statistix Version 4.0 (Analytical Software, St. Paul, Minesota).

RESULTS

Larval Growth

Growth of *Holothuria nobilis* larvae varied among diet treatments. However, there was also a considerable variation in the timing of developmental events; hence, several larval stages were found in most of the samples (Fig. 1). I measured equal numbers of larvae for each sample, but the larvae were not all at the same stage of development. Because of this, the stage-specific analysis of growth are based on unequal sample sizes.

Experiment 1

Over the entire study period the unfed larvae were generally smaller than those raised on either natural seawater or T-*Isochrysis* (Fig. 2). On day four after fertilization, which corresponds to one day after the larvae had been exposed to the diets, there were no significant differences in mean length of first auricularia larvae among the three diets treatments ($F_{2,12}$ =1.01, p=0.3925) (Fig. 3). Mean lengths for the larvae raised on the unfed treatment, natural seawater, and T-*Iso* were 488 µm, 504 µm, and 500 µm, respectively. For the secondary auricularia stage, measurements on total length of the larvae were compared on day twelve after fertilization (on this day most larvae exhibited the maximum size). Larvae raised on natural seawater and T-*Isochrysis* treatments had grown significantly larger (579 µm and 603 µm respectively) compared to those raised on the unfed treatment (456 µm), which

actually shrank. There were significant differences among treatments ($F_{(1,155)}$= 43.36, p= 0.0000; Fig. 4) and according to the Tukey's comparisons of means test, larvae raised on the unfed treatment were significantly smaller than those raised in the T-*Isochrysis* and natural seawater treatments.

Auricularia larvae displayed the characteristic reduction in body length after 12 days of development. The result was that larvae were close in size despite the diet, with those raised on the T-*Isochrysis* diet remaining slightly larger. Mean lengths of the larvae at the doliolaria stage on day 28, were not significantly different ($F_{2,9}$=0.20 p=0.8195) among diet treatments. Mean lengths of the larvae were: 275 µm; 277 µm; and 283 µm for the unfed, natural seawater, and T-*Isochrysis* diets, respectively (Fig. 5).

Experiment 2

On day four after fertilization, which corresponds to one day after larvae were exposed to the diets, all of the larvae were in the first auricularia stage. First auricularia larvae fed *Pavlova salina* had a mean length of 464 µm; T-*Iso*-fed larvae 458 µm, and the mixed treatment fed larvae were the smallest with a mean length of 437 µm. The differences in size were statistically significant ($F_{(2,222)}$= 4.03, p=0.01; Fig. 6).

Mean lengths of larvae on day twelve, when the three treatments had secondary auricularia stages present, were: 503 µm on the *P. salina* diet, 54 µm on the T-*Isochrysis* diet, and 50 µm on the mixed diet. There were no significant differences among treatments ($F_{(2,84)}$= 1.77, p=0.175; Fig. 7). From this day on, the larvae fed

the T-*Iso*chrysis and *P. salina* diets underwent a gradual reduction in size, whereas larvae fed the mixed diet exhibited an increase in size toward the end of the experiment (Fig. 7).

Very few individuals underwent metamorphosis to the doliolaria stage in any of the treatments. For those larvae that reached the doliolaria stage, the time of occurrence was irregular, therefore comparisons of doliolaria size were not made.

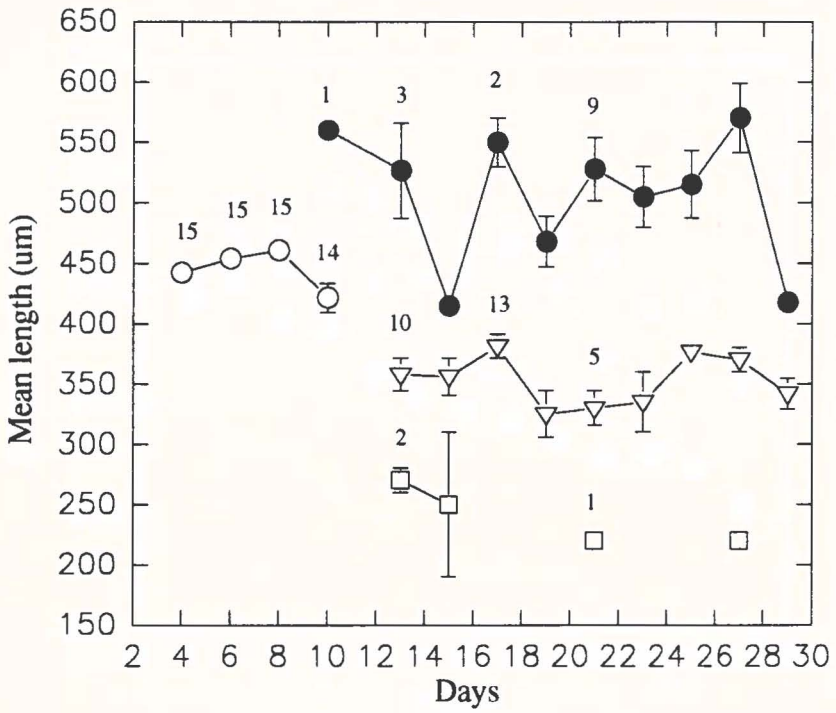

Figure 1. Growth of *Holothuria nobilis* larvae . Symbols represent stages of development; ○ First Auricularia ● Secondary Auricularia, ▽ Srinking Auricularia, and □ Doliolaria. Numbers indicate individuals at different larval stages. Data demonstrate the variation in growth and development from one representative bottle of the mixed diet. Measurements were made on 15 larvae on alternate days.

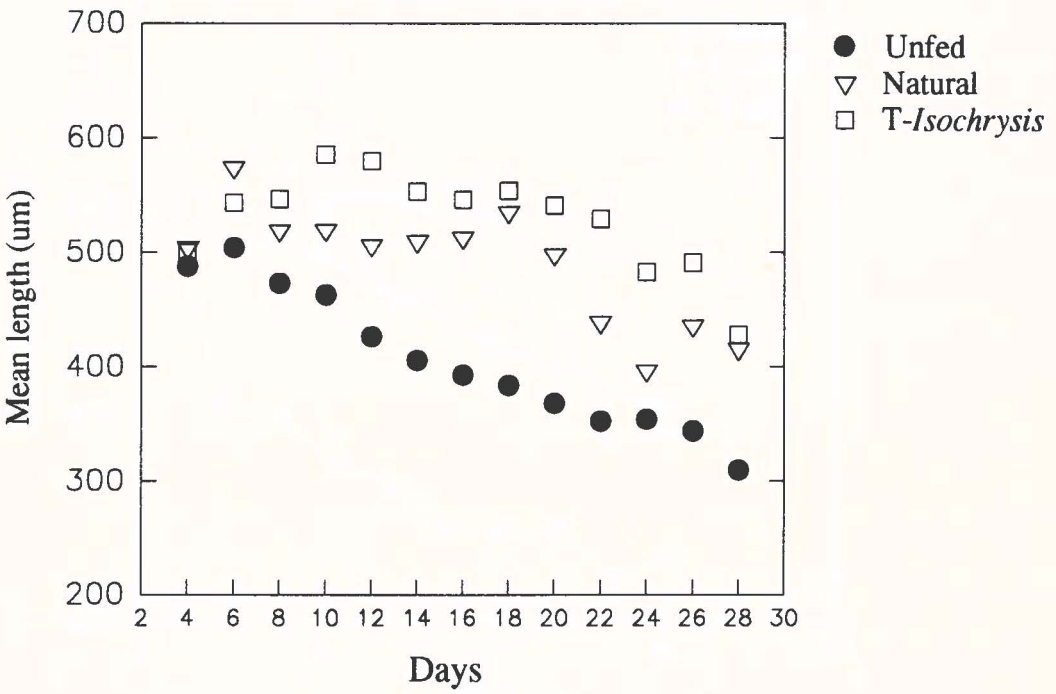

Figure 2. Growth of *Holothuria nobilis* larvae raised on three different diets during a thirty day period. Means are lengths from pooled data of all larvae measured at all stages of development.

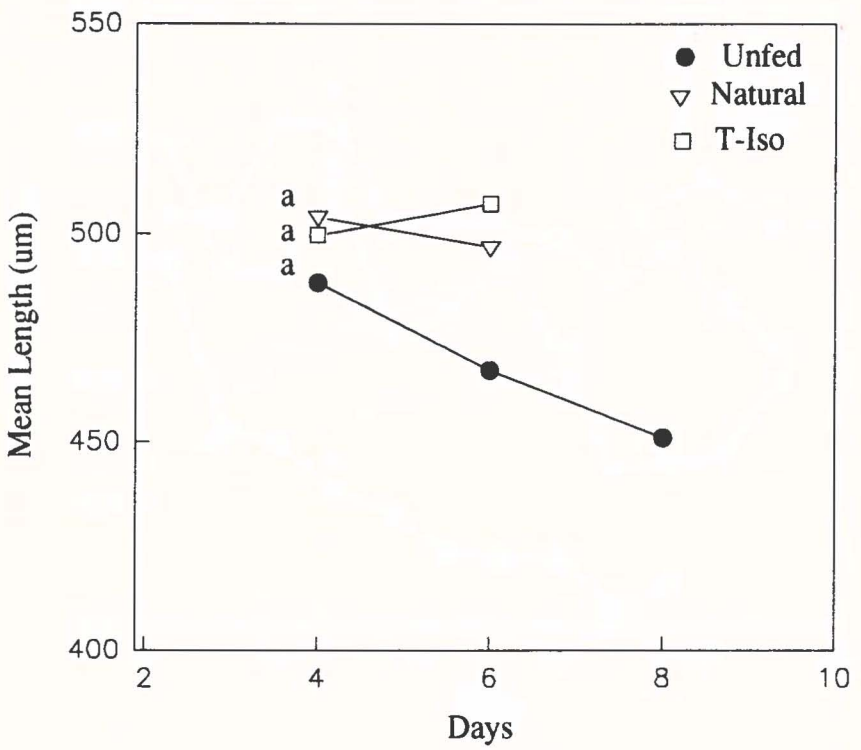

Figure 3. Length of *Holothuria nobilis* larvae at the first auricularia stage. Symbols represent the three different diet treatments. Same letters indicate means were not significantly different on day 4, (initial growth) one day after larvae were exposed to experimental diets. n= 5 bottles / treatment.

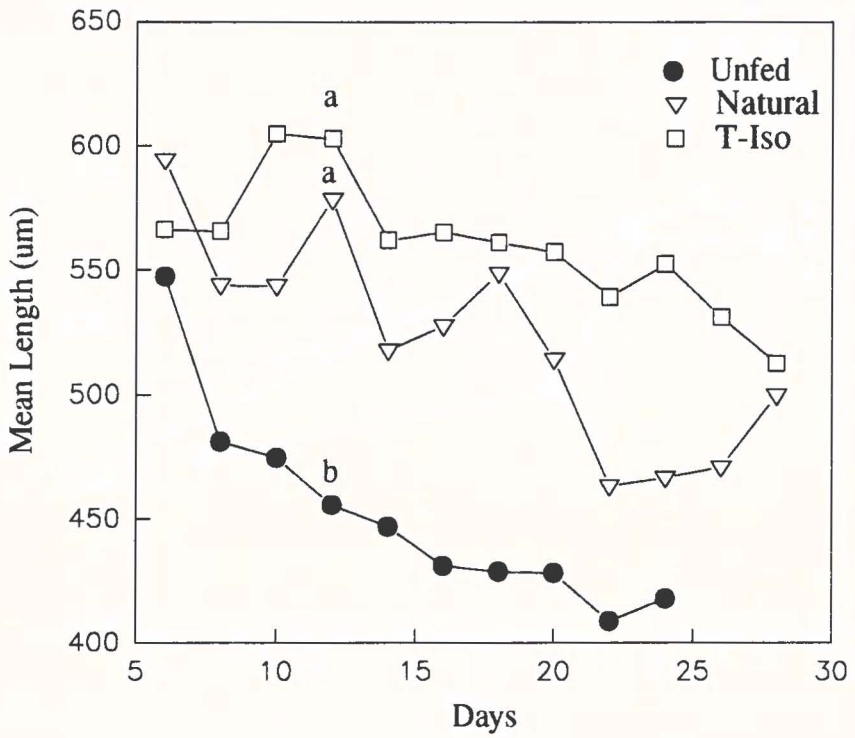

Figure 4. Length of *Holothuria nobilis* larvae at the secondary auricularia stage. Symbols represent the three different diet treatments. Means with different letters are significantly different on day 12 ($p<0.05$, Tukey's test). n= 5 bottles/ treatment

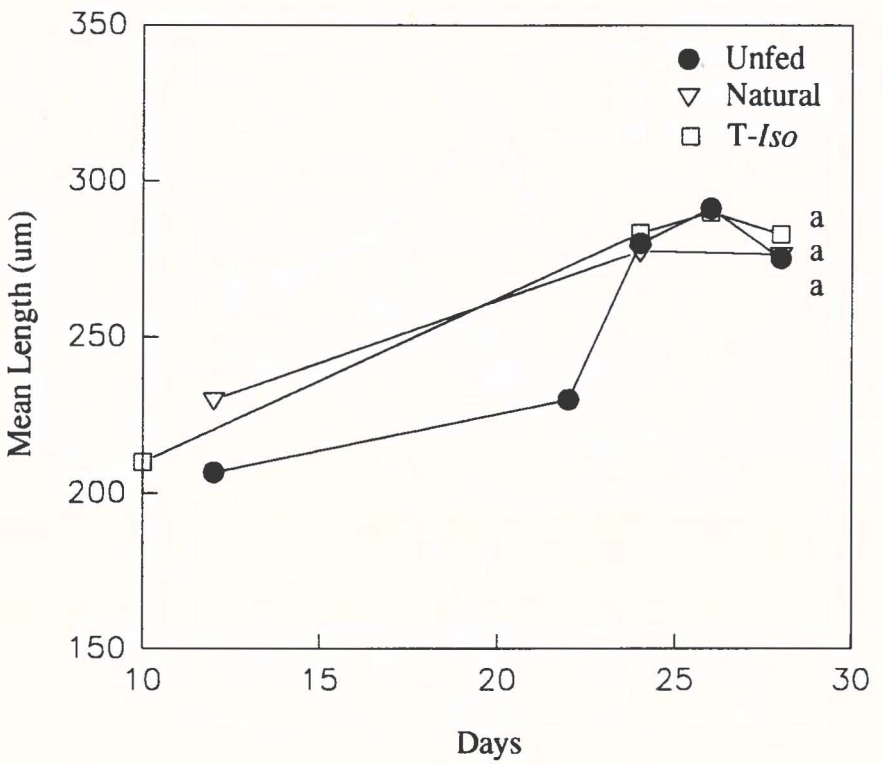

Figure 5. Length of *Holothuria nobilis* larvae at the doliolaria stage. Symbols represent the three different diet treatments. Same letters indicate means were not significantly different on day 28. n = 5 bottles / treatment.

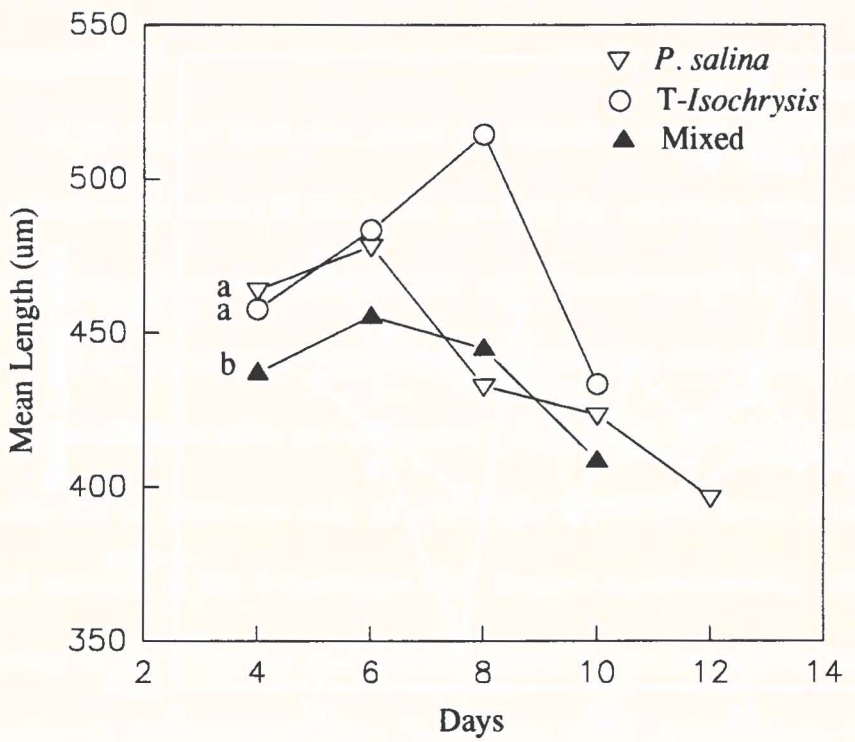

Figure 6. Length of *Holothuria nobilis* larvae at the first auricularia stage. Symbols represent diets. Means with different letters are significantly different at day 4, (initial growth) one day after larvae were exposed to diets ($p<0.05$, Tukey's test). n= 5 bottles / treatment.

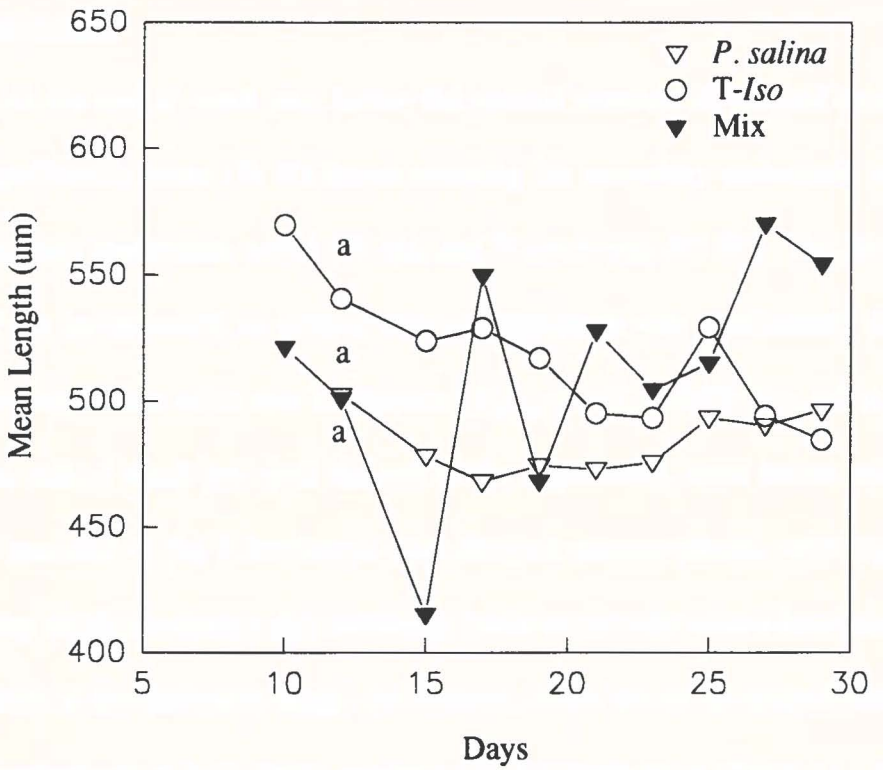

Figure 7. Length of *Holothuria nobilis* larvae at the secondary auricularia stage. Symbols represent the three different diet treatments. Same letters indicate means were not significantly different on day 12. Number of bottles was 5 for *Pavlova salina*; 4 for T- *Isochrysis*; and 3 for Mixed.

Effect of Diet on time of Development

Four stages of development were observed in this study: first auricularia (Fig. 8), secondary auricularia (Fig. 9), shrinking auricularia (Fig. 10), and doliolaria (Fig. 11). The percentages of larvae at each stage of development are shown in tables 1 and 2, for experiments 1 and 2 respectively.

Experiment 1

At the end of week one, larvae fed natural seawater developed most rapidly, with significantly more (78 %) larvae reaching the secondary auricularia stage than in the other two treatments, unfed (50 %), and T-*Isochrysis* (57 %). The development time of the larvae raised on natural seawater treatment was significantly different from that observed in the other two treatments ($F_{(2,12)}$= 3.87, p=0.05; Fig. 12A). However, from the end of week two until the end of the experiment, most larvae raised on either natural seawater or the T-*Iso*chrysis diets delayed metamorphosis, with high numbers remaining at the secondary and shrinking larval stages and with a low percentage of individuals at the doliolaria stage. In contrast, unfed larvae developed faster and had a higher percentage of individuals reaching the doliolaria stage (Table 1). At the end of week four, larvae raised on the natural seawater diet had a lower mean percentage of doliolaria stage (4 %) than larvae from either the unfed group (37 %), or the T-*Iso*chrysis fed larvae (14 %). Larvae raised on natural seawater developed significantly slower than larvae raised on the unfed and T-*Isochrysis* treatments ($F_{(2,12)}$= 4.85, p= 0.0286; Fig. 12B).

Figure 8. Larva of *Holothuria nobilis* at the first auricularia stage.

Figure 9. Larva of *Holothuria nobilis* at the secondary auricularia stage.

Figure 10. Larva of *Holothuria nobilis* at the shrinking auricularia stage.

Figure 11. Larva of *Holothuria nobilis* at the doliolaria stage.

Table 1. Larval development of *Holothuria nobilis* larvae raised at three different diet treatments. Values indicate percentage of larvae attaining the various stages at the end of each week during a month study period.

	First Auricularia	Secondary Auricularia	Shrinking Auricularia	Doliolaria
Week 1				
Unfed	49	50	0	0
Natural	21	78	0	0
T-Iso	42	57	0	0
Week 2				
Unfed	0	49	50	0
Natural	0	82	17	0
T-Iso	0	93	6	0
Week 3				
Unfed	0	16	81	2
Natural	0	82	17	0
T-Iso	0	89	10	0
Week 4				
Unfed	0	0	62	37
Natural	0	28	21	4
T-Iso	0	48	37	14

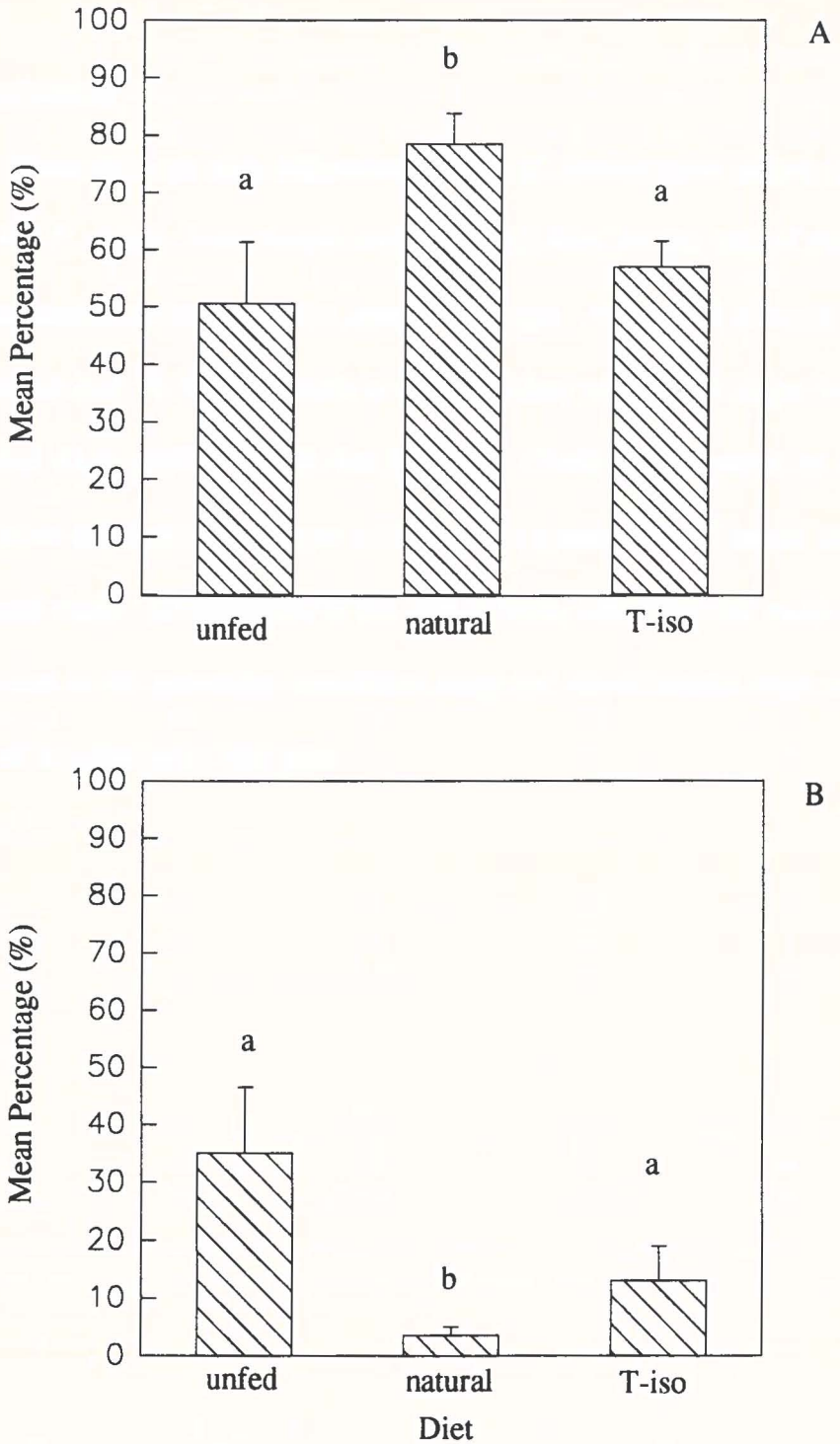

Figure 12. Larval development of *Holothuria nobilis* under three different diet treatments (unfed, natural, and T- *Isochrysis*). Graph A shows percentage of individuals that reached secondary auricularia stage at week one. Graph B shows the percentage of individuals that were at the doliolaria stage at week four.

Experiment 2

There were no clear differences in rate of development among the groups of larvae fed *P. salina*, T-*Isochrysis* and the mixed diet. At the end of week one, larvae in all treatments were at the first auricularia stage. At the end of week two, larvae fed T-*Isochrysis* had 70 % individuals at the secondary auricularia stage, whereas *P. salina* had 43 % and the mixed diet had 28 % (Table 2). However, at the end of weeks three and four, all cultures still had high percentages of larvae in the secondary auricularia stage. Comparisons at the end of week four were not made since most larvae were in the secondary auricularia stage and the doliolaria stage was not present in any of the groups by this time.

Table 2. Larval development of *Holothuria nobilis* larvae raised at three different diet treatments. Values indicate percentage of larvae attaining the various stages at the end of each week during a month study period.

	First Auricularia	Secondary Auricularia	Shrinking Auricularia	Doliolaria
Week 1				
T-Iso	100	0	0	0
P. salina	100	0	0	0
Mixed	100	0	0	0
Week 2				
T-Iso	1	70	20	8
P. salina	40	42	13	4
Mixed	0	28	57	13
Week 3				
T-Iso	0	80	20	0
P. salina	0	68	30	1
Mixed	0	73	26	0
Week 4				
Unfed	0	85	13	1
Natural	0	78	21	0
T-Iso	0	86	13	0

Larval survival

Survival values from each of the five replicate bottles per treatment that were collected at the end of the experiment, were corrected by substracting the number of larvae removed for measurements throughout the study period.

Experiment 1

There were highly significant differences in larval survival among diet treatments ($F_{2,12}$= 11.1, p=0.0019; Fig. 13). Survival was highest for larvae fed the T-*Isochrysis* diet, with a mean survival of 52 % (range 34 to 66.2 %), followed by the unfed diet with 26 % (range 18 to 36.2 %), and lowest for larvae raised in the natural seawater diet with a mean survival of 5 % (range 0 % to 12 %). According to Tukey's pairwise comparison of means test, mean survival for larvae fed the natural seawater diet was signicantly different than that of T-*Isochrysis* fed larvae, but there were no significant differences in survival, either between T-*Isochrysis* and unfed or between unfed and natural seawater diets.

Experiment 2

There were no significant differences in survival among diet treatments ($F_{2,12}$= 1.98, p= 0.1806; Fig. 14). At the end of this experiment, the larvae reared on the T-*Isochrysis* diet had a mean survival of 20 % (range 0 to 52.5 %), *P. salina* fed larvae had a 13 % mean survival (range 0 to 26 %), and the mixed treatment fed larvae had a mean survival of 1.4 % (range 0 to 7 %). Nevertheless large differences were seen in survivorship between the T-*Isochrysis* and mixed treatments. However, the lack of

statistically significant differences may be due to the low power of the test, resulting from the small sample size. A summary of the results obtained in this study is shown in Table 3.

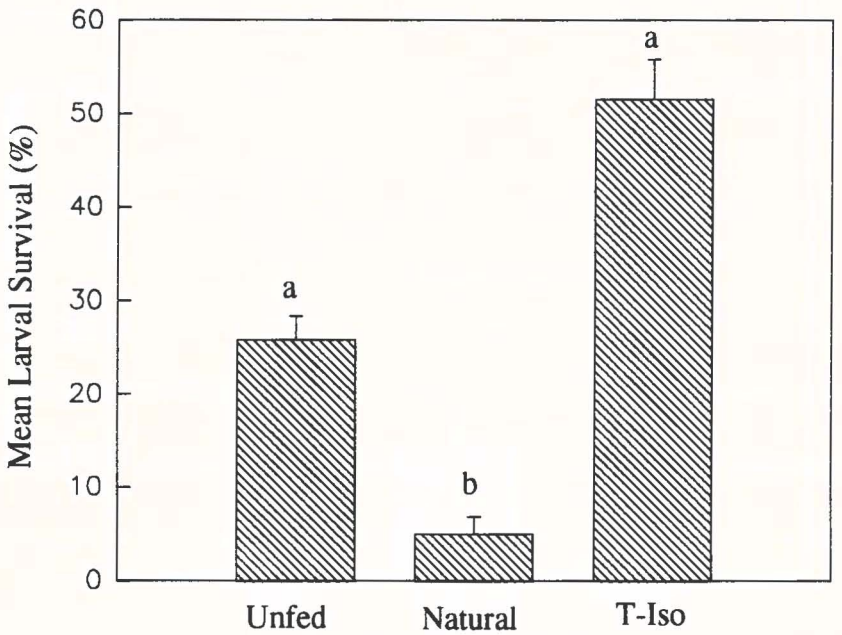

Figure 13. Survival of *Holothuria nobilis* larvae raised on three different diets. Bars are standard errors. Means with different letters are significantly different ($p<0.05$, Tukey's test). n = 5 bottles / treatment.

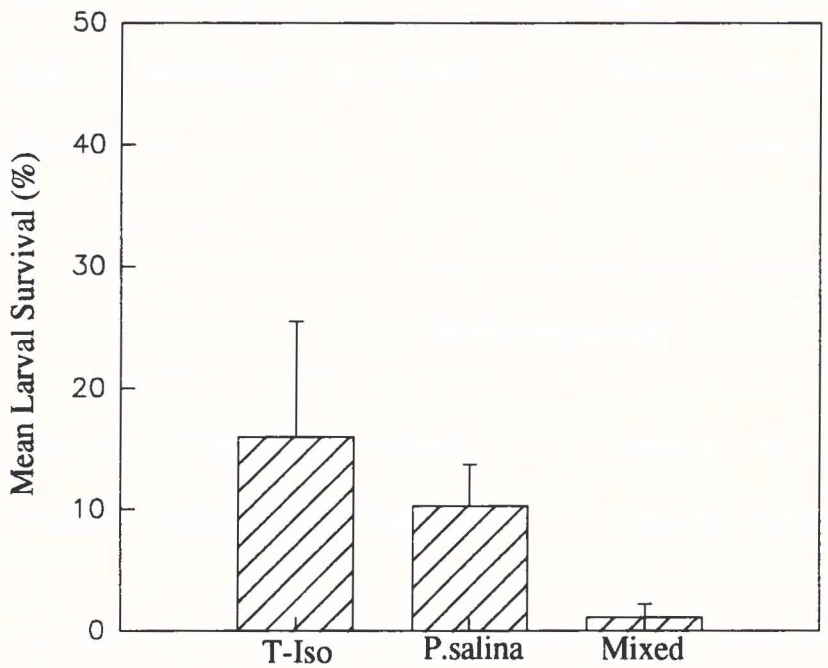

Figure 14. Suvival of *Holothuria nobilis* larvae raised on three different diets. Bars are standard errors. Means are not significantly different (p= 0.1806, ANOVA). n= 5 bottles / treatment.

Table 3. Summary results of this study.

Experiment 1	Experiment 2
Growth (size)	
day 4 : ns (among diets)	day 4 *P. salina*= T- *Iso* > Mixed
day 12 : natural = T- *Iso* > Unfed	day 12 : ns
day 28 : ns	day 28 : not made
Development Rate	
week 1 : natural > T-*Iso* = unfed	week 1 : ns
week 4: unfed > T-*Iso* > natural	week 4 : not made
Survival	
T-I*so* > unfed > natural	ns

ns = non significant differences.

DISCUSSION

The results of this study indicate that diet is an important factor in rearing larvae of *Holothuria nobilis*. The larvae were capable of developing to the doliolaria stage and surviving for at least 30 days in the absence of food. However, when larvae were raised on cultured microalgae, both their growth and survival increased.

Effects of Diet on Larval Size

Sea cucumber larvae reach their maximum size at the secondary auricularia stage, when the larvae develop conspicuous lobes along the body. At this stage unfed larvae were small, and the elongations along the body margins were not well-developed. Their steady reduction in size throughout the experiment might be the result of starvation. The deprivation of food for an extended period of time may produce "negative growth" (Féral, 1985). Reduction in size has been observed in other echinoderm larvae deprived of food. For example, Allison (1994) found that larvae of the asteroid *Asterina miniata* exhibited a reduction in size when starved. Paulay et al., (1985) reared larvae of the echinoid *Dendraster excentricus* with a reduced diet of natural seawater, in which part of the water volume was filtered through a 0.45-µm membrane filter, and observed that these larvae were smaller than larvae fed natural seawater enhanced with cultured algae. Strathmann et al. (1992) raised larvae of the sea urchin *Paracentrotus lividus* on natural seawater combined with 0.45-µm filtered

seawater and obtained larvae with slower growth rates and smaller body parts compared to sibling larvae fed a diet of natural seawater enhanced with cultured algae. These consistent results in planktotrophic echinoderm larvae of reduced larval size in response to starvation or scarcity of food, demonstrates that although larvae may be able to use stored energy from the yolk, these reserves do not provide enough energy to allow for normal larval growth.

Coral reef waters are generally oligotrophic; nevertheless, phytoplankton blooms occur in Guam around the beginning of the rainy season, as a result of the increase in nutrients derived from terrestrial runoff (Birkeland, 1982). These events might have some influence on *H. nobilis*, in that although this species spawns throughout the year, the peak spawning is in the summer. The present study was conducted during these months, and I found that larvae raised with natural seawater developed larger secondary auricularia larvae than those raised on the unfed treatment (Fig 3). Although there have been some controversies regarding this study, Olson (1987) reported that in the Great Barrier Reef, larvae of the starfish *Acanthaster planci* reared (in situ) in natural seawater exhibited maximal rates of development. His assumption was that the crown-of-thorns starfish does not obtain all of its nutritional requirements from phytoplankton but also from other resources such as dissolved organic matter and bacteria. Other studies have demonstrated that dissolved organic matter can provide as much as 79 % of the total nutrition of echinoplutei larvae (Boidron-Metairon, 1988).

An interesting finding in the present study was that most larvae fed on the natural seawater diet exhibited longer cilia than those observed by sibling larvae from the unfed and T-*Isochrysis* diets. This is a qualitative observation, and no measurements of cilia length were included in this study. However, the allocation of energy into increasing growth of certain body parts has been demonstrated in echinoid larvae (Boidron-Metairon, 1988; Strathmann et al. 1992), as well as in asteroid larvae (Allison, 1994) when subjected to food limitation. An interpretation of this developmental plasticity might be that this is an adaptive response when feeding conditions are adverse. Longer cilia may increase the rate of food capture and ingestion. When food is scarce, growth may be allocated to the structures for clearing particles (Strathmann, 1993).

Effect of Diet on the Temporal Pattern of Development

Holothuria nobilis larvae exhibited variation in timing of development to the different larval stages. This variation did not only occur among diet treatments but also among replicates. Previous studies have shown that larvae from single cultures develop at different rates (Pechenik, 1990).

Experiment 1

After the first week of the experiment, unfed larvae reached the shrinking auricularia and doliolaria stages more quickly than larvae raised on the natural seawater and T-*Isochrysis* diets (Table 1). These results differ from those obtained in other studies of echinoderm larvae (Paulay et al., 1985; Young et al.,1989; Strathmann,

1992; Allison, 1994), where development was delayed when larvae were exposed to starvation or low food availability. In the case of *H. nobilis,* this rapid development to the doliolaria stage might be interpreted as a survival strategy when larvae are exposed to extreme conditions of food scarcity. By moving to the benthic stage, the larvae may have access to a higher quantity and quality of food.

Experiment 2

The combination of two or more algal cultures has often been recommended as a better diet than monoalgal cultures for planktotrophic larvae (Smiley, 1986; Strathmann, 1987; Pechenik, 1987). However, in this experiment, the mixed diet of T-*Isochrysis* and *P. salina* did not enhance the development of the larvae. Larvae developed at very similar rates among the three diet treatments. Similar responses of unenhanced growth when feeding on a mixed diet rather than on single species diet were reported for adults of the sea hare *Aplysia californica* and the sea urchins *Strongylocentrotus droebachiensis* and *Tripneustes gratilla* (Pennings et al., 1993).

No juveniles were obtained in any of the treatments tested in this study. The reason for this may be attributed to several factors: 1) *H. nobilis* larvae may have a growth rate that requires longer development times in the plankton, therefore the 30-day duration of this experiment might have been insufficient for the larvae to reach the pentacula or juvenile stage; 2) the diets lacked certain nutrients required by the larvae during the later stages of development, and nutritional requirements may change during larval development (Pechenik, 1987); 3) Absence of the triggering cues needed for the larvae to metamorphose. Some invertebrate larvae have been observed

to develop better when their culture medium had been previously conditioned by conspecific adults (Wilson, 1981). Laboratory studies of the seastar *Mediaster aequalis* demonstrated that larvae can delay metamorphosis for 128 days in the absence of appropiate substratum, but 32 of the 33 larvae tested in this experiment settled and metamorphosed nine days after exposure to a metamorphic inducer, whereas larvae that remained for the same period of time in clean glass bowls without inducers did not undergo metamorphosis (Birkeland et al., 1971).

Mortensen (1937) raised several species of tropical holothurians in the laboratory; and out of the 15 species raised, he was able to rear 7 of them to the juvenile stage and seven more near metamorphosis. The only species he was not able to raise beyond the first auricularia stage was *H. nobilis*. He concluded ".....it will probably be a very difficult task to rear them beyond this stage". Hopper (1990) raised larvae of another tropical holothurian, *Actinopyga mauritiana*, on various algal cultures and was only able to rear them to the doliolaria stage. All of these examples demonstrate that although larvae may be competent, this state does not guarantee that larvae will reach the juvenile stage. Studies have shown that for certain benthic species, larvae will not metamorphose in the absence of the appropiate inducer (Hadfield, 1978; Pawlik, 1986; Rodriguez et al.., 1993).

Effect of Diet on Survivorship

The results of both experiments 1 and 2 suggest that the alga T-*Isochrysis* was the best diet among those tested. The poor survival of larvae raised on natural seawater might be related to contamination of the culture, as the seawater used for this treatment was only filtered through a 60 µm mesh, which could have allowed the introduction of a wide variety of organisms that could compete with *H. nobilis* for food. For example, ciliates were often seen swimming inside the esophagus and stomach of the larvae. Contamination with ciliates in rotifer cultures is known to deplete the microalgae and to inhibit the growth of the rotifers causing low production or collapse of the cultures (Lim, 1991). Also, the larvae may have been exposed to metabolites released by other organisms in the culture. The sensitivity of larvae to certain metabolites has been found to vary during of development (Wilson, 1981).

The 30-day survival of the unfed larvae exceeds the time other echinoderm larvae have lasted without food in laboratory experiments. Echinoderm larvae from temperate waters die within few days if not fed (Young et al., 1989). This suggests that planktotrophic larvae from tropical environments may be better adapted to limited food resources for longer periods due to the fact that tropical reef waters are generally oligotrophic.

The low survival obtained with the mixed diet is quiet contrary to what the literature reports for larvae raised with mixed algal cultures. Smiley (1986) was able to raise the sea cucumber *Stichopus californicus* on a mixture of the unicellular algae *Isochrysis galbana, Pavlova lutheri,* and *Dunaliella tertiolecta.* However, when he fed

the larvae on monocultures of these species, the larvae did not develop well and few completed metamorphosis.

In conclusion, this study has demonstrated that diet has a major influence on the growth, development, and survival of *Holothuria nobilis* larvae. Thus far, I have only been able to raise the larvae to the late doliolaria stage. Future research efforts should concentrate in looking at chemical inducers and in finding a diet that will allow development to the juvenile stage. This phase of the study is of critical importance in developing techniques for the mass rearing of this and other holothurians.

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