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Fecundity and Survival of the Calanoid Copepod *Acartia tonsa* Fed *Isochrysis galeana* (Tahitian Strain) and *Chaetoceros mulleri*

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FECUNDITY AND SURVIVAL OF THE CALANOID COPEPOD *ACARTIA TONSA* FED *ISOCHRYSIS GALBANA* (TAHITIAN STRAIN) AND *CHAETOCEROS MULLERI*

by

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ABSTRACT

FECUNDITY AND SURVIVAL OF THE CALANOID COPEPOD *ACARTIA TONSA* FED *ISOCHRYSIS GALBANA* (TAHITIAN STRAIN) AND *CHAETOCEROS MULLERI*

Historically, red snapper (*Lutjanus campechanus*) larviculture at the Gulf Coast Research Lab (GCRL) used 25 ppt artificial salt water and mixed, wild zooplankton composed primarily of *Acartia tonsa*, a calanoid copepod. *Acartia tonsa* was collected from the estuarine waters of Davis Bayou and bloomed in outdoor tanks from which it was harvested and fed to red sapper larvae. We are developing a more controlled copepod mass culture system to support finfish larviculture. To that end, I examined the effect of single species versus mixed species of algae as food for the copepods. Wild *A. tonsa* were isolated and reared in a controlled environment. Diets used for culture of *A. tonsa* were T- Iso (Tahitian strain *Isochrysis galbana*) and *Chaetoceros* (*Chaetoceros mulleri*). Mated female copepods (n=100) were isolated from the laboratory population and placed individually in 10-ml containers. Twenty-five were fed T-Iso only at a cell density of 300,000 cells/ml, 25 were fed *C. mulleri* only at a cell density of 300,000 cells/ml, and 25 were fed a mixture of both T-Iso and *C. mulleri* at a cell density of 150,000 cells/ml each and 25 were fed no algae. Eggs were collected and counted to quantify fecundity over a 72-hour period. The experiment was repeated. Fecundity was higher in the mixed diet treatment where 17 of the 25 female *A. tonsa* survived (68% survival) and produced a total of 520 eggs.

Fecundity of *A. tonsa* in the small-scale experiment fell within the published range of 20-150 eggs/female/day for all the treatments offered a diet. Egg production

in treatments receiving a mixed diet was higher than production from any of the single diet fed treatments in all small-scale experiments. Production in the unfed treatment was significantly lower ($p<0.05$) than in all small-scale experiments. Diet did not affect survival ($p>0.05$) in any fed or unfed treatments over the 72-hour duration of the experiments. In a larger scale experiment, a total of 20, 1-liter beakers involving 5 replicates per treatment were used. Copepod density, algal density, water temperature, and salinity were the same as in the small-scale experiments. This larger scale experiment also was replicated. Individual egg production for the larger scale system fell within the published range of 20-150 eggs/female/day for the algal fed treatments. Individual production in the unfed control was below 3 eggs/female/day. The hatch rate of eggs produced from a larger scale experiment was not significantly different between treatments indicating that diet had no effect on the hatch rate and that eggs produced from the experiment were fertile.

Overall, the copepods produced eggs at a rate comparable to both published results and previous experiments at GCRL in all the algal fed treatments while few eggs were produced in the unfed controls. However, egg production was more consistent in the mixed diet than in the single species diet both when the copepods were tested singly and in a larger scale. There was no effect of diet on survival within each experiment when the copepods were tested singly and no effect of time on egg production. Between experiment variability may be attributed to variability in algal quality, specifically in single diet treatments.

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LIST OF ABBREVIATIONS

%.....percent

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ASW.....Artificial salt water

CCMP.....Center for Culture of Marine Phytoplankton

Chaet.....*Chaetoceros mulleri*

DHA.....Docosahexaenoic Acid

EFA.....Essential Fatty Acids

EPA.....Eicosapentaenoic Acid

GCRL.....Gulf Coast Research Laboratory

hrs.....hours

HUFA.....Highly Unsaturated Fatty Acid

m^2.....square meters

m^3.....cubic meters

mg.....milligrams

ml.....milliliters

®.....Registered Trademark

°C.....degrees Celsius

ppm.....parts per million

ppt.....parts per thousand

SE.....Standard error

spp.....species

T-Iso.....Tahitian strain *Isochrysis galbana*

µm.....micrometers

CHAPTER I

INTRODUCTION

Nutritional Value of Copepods in Larviculture

Typically, *Artemia* nauplii and rotifers (*Brachionus* spp.) are offered to teleost larvae at first feeding. Marine finfish larvae require first feeds that are high in the essential fatty acids (EFAs) eicosapentaenoic acid (EPA 20:5ω-3) and docosahexaenoic acid (DHA 22:6ω-3) (Watanabe, 1991) and investigations of the essential fatty acid (EFA) content of *Artemia* and rotifers used in the rearing of marine larvae suggests that even though these organisms are easy to produce, they may not be suitable for first feeding without enrichment with fatty acids to yield high larval survival (Watanabe *et al.*, 1983; Støttrup *et al.*, 1998). Copepods, on the other hand, are naturally rich in EFAs and may be more suitable as a first food. Watanabe (1991) investigated the highly unsaturated fatty acid (HUFA) content of *Acartia clausi* collected from the wild and determined that it had a relatively high percentage of total omega-3 HUFAs (30-60%). *Acartia clausi* fed marine *Chlorella* spp. had an EPA content of 20-30% of total EFA content. Thus, the high levels of EFAs in marine calanoid copepods like those in the genus *Acartia* make them prime candidates for rearing marine fish larvae (Watanabe *et al.*, 1983, 1991), particularly for those larvae of marine finfish that are either very small and cannot consume enriched *Artemia* nauplii and rotifers due to their small mouth gape or are slow growing or have very unique nutritional requirements. Finfish whose larval stages match any of these conditions are known as "difficult to rear" and include larvae of fish like the flame angel (*Centopyge* spp.), yellow tang (*Zebrasoma flavescens*) and the Gulf of Mexico red snapper (*Lutjanus campechanus*).

Standard hatchery techniques for snappers in the family Lutjanidae using the smaller ss-rotifers and *Artemia* as food have failed to yield adequate numbers of juvenile red snapper (*Lutjanus campechanus*) (Shields *et al.*, 2001; Laidley *et al.*, 2004). Similar results were obtained with the Australian golden snapper (*Lutjanus johnii*), where ingested rotifers were not digested within 5 days of commencement of feeding (Schipp *et al.*, 1999). Larvae of mangrove jack (*Lutjanus argentimaculatus*) reared in Thailand however, survived better when fed a calanoid copepod of the genus *Acartia* harvested from the wild and bloomed in outdoor tanks. Success was attributed primarily to the copepod's smaller size and easier digestibility (Doi and Singhagrawan, 1993; Schipp *et al.*, 1999). The high content of HUFAs in marine copepods and the importance of these fatty acids, specifically eicosapentaenoic acid (EPA 20:5ω-3), contribute to the successful development and rearing of marine "difficult to rear" teleost larvae (Watanabe, 1991; Mauchline, 1998c).

Techniques similar to those described by Schipp *et al.* (1999), in which wild sea water is pumped and sieved to collect zooplankton, have been implemented to rear red snapper larvae at GCRL. Ogle (1979) and Ogle and Lotz (2000) developed production of copepods using a "brown water" approach which involved transferring coarsely filtered estuarine water from Davis Bayou into a 70-m^3 (100-m^2 surface area) outdoor pond. The incoming water contained abundant zooplankton of which *Acartia tonsa* was the predominant species. The zooplankton was harvested 4-7 days post-filling when the copepod densities had peaked. Zooplankton was collected by removing approximately 50% of the pond's water, sieving it into various size fractions, and feeding the appropriate size fraction to larvae. A sub-sample of the daily harvest was counted to estimate abundance of individuals in the size class of interest. The water removed from a

harvested pond was replaced with Davis Bayou water to feed the remaining stock of copepods. The water in the outdoor tank was allowed to bloom for 48 hours before the next harvest.

Relying on the brown water technique for culturing copepods is likely to result in variable zooplankton quality, quantity and composition, and may result in variable fish larval survival. Consistency in the supply of zooplankton (calanoid copepods) and space are primary issues when using the "brown water" method. To acquire the desired zooplankton biomass, large volumes of water have to be pumped and sieved requiring a great deal of effort, staff and infrastructure. Currently, only 40,000 *L. campechanus* larvae can be fed by this method at the GCRL. Less than 40% of the larvae produced from induced spawning efforts are used for initial stocking (Day 0 –Day 1 post hatch). Survival from stocking to day 25 is less than 1%. Additionally, brown water-produced copepods are subjected to abrupt changes in salinity, temperature, pH and dissolved oxygen during their harvest and transfer to the larval fish tanks. These stressors can reduce copepod quality and also cause copepod mortalities. In order to reduce variability in copepod quality and survival, these stresses have to be minimized or eliminated.

One way to reduce variability in copepod nauplii production and quality and also reduce the risk of introducing pathogens to fish larvae is through mass culture of the copepods in a controlled environment. However, transitioning from harvesting wild zooplankton to indoor culture under controlled conditions demands a great deal of effort. It also requires infrastructure and specialized staff, but the benefits of developing a reliable copepod mass culture system capable of meeting live feed demand outweigh the costs. Controlled, intensive mass cultures of rotifers and artemia have been supplying researchers and culturists with a reliable and consistent source of those live feeds. The

development of a similar copepod culture system will produce live food of potentially higher quality than rotifers and *Artemia* due to the high HUFA content of copepods (Watanabe *et al.*, 1983; Watanabe, 1991) and provide a tremendous service to fish culturists and scientists.

Acartia tonsa (Copepoda: Calanoida)

Acartia tonsa is the predominant copepod in the brown water system. *Acartia tonsa* currently is being cultured at GCRL and is one of many candidate marine zooplankton species being evaluated for intensive mass culture. *Acartia tonsa* is a euryhaline, pelagic, calanoid copepod with a worldwide distribution. It is an opportunistic feeder that consumes primarily algae, but also diatoms (Roman, 1984), dinoflagellates (Kleppel, 1993), ciliates, rotifers (Stoecker and Egloff, 1987) and copepod nauplii (Tackx and Polk, 1982; Kleppel, 1993; Uye and Liang, 1998). *Acartia tonsa* is a broadcast spawner with documented fecundities ranging from 20 to 150 eggs/female/day (Parrish and Wilson, 1978; Sullivan and Ritacco, 1985; Mauchline, 1998a; McManus and Foster, 1998). Female *A. tonsa* are fertilized when the male attaches a single spermatophore to the genital somite that develops during the fifth copepodite stage (Mauchline, 1998b). The spermatophore adheres to the female by adhesive secretions extruded during transfer from male to female (Blades-Eckelbarger, 1991). Even though Uye (1981) found that female *A. clausi* fertilize all the eggs they will ever produce from a single mating event, *A. tonsa* must re-mate to produce successive broods of fertile eggs (Wilson and Parrish, 1971; Parrish and Wilson, 1978). *Acartia tonsa* produces eggs that sink and therefore isolate themselves from the adults during culture. Settled eggs can be easily collected without disturbing the adult population (Støttrup *et al.*, 1986). A

continuous supply of *A. tonsa* nauplii can be produced from those eggs in an intensive culture system.

Acartia tonsa's small naupliar size (50-60 μm) makes it a candidate food species for small "difficult to rear" marine finfish species. Recent advances in the rearing of larvae of marine ornamentals like potter's and flame angelfish (*Centropyge* spp.) and the popular yellow tang (*Zebrasoma flavescens*) show that using copepods as a first live food can increase larval fish survival (Laidley, 2004). Because *Acartia tonsa* is the dominant species in our harvested live feed we assume that *A. tonsa* is an appropriate first food for the *L. campechanus* larvae; therefore, we are investigating its intensive culture potential. Development of an efficient intensive culture system for *A. tonsa* may help improve existing larval rearing technologies for many species including cobia (*Rachycentron canadum*), amberjack (*Seriola dumerili*), red drum (*Sciaenops ocellatus*), spotted seatrout (*Cynoscion nebulosus*) and southern flounder (*Paralichthys lethostigma*). These fish species are potential candidates for stock enhancement programs as well as commercial aquaculture.

Copepod Diet

The importance of diet in copepod production is well documented (Dagg, 1978; Landry, 1978; Checkley, 1980; Durbin *et al.,* 1983; Donaghay, 1985; Sullivan and Ritacco, 1985; Kleppel, 1992; Dam *et al.*, 1994; Kleppel and Burkart, 1995; Mauchline 1998b). In the wild, the density of available food and that food's nutritional quality are two of the most important factors that determine the success of wild copepod production (Roman, 1984; Kleppel, 1993). The feeding rate of copepods is influenced by the density of the available food and container size (Anraku, 1964) as well as diet quality and particle

size (Fuller, 1937; Gauld, 1951; Paffenhöfer, 1976; Frost, 1977; Reeve and Walter, 1977; Kiørbe *et al.*, 1985).

In culture, a mixed diet may increase copepod fecundity and enhance production in existing intensive mass culture systems (Marshall and Orr, 1952; 1972). The current diet used in the intensive mass culture system for *A. tonsa* under development at GCRL is the Tahitian strain of the flagellate *Isochrysis galbana* (T-Iso) fed at a density of 50,000-100,000 cells/ml (Zillioux, 1969; Klein Breteler, 1980; Støttrup *et al.*, 1986; Bergreen *et al.*, 1988; Sun and Fleeger, 1995; Tiselius, 1998; Schipp *et al.*, 1999; Payne, 2000; Rippingale, 2000; Shields and Tang, personal communication). T-Iso also contains a high concentration of EFA especially EPA (20:5ω-3) a requirement for the successful propagation of larvae of marine finfish (Watanabe, 1991).

Copepod Fecundity

Egg production of *A. tonsa* is influenced by diet quality and quantity (Parrish and Wilson, 1978; Donaghay, 1985; Støttrup and Jensen, 1990; Kleppel, 1992; Kleppel and Burkart, 1995). Marshall and Orr (1953; 1972) found that the number of eggs produced was proportional to the food quantity available to mated female *A. tonsa*. Egg production per female per day increased as food availability increased (Durbin *et al.*, 1983; Dam *et al.*, 1994). Egg production also increased with temperature to an optimum temperature above which production decreased (Mauchline, 1998b). Optimal temperature for egg production of *A. tonsa* was reported as 15 °C and 20 °C and production was estimated at 34 eggs/day/female (Kiørbe *et al.*, 1985) and 40-50 eggs/day/female (Saiz *et al.*, 1992), respectively. However, Lemus (2004) reported that adult *A. tonsa* were just as productive at temperatures up to 25 °C.

Currently, copepod fecundity is assessed in the GCRL intensive mass culture system by estimation, assuming a sex ratio of 1:1. Estimates have high variance and evaluating the effect of treatments on fecundity and survival in mass culture systems is difficult. Lemus (2004) partially demonstrated the relationship between copepod density and total fecundity as well as individual fecundity. He examined six densities ranging from 50 to 1500 copepods/liter. Even though there was a significant positive linear relationship between total fecundity and copepod density, the individual fecundities decreased and the optimal density was not determined. Støttrup *et al.* (1986) observed mortalities and low egg production in the higher densities.

Objectives

Development of a reliable and consistent source of nauplii of the calanoid copepod *Acartia tonsa* for rearing larvae of "difficult to rear" marine finfish species has become a priority because lack of a suitable food has been identified as one of the big bottlenecks in the larviculture of these species. This research will investigate the hypothesis that a combination of two algal species for broodstock copepod husbandry will yield greater fecundities and higher survival than single species diets. A variety of diets for *A. tonsa* have been evaluated singly including the algae *Isochrysis galbana* (Tahitian strain) (Lemus, 2004), *Rhodomonas baltica* (Støttrup *et al.*, 1986; Berggreen *et al.*, 1988), diatoms and dinoflagellates (Dagg, 1977). Even though combined diets have been offered to maintain cultures they have not been evaluated thoroughly. *Isochrysis galbana* and *Rhodomonas baltica* in combination are the only two algal diets currently under evaluation (Marcus 2004, personal communication). Roman (1984) documented that *A. tonsa* also feeds on diatoms like *Chaetoceros mulleri.* Ianora et al. (1996) suggested that *Acartia clausi* fed the diatom *Thallasiosira rotula* produced significantly

fewer eggs than *A. clausi* fed the dinoflagellate *Prorocentrum minimum* and that the diatom diet produced a compound that inhibited the embryogenesis of *A. clausi*. However, there is no literature on the evaluation of *Chaetoceros mulleri* as a diet for *A. tonsa*.

The objectives of this study are to evaluate the (1) effect of the chrysophyte T-Iso and the diatom *C. mulleri* on individual and total fecundity of *A. tonsa* singly and in combination at high densities (300,000 cells/ ml), (2) survival of *A. tonsa* fed different diets and (3) effect of increased scale on the fecundity of *A. tonsa.*

CHAPTER II

MATERIALS AND METHODS

Copepod Acquisition

There were three prerequisites for copepods used in this study: 1) they had to originate from one culture batch to ensure that all the adults were of the same age and quality, 2) they had to be isolated and fasted for 24 hours to clear their guts of residual food, and 3) they had to be mated females to ensure that they were ready to reproduce.

Algae Preparation

Stock solutions of *Chaetoceros mulleri* and *Isochrysis* sp. (T-Iso) were purchased from Bigelow Laboratory for Ocean Sciences, Center for Culture of Marine Phytoplankton (CCMP), West Boothbay Harbor, Maine. Culture vessels were maintained under continuous fluorescent lighting (>10,000 Lux). Algae were cultured in batches at 22 ± 2 °C in 25 ± 1 ppt artificial salt water (ASW) prepared from reverse osmosis tap water that had been chlorinated at 50 ppm and de-chlorinated using sodium thiosulphate prior to use and mixed with Marine Mix salt™, Aqua Craft®, Inc., Hayward, California, an artificial marine salt. Cultures were continuously aerated and carbon dioxide was added for five seconds every five minutes at a differential pressure of 8 psi. Each batch was fed F/2 A and Fritz F/2 B culture media and silicate at 0.132ml/L/day and 0.23grams/L/day, respectively. To maintain adequate pH (7.8-8.4) and alkalinity levels (120-180 mg/L $CaCO_3$), soda ash was added at a rate of 0.2 grams/L. To start a batch culture of algae, algal stock was diluted at 1 part algae to 9 parts culture water. The algal incubation period prior to harvest was 7-10 days.

Testing Copepod Fecundity Individually

Fertilized female *A. tonsa* were placed individually in each of six flat-bottomed 10-ml sterile polystyrene wells on a FALCON® multiwell™ tissue culture plate (Becton Dickinson Labware, New Jersey), and algae were added to the wells. The water used was 25 ppt ASW as in the algae culture. The water was passed through an activated carbon filter (0.5-1.0 micron). Each well was filled with 10 ml of filtered ASW and a fasted, mated female *A. tonsa* was placed singly into each well. Diets were added to achieve total cell densities of 300,000 cells/ml either singly or in combination at a 1:1 ratio. Diet densities were high to ensure continuous availability. The treatments included T-Iso (4-8 μm cell diameters), *Chaetoceros mulleri* (4-9 μm cell diameter), a combination of T-iso and *C. mulleri* at a 1:1 ratio, and an unfed control. Each treatment tested 25 individual *A. tonsa* females. The experiment was performed indoors under continuous light at a temperature of 22±0.3 °C. The 10-ml multiwells were covered with their respective low evaporation lids and placed on an orbital shaker to maintain the diets in suspension. Fecundity was assessed every 24 hours. Eggs were removed every 24 hours. At the 24-hour check, residual diet density was measured and algae were added to maintain the desired densities of 300,000 cells/ml (Shields *et al.*, 2001). Survival of the adult *A. tonsa* also was assessed every 24 hours and dead females were removed. The duration of each of three replicate experiments was 72 hours.

Large scale

Twenty 1-liter beakers were stocked with the same batch of 25 ppt ASW and approximately 50 mated females held in a custom-made 200-micron, 2-inch diameter PVC cage at the same density as in the small scale experiments (1female/10 ml, for a cage volume of 500 ml). There were a total of five replicates per treatment. Fecundity in

these experiments was assessed every 12 hours to expedite egg counting, prevent eggs from hatching and prevent nauplii from being cannibalized. This strategy also helped monitor algal cell densities and adult copepod survival. The large scale experiments were replicated twice. Eggs collected from the second large scale trial were allowed to hatch to ensure fertilization and hatch rates comparable to previously unpublished trials.

Statistical Analysis

Eggs collected from each 10-ml well were counted every 24-hour period for a total of 72 hours. Mortalities also were recorded in 24-hour intervals. ANOVA with Bonferroni post-hoc tests was used to compare fecundity of individual copepods as well as total fecundity between the four algal diets (α=0.05). Since female copepods were used to collect 3 data points 24 hours apart, a repeated measures analysis also was conducted. Survival analysis also was performed to detect the effect of the different diets on survival of *A. tonsa.* All statistical analyses were performed using SYSTAT® Version 11 (Copyright © SYSTAT Inc., 2004).

CHAPTER III

Results

Testing Copepod Fecundity Individually

Eggs from all 3 experiments were collected and counted. A summary of the total fecundity in all 3 experiments is depicted in Table 1.

Table 1

Average and total individual fecundity of *A. tonsa* females fed one of three algal diets or and unfed control in 24-hour increments over a 72-hour period for Experiments 1-3.

Experiment	Alga	Fecundity 24 hrs	Fecundity 48 hrs	Fecundity 72 hrs	Total fecundity	Average fecundity
1	***I. galbana***	182.00	286.00	393.00	861.00	287.00 ± 60.91 [a, b]
	C. mulleri	250.00	53.00	66.00	369.00	123.00 ± 63.61 [a,]
	Mixed	395.00	472.00	520.00	1,387.00	462.30 ± 36.41 [b]
	None	89.00	35.00	9.00	133.00	44.33 ± 23.56 [a]
2	***I. galbana***	167.00	217.00	187.00	571.00	190.33 ± 14.53 [a]
	C. mulleri	437.00	382.00	405.00	1,224.00	408.00 ± 15.95 [b]
	Mixed	567.00	632.00	603.00	1,802.00	600.67 ± 18.80 [c]
	None	28.00	19.00	4.00	51.00	17.00 ± 7.00 [d]
3	***I. galbana***	179.00	328.00	302.00	809.00	269.67 ± 45.95 [a]
	C. mulleri	212.00	655.00	586.00	1,453.00	484.33 ± 137.62 [a]
	Mixed	343.00	830.00	828.00	2,001.00	667.00 ± 162.00 [a, b]
	None	9.00	17.00	10.00	36.00	12.00 ± 2.52 [a]

I. galbana = T-Iso, Mixed = 50%: 50% T-iso and *C. mulleri*
[a, b, c] Numbers sharing the same letter are not statistically different (ANOVA, Bonferroni post hoc test α=0.05)

Total fecundity in treatments containing a mixed diet was higher than total fecundities in treatments containing single diets. The unfed controls in all trials had the lowest total fecundities (Table 1; Figures 1-3).

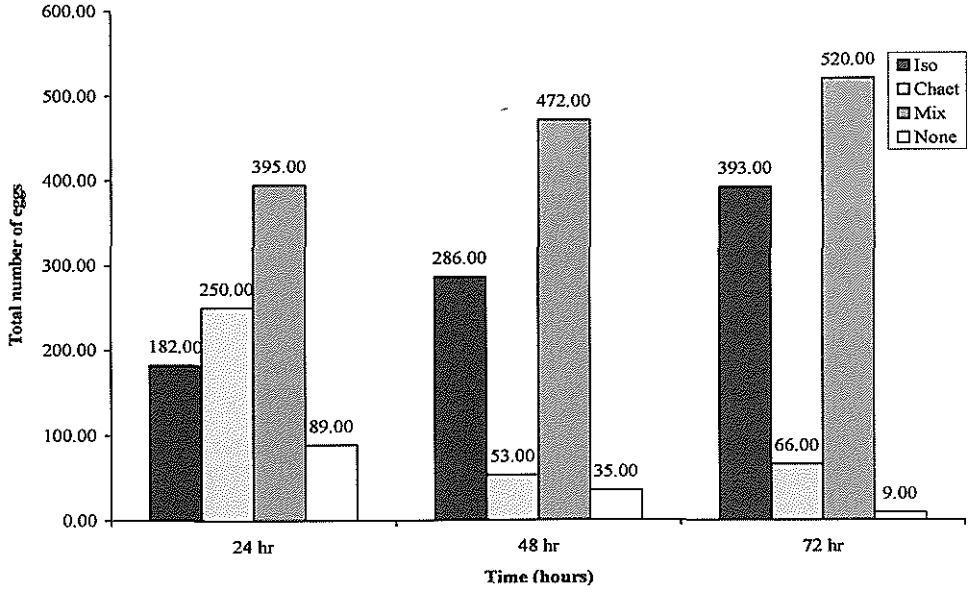

Figure 1 Total fecundity of individual *A. tonsa* fed three algal diets and an unfed control collected in 24-hour intervals in Experiment 1. Numbers on top of bars represent data values.

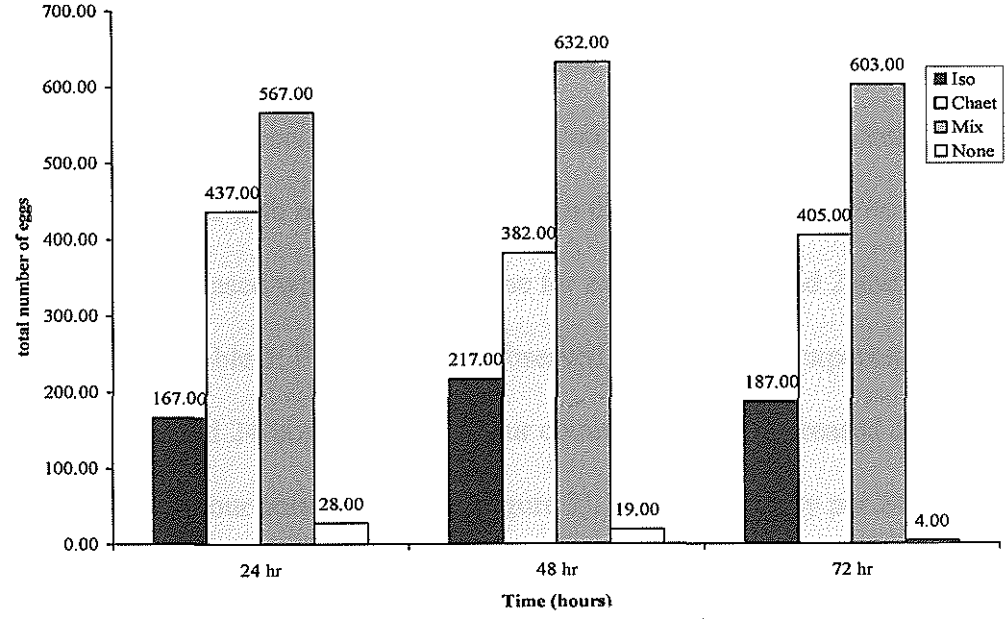

Figure 2 Total fecundity of individual *A. tonsa* fed three algal diets and an unfed control collected in 24-hour intervals in Experiment 2. Numbers on top of bars represent data values.

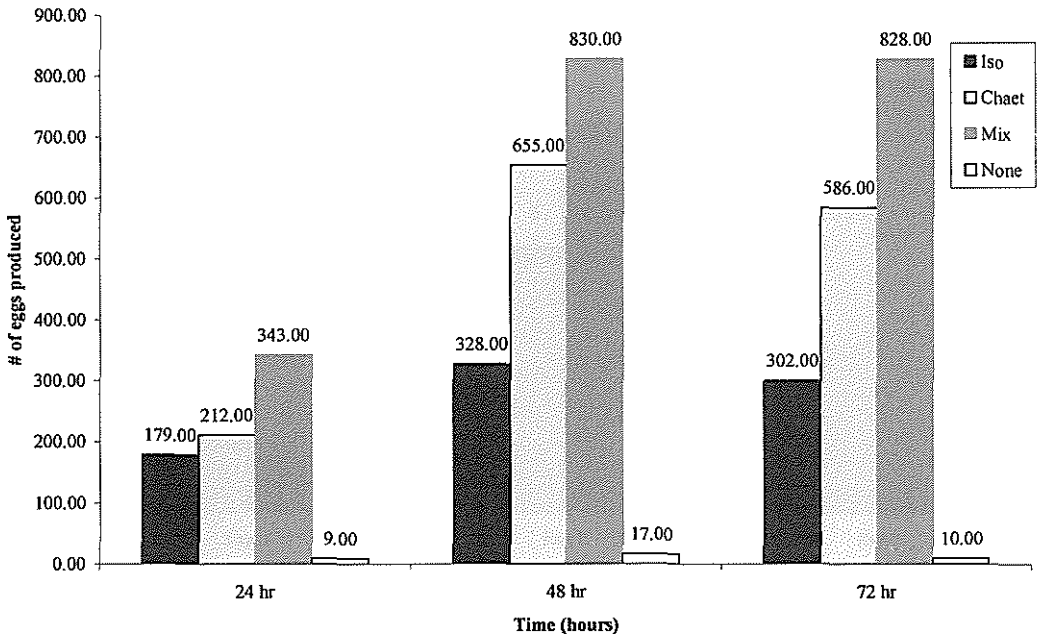

Figure 3 Total fecundity of individual *A. tonsa* for fed three algal diets and an unfed control collected in 24-hour intervals in Experiment 3. Numbers on top of bars represent data values.

Total fecundities increased as a function of time in all three trials of either T-Iso or mixed diet treatments. Total observed fecundities in the unfed controls in experiments 1 and 2 decreased with respect to time, and in the *Chaetoceros mulleri* treatment, total fecundities decreased with respect to time for experiments 1 and 2 and increased for experiment 3 (Table 1; Figures 1, 2). Observed fecundities in experiment 3 increased during the second 24-hour count (48-hour count), and then decreased during the third 24-hour count (72-hour count) (Table 1, Figure 3). Mean fecundity per 24 hours increased for *C. mulleri* and mixed diet fed treatments in all three experiments, decreased for the unfed controls and varied for the T-Iso fed treatment (Table 1, Figure 4).

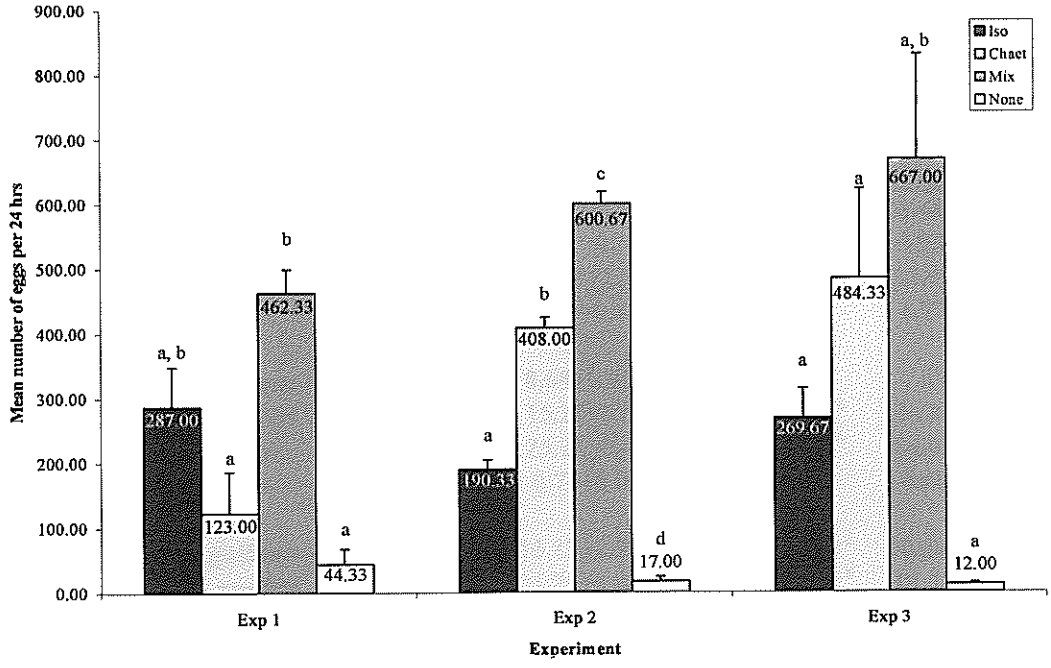

Figure 4 Mean fecundity of individual *A. tonsa* females fed three algal diets and an unfed control per 24 hours for Experiments 1, 2 and 3. Numbers on top of bars represent data values. Vertical lines represent the standard error of the mean. [a, b, c] Numbers sharing the same letter are not statistically different (ANOVA, Bonferroni post hoc test α=0.05)

Mean fecundity per female *A. tonsa* for the T-Iso fed treatments and the mixed diet fed treatments increased as a function of time in all three experiments. Mean fecundity per female *A. tonsa* for treatments fed *Chaetoceros mulleri* showed a decline as a function of time for Experiment 1, remained somewhat stable in Experiment 2 and increased in Experiment 3 (Table 2).

Table 2

Mean egg/female production of individual *A. tonsa* ± SE fed three algal diets and an unfed control per 24-hour interval and mean egg/female production ± SE per female for the 72-hour period (n=300)

Experiment	Alga	24 hr	48 hr	72 hr	Mean ± SE
1	***I. galbana***	7.28±0.55 [a]	11.44±0.85 [a, b]	15.72±1.08 [a, b]	**11.48±2.43 [a, b]**
	C. mulleri	10.00±0.88 [a]	2.12±0.25 [a]	2.64±0.51 [a]	**4.92±2.54 [a]**
	Mixed	15.8±0.98 [a]	18.88±1.20 [b]	20.8±1.51 [b]	**18.49±1.46 [b]**
	None	3.56±0.23 [a, b]	1.40±0.13 [a]	0.36±0.06 [a]	**1.77±0.94 [a]**
2	***I. galbana***	6.68±1.52 [b]	8.68±0.31 [a, c]	7.48±0.39 [c]	**7.63±0.58 [a]**
	C. mulleri	17.48±3.26 [a]	15.28±0.50 [a]	16.20±0.44 [a]	**16.32±0.64 [b]**
	Mixed	22.68+4.90 [a]	25.28±0.48 [b]	24.12±0.86 [b]	**24.03±0.75 [c]**
	None	1.12±0.14 [b]	0.76±0.06 [c]	0.16±0.07 [c]	**0.68±0.28 [d]**
3	***I. galbana***	7.16±1.62 [a]	13.12±2.97 [a, c]	12.08±3.19 [a, c]	**10.79±1.84 [a, c]**
	C. mulleri	8.48±1.32 [a, b]	26.20±4.84 [a, b]	23.44±6.88 [a, b]	**19.37±5.5 [a, c]**
	Mixed	13.72±2.31 [b]	33.20±5.66 [b]	33.12±8.32 [b]	**26.68±6.48 [a, b]**
	None	0.36±0.20 [c]	0.68±0.38 [c]	0.40±0.67 [c]	**0.48±0.10 [c]**

[a, b, c] Numbers sharing the same letter are not statistically different (ANOVA, Bonferroni post hoc test α=0.05)

The mean fecundities per copepod for every 24-hour interval for each experiment are depicted in figures 5, 6, and 7 respectively. Mean fecundities were computed using only copepods that were alive at the time of the count.

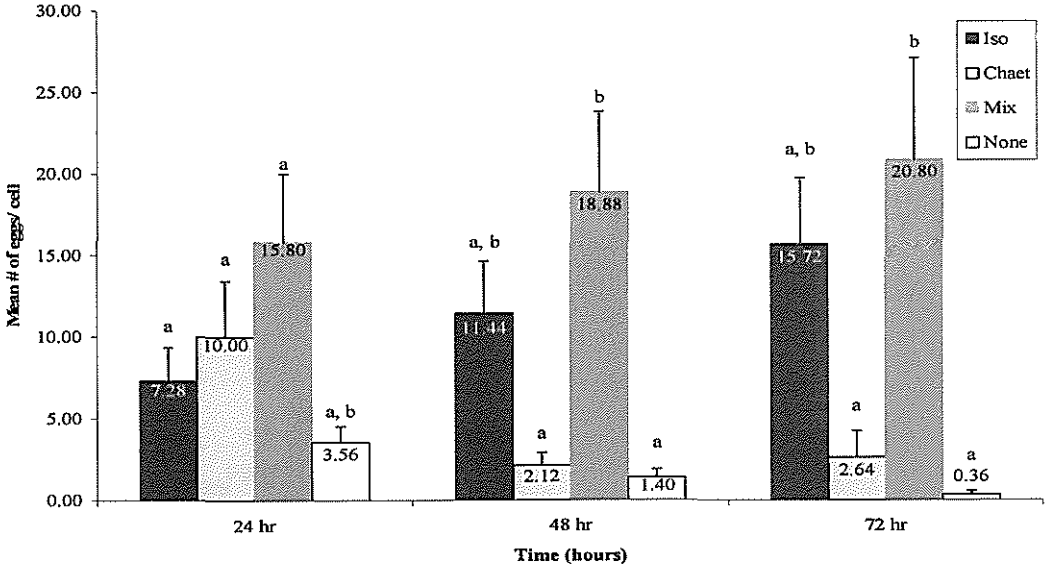

Figure 5 Mean number of eggs produced per female *A. tonsa* per 24-hour period fed three algal diets and an unfed control in Experiment 1. Vertical lines represent standard error of the mean (ANOVA α=0.05). [a, b, c] Numbers sharing the same letter are not statistically different (ANOVA, Bonferroni post hoc test α=0.05)

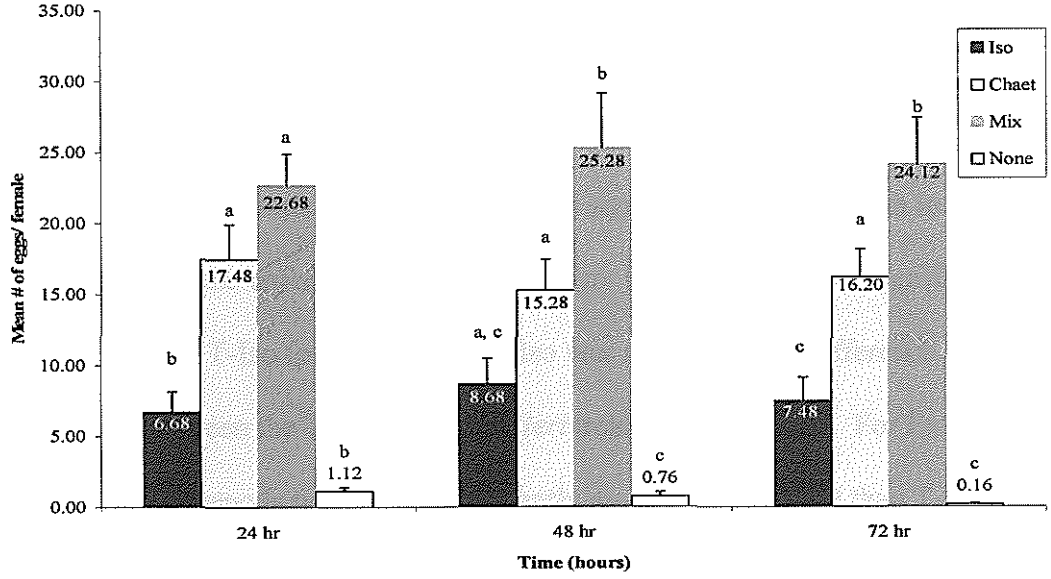

Figure 6 Mean number of eggs produced per female *A. tonsa* per 24-hour period fed three algal diets and an unfed control in Experiment 2. Vertical lines represent standard error of the mean (ANOVA α=0.05). [a, b, c] Numbers sharing the same letter are not statistically different (ANOVA, Bonferroni post hoc test α=0.05)

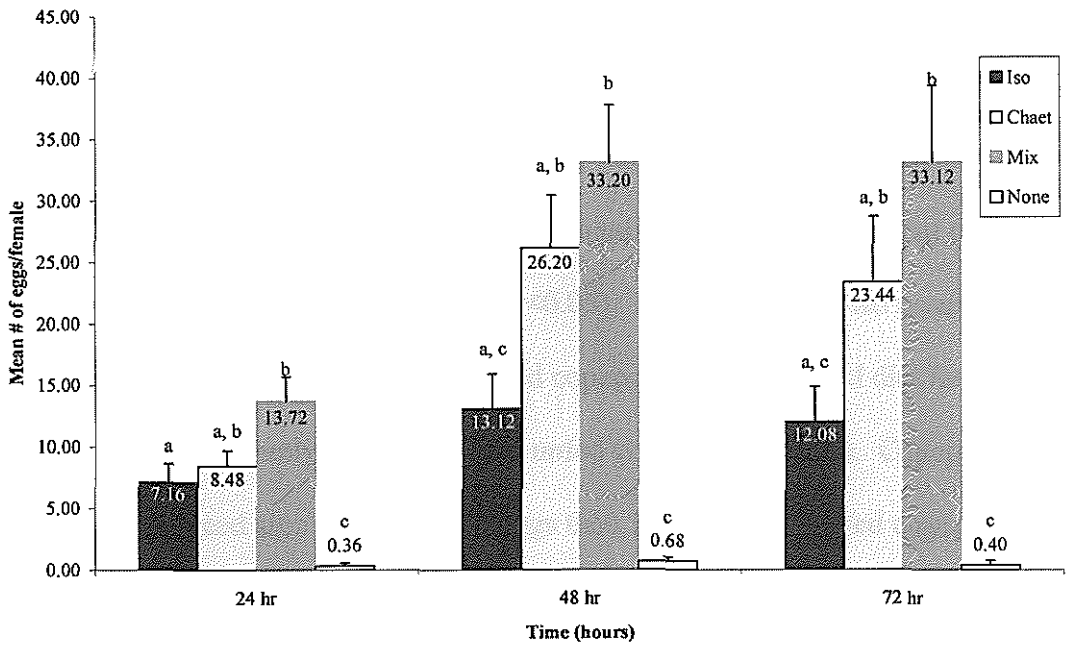

Figure 7 Mean number of eggs produced per female *A. tonsa* per 24-hour period fed three algal diets and an unfed control in Experiment 3. Vertical lines represent standard error of the mean (ANOVA α=0.05). [a, b, c] Numbers sharing the same letter are not statistically different (ANOVA, Bonferroni post hoc test α=0.05)

There was a significant difference ($p < 0.05$) in fecundity among treatments in all three experiments (Table 2). Results from Experiment 1 indicated that individual fecundities in the mixed diet treatment were significantly greater than in those fed *C. mulleri* and in the unfed control treatments ($p< 0.05$) but were not significantly different from those fed T-Iso ($p=0.31$). There also was no significant difference in individual fecundity between the T-Iso and *C. mulleri* treatments ($p=0.12$). For Experiment 2, *C. mulleri* fed copepods produced significantly more eggs ($p<0.05$) than T-Iso fed and unfed control copepods. Copepods fed T-Iso produced a significantly higher number of eggs than unfed copepods ($p<0.05$) but a significantly fewer eggs than *C. mulleri* ($p=0.001$) and mixed diet fed copepods ($p<0.05$).

Copepods fed a mixed diet produced significantly more eggs than T-Iso fed ($p<0.05$) and unfed ($p<0.05$) copepods and there was no significant difference in the number of eggs produced between mixed diet fed copepods and copepods fed *C. mulleri* ($p=0.277$). Results of Experiment 3 were very similar to those of experiment 2 where the fecundity of *C. mulleri* fed copepods was significantly higher than that of T-Iso fed copepods ($p<0.05$) and unfed copepods ($p<0.05$). Copepods fed T-Iso produced significantly fewer eggs than *C. mulleri* fed copepods and the mixed diet fed copepods ($p<0.05$) and significantly more eggs than unfed copepods ($p<0.05$). Copepods fed a mixed diet produced significantly more eggs than T-Iso fed and unfed copepods ($p<0.05$) but production was not significantly different than in *C.mulleri* fed copepods ($p=0.09$).

Survival of female copepods was best in treatments receiving a single or a mixed diet during the 72-hour duration of each experiment. Treatments receiving T-Iso had higher survival than the unfed control treatments which had the lowest survival. However, the difference in survival between treatments was not significant (Table 3, Figure 8) indicating no effect of algae on the survival ($p>0.05$) of *A. tonsa* for the 72-hour duration of the experiment.

Table 3

Mean survival time (hours) of *A. tonsa* females fed three algal diets and an unfed control in three experiments testing individual fecundity.

Experiment	Alga	Mean Survival Time (hr)± SE
1	*I. galbana*	50.88 ± 0.09 [a]
	C. mulleri	48.00 ± 0.09 [a]
	Mixed	57.60 ± 0.09 [a]
	None	53.76 ± 0.09 [a]
2	*I. galbana*	69.12 ± 0.06 [b]
	C. mulleri	71.04 ± 0.04 [b]
	Mixed	68.16 ± 0.06 [b]
	None	71.04 ± 0.04 [b]
3	*I. galbana*	66.24 ± 0.06 [b]
	C. mulleri	65.28 ± 0.08 [b]
	Mixed	58.56 ± 0.09 [a]
	None	69.12 ± 0.05 [b]

[a,b,c], Numbers sharing the same letter are not statistically different (Survival analysis α=0.05)

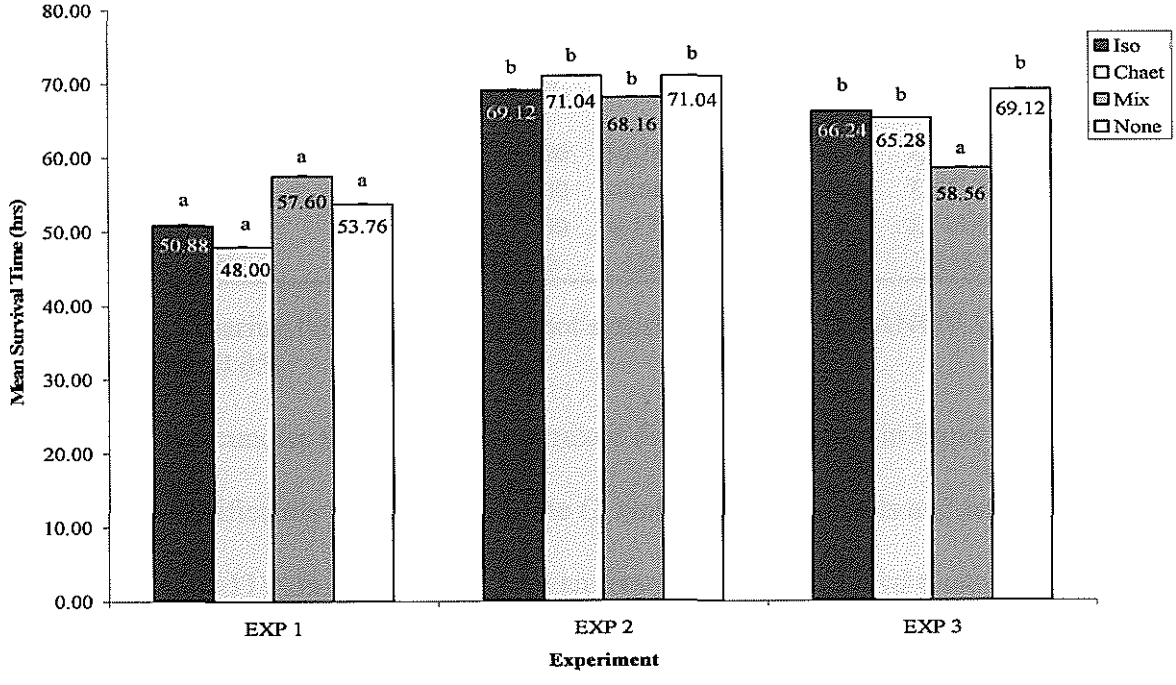

Figure 8 Mean survival time (hours) of individual *A. tonsa* fed three algal diets and an unfed control in three experiments. There was no statistical difference between mean survival time within each experiment (Survival analysis α=0.05).

Testing Copepod Fecundity at the Large-scale

Eggs from Experiments 4 and 5 were collected in 12-hour intervals. A summary of total fecundity per treatment is depicted in Table 4. Copepods fed *I. galbana* (T-Iso) produced more eggs compared to those fed *C. mulleri* and the mixed diet (Table 4, Figure 9). The unfed controls produced the fewest eggs. In Experiment 5 copepods fed a mixed diet produced fewer eggs than copepods fed single diets, and the unfed controls produced the fewest eggs (Table 4, Figure 10).

Table 4

Mean ± SE and total fecundity of *A. tonsa* for each of three algal diets and an unfed control in two large-scale experiments in 24-hour intervals for 72 hours

Experiment	Alga	fecundity 24 hrs	fecundity 48 hrs	fecundity 72 hrs	Total fecundity	Mean fecundity
4	***I. galbana***	3,111.00	10,189.00	14,866.00	28,166.00	9,388.67 + 3,416.89 [b]
	C. mulleri	2,559.00	4,131.00	2,402.00	9,092.00	3,030.67 + 552.03 [a]
	Mixed	3,223.00	8,943.00	13,024.00	25,190.00	8,396.67 + 2,842.46 [b]
	None	387.00	894.00	1,056.00	2,337.00	779.00 + 201.50 [a]
5	***I. galbana***	3,471.00	7,739.00	10,741.00	21,951.00	7,317.00 + 2,109.25 [a]
	C. mulleri	3,024.00	7,932.00	10,666.00	21,622.00	7,207.33 + 2,235.61 [a]
	Mixed	2,791.00	6,309.00	8,131.00	17,231.00	5,743.67 + 1,567.23 [a]
	None	393.00	223.00	120.00	736.00	245.33 + 79.60 [b]

[a,b,c], Numbers sharing the same letter are not statistically different (ANOVA, Bonferroni post hoc test α=0.05)

Mean fecundity for the T-Iso and mixed diet fed treatments in Experiment 4 was similar and significantly higher than for the *C. mulleri* fed treatments and unfed controls (Table 4, Figure 11). In Experiment 5, mean fecundities of all the fed treatments were similar and the mean fecundity of the unfed control treatment was significantly lower (Table 4, Figure 11).

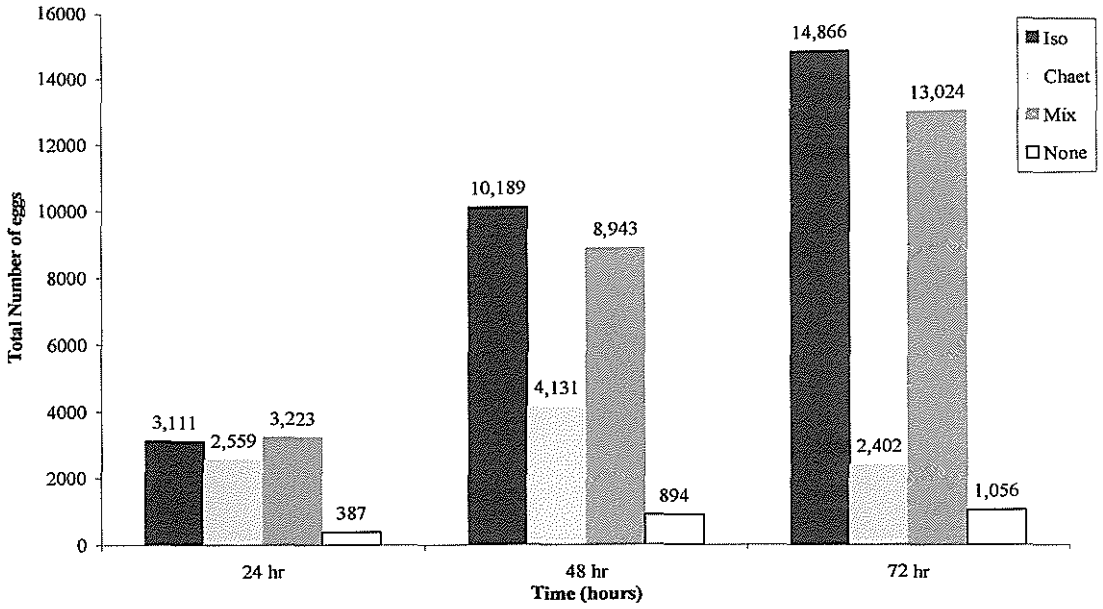

Figure 9 Total fecundities of *A. tonsa* at the large scale (Experiment 4) fed three algal diets and an unfed control in 24-hour increments over 72 hours. Numbers over bars represent data values

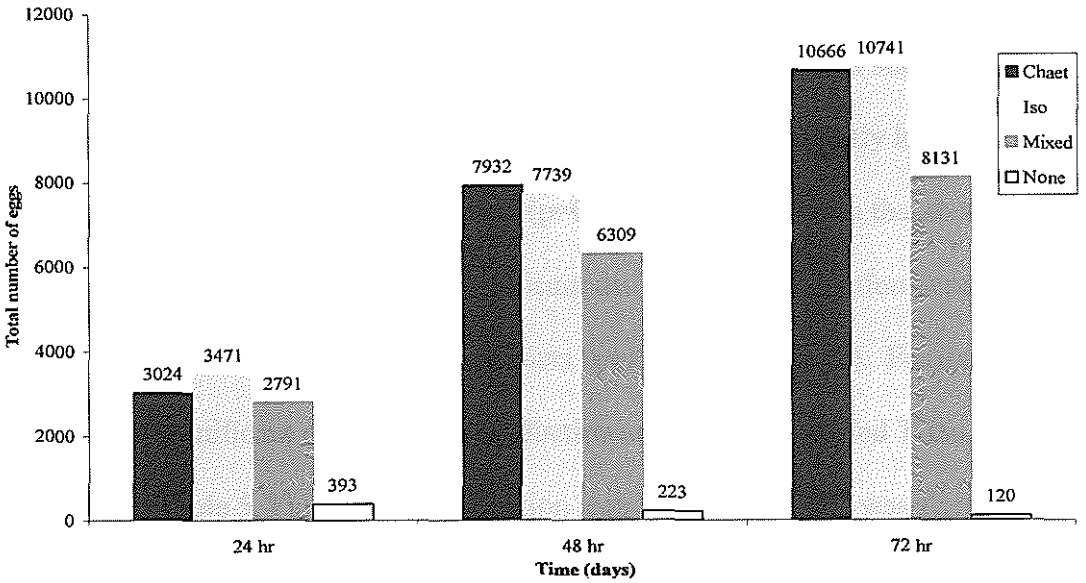

Figure 10 Total fecundities of *A. tonsa* at the large scale (Experiment 5) fed three algal diets and an unfed control in 24-hour increments over 72 hours. Numbers over bars represent data values

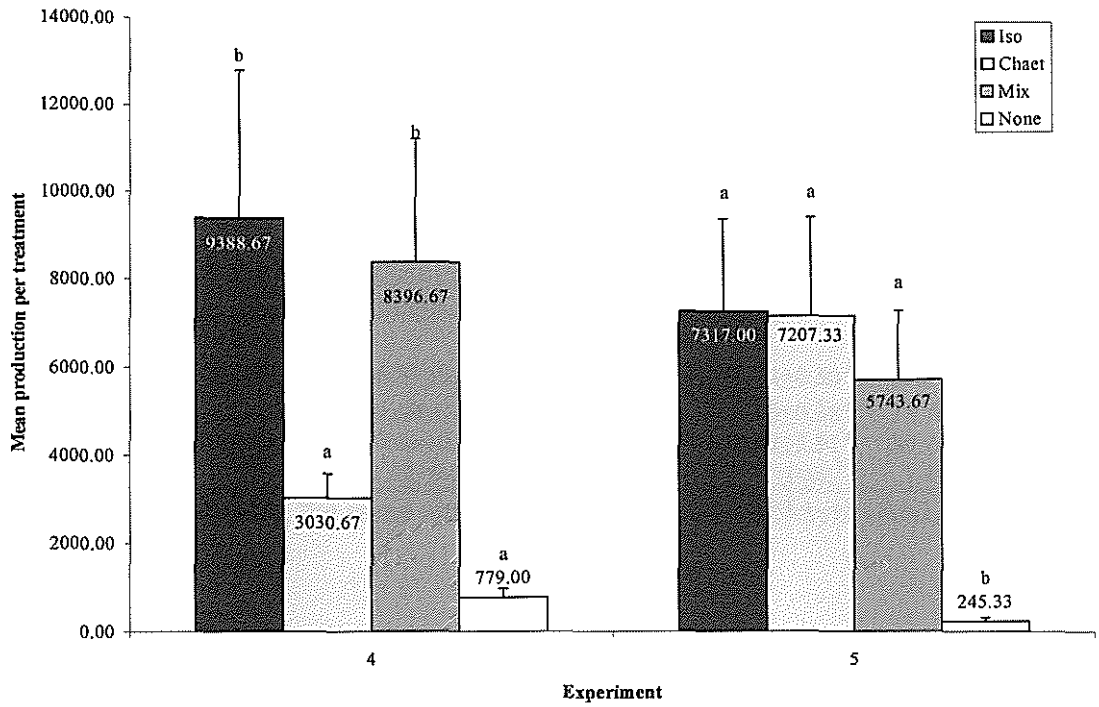

Figure 11 Mean fecundities of *A. tonsa* fed three algal diets and an unfed control in two large-scale experiments over 72 hours. Vertical lines represent standard error (ANOVA α=0.05)

Mean production of eggs per female *A. tonsa* per 24 hours is summarized in Table 5. Production of eggs from copepods fed *C. mulleri* was significantly lower than that from copepods fed T-Iso and mixed diets (p=0.002) and higher than that from unfed copepods (p=0.000). There was no significant difference in production between copepods fed T-iso and those fed mixed diets (p=1.000) (Figure 12). For experiment 5 there was no significant difference in production among copepods fed T-Iso, *C.mulleri* or mixed diets (Table 5, Figure 13). Production from unfed copepods was significantly lower (p=0.000) in both experiments.

Table 5

Mean production ± SE of eggs per female *A. tonsa* fed three algal diets and an unfed control in two large-scale experiments in 24-hour increments over 72 hours (n=1,557).

Experiment	Alga	24 hr	48 hr	72 hr	Mean
4	***I. galbana***	15.25±14.38 [b]	49.94±35.72 [b]	72.87±42.86 [b]	36.96±13.45 [b]
	C. mulleri	12.19±25.89 [a]	16.67±10.84 [a]	11.44±6.49 [a]	11.35±2.07 [a]
	Mixed	15.57±12.94 [b]	43.20±27.46 [b]	62.92±33.22 [b]	34.55±11.70 [b]
	None	2.13±3.58 [c]	4.91±5.85 [c]	5.80±10.37 [c]	2.60±0.67 [c]
5	***I. galbana***	16.97±0.18 [a]	38.00±0.29 [a]	52.84±0.50 [a]	35.94±10.41 [a]
	C. mulleri	14.48±0.34 [a]	37.04±0.50 [a]	49.66±0.38 [a]	33.73±10.29 [a]
	Mixed	17.63±0.85 [a]	40.29±1.11 [a]	52.36±1.91 [a]	36.76±10.18 [a]
	None	2.32±0.04 [b]	1.33±0.06 [b]	0.72±0.02 [b]	1.45±0.47 [b]

[a,b,c], Numbers sharing the same letter are not statistically different (ANOVA α=0.05)

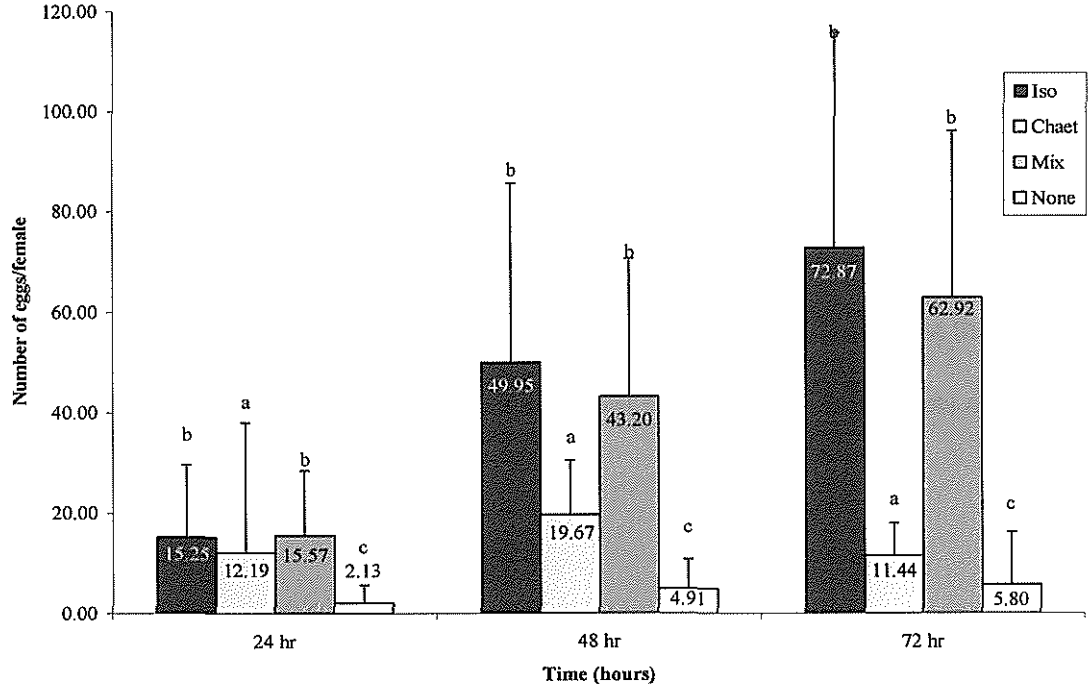

Figure 12 Mean number of eggs produced per female *A. tonsa* per 24-hour period fed three algal diets and an unfed control in Experiment 4. Vertical lines represent standard error (ANOVA α=0.05)

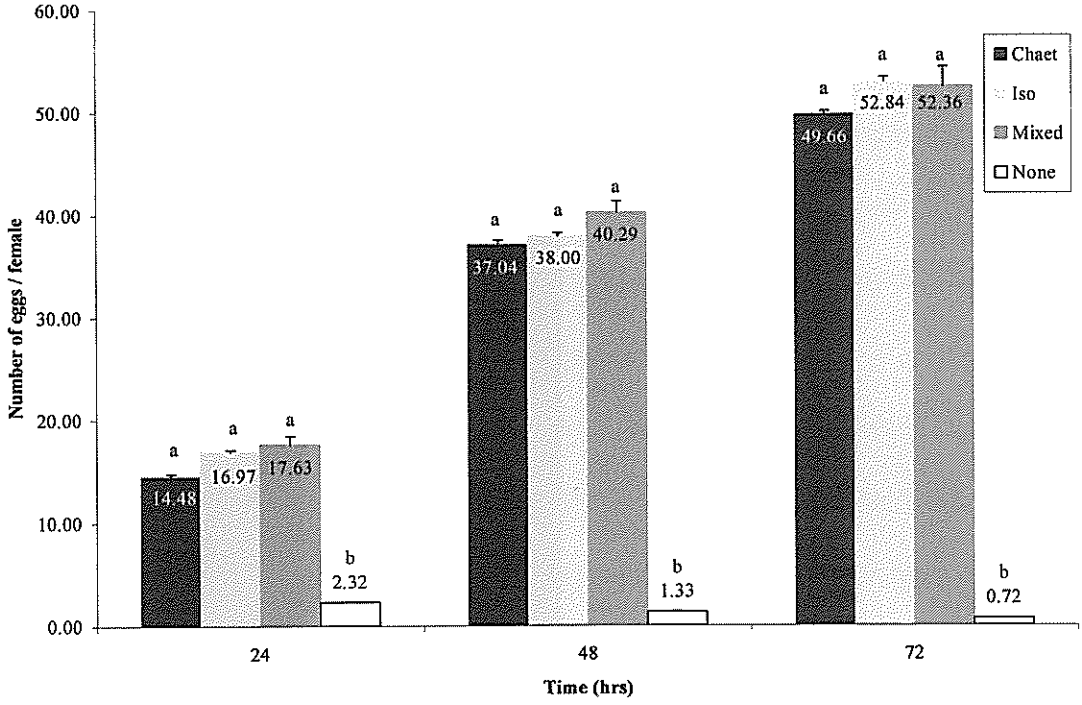

Figure 13 Mean number of eggs produced per female *A. tonsa* per 24-hour period fed three algal diets and an unfed control in Experiment 5. Vertical lines represent standard error (ANOVA α=0.05)

The hatch rate of eggs collected from experiment 5 was examined. The hatch rate was not significantly different amongst the different diets indicating that there is no effect of diet on the hatch rates and that eggs produced from females in all diets and the unfed control were fertile (Figure 14).

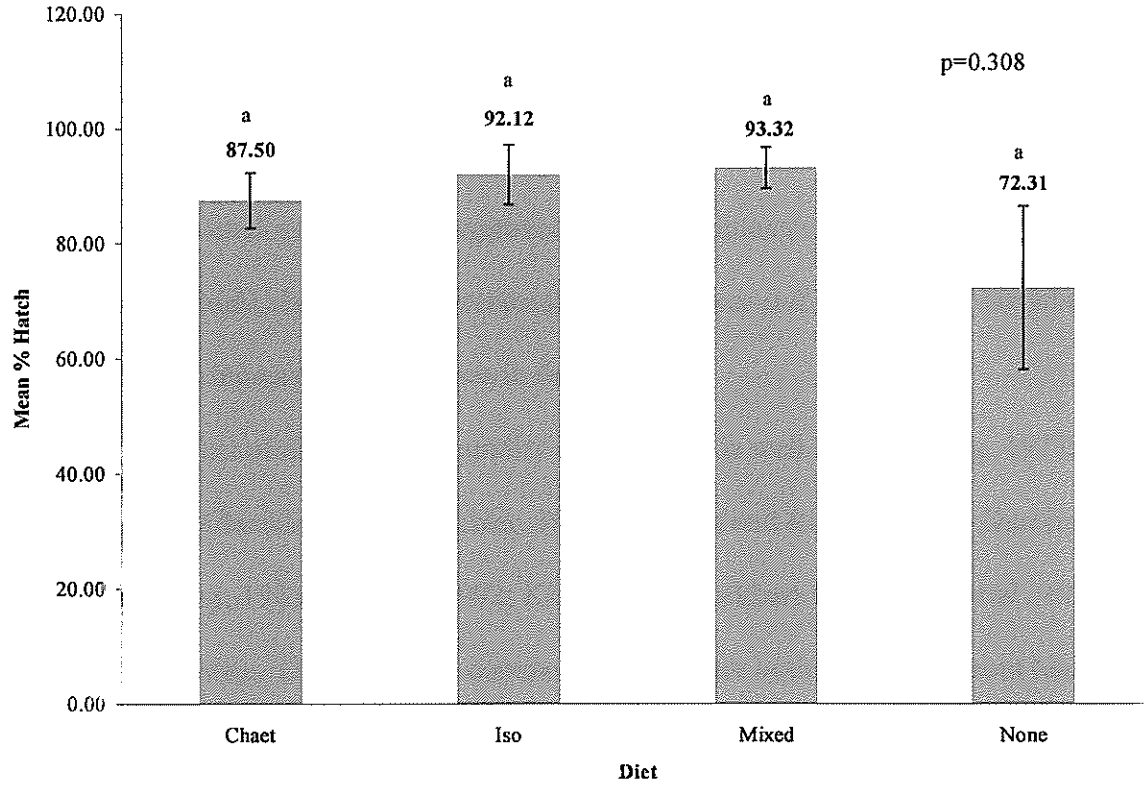

Figure 14 Mean hatch rate of eggs produced in Experiment 5 from *A. tonsa* fed three algal diets and an unfed control. Vertical bars represent standard error between means. [a,b,c], Numbers sharing the same letter are not statistically different (ANOVA α=0.05)

CHAPTER IV

DISCUSSION

A system allowing evaluation of single, mated female copepods would help accelerate the development of an improved diet for an intensive mass culture system. Testing individuals also provides greater statistical power and helps determine whether or not diet effects increases or decreases in fecundity.

The effect of diet on the fecundity of copepods is well known (Dagg, 1978; Landry, 1978; Checkley, 1980; Ambler, 1982; Sullivan and Ritacco, 1983; Durbin *et al.*, 1983; Donaghay, 1985; Kleppel, 1992; Kleppel and Burkhart 1995; Dam *et al.*, 1994; Mauchline 1998b). The need for a copepod mass culture system that produces high numbers of nauplii to rear marine finfish also has been identified as a priority to improve the survival of larvae of "difficult to rear" marine finfish such as he red snapper *L. campechanus* in a culture system. Lemus (2006) found that for a 500-L culture system fed a T-Iso diet, densities of adult *A. tonsa* on the order of 1500/L produced more than 10,000 eggs/L at a per capita production of 7.172 eggs. In this study, copepod densities were on the order of 100/L and the individual fecundity of *A. tonsa* was highest in copepods fed a mixture of T-Iso and *C. mulleri* diets at a 50:50 ratio in all three experiments. Despite the higher production of eggs in the mixed diet treatments, production of eggs was not significantly different from T-Iso fed copepods in Experiment 1 and *C. mulleri* fed copepods in Experiments 2 and 3. The results of all three experiments also demonstrate that lack of food reduces fecundity but does not eliminate survival for at least 72 hours. Even though there was no significant difference in copepod survival between the unfed and the algal diets in all experiments for the 72-hour period, there was a significant difference in egg production ($p<0.05$) in all three experiments.

Unfed copepods produced significantly fewer eggs in all three experiments (Table 1; Figures 1-3). Copepod fecundity individually and per treatment also decreased as a function of time for unfed copepods in Experiments 1 and 2, and remained unchanged over time in Experiment 3. (Tables 1-2; Figures 1-7). Both total individual fecundity and treatment total fecundities of copepods fed the mixed and T-iso diets increased as a function of time for Experiment 1(Tables 1-2; Figures 1 and 5). Total individual and treatment total fecundities of copepods fed *C. mulleri* decreased in experiment 1 (Tables 1-2; Figures 1 and 5). Total Individual and treatment total fecundities of all copepods receiving an algal diet increased as a function of time for Experiments 2 and 3(Tables 1-2; Figures 2, 3, 4, 6, and 7).

Anraku (1964) documented the effect of available food density and the container size on the feeding rate of copepods in culture. Residual algae counts taken in 24-hour intervals indicate that the density of copepods chosen (0.1/ml or 100/L) for all experiments was adequate to allow *A. tonsa* to feed and reproduce within the 20 to 150 eggs/female/day range documented by Mauchline (1998b), McManus and Foster (1998), Sullivan and Ritacco (1985) and Parrish and Wilson (1978). Residual algae in the fed treatments in all experiments were at a density of 20,000 cells/ml or greater indicating substantial consumption of the original 300,000 cells/ml fed to each experimental unit in every treatment. Observations of settled fecal matter in every fed unit also indicate consumption of the algae. Egg production of *A. tonsa* also is influenced by diet quality (Parrish and Wilson, 1978; Donaghay, 1985; Støttrup and Jensen, 1990; Kleppel, 1992; Kleppel and Burkart, 1995,) and quantity (Marshall and Orr, 1953, 1972). Marshall and Orr (1953, 1972) documented that production of eggs was proportional to the food quantity available to mated female *A. tonsa*, and Dam *et al.* (1994) and Durbin *et al.*,

(1983) documented that egg production per female per day increases as food availability increases. Støttrup and Jensen (1990) documented thresholds of algal concentrations below and above which the concentration of available algae did not decrease or increase production of eggs. The 300,000 cell/ml algal density used in this experiment was higher than the upper threshold density of approximately 150,000 cells/ml reported by Støttrup and Jensen (1990) and production did not exceed typical documented production/female/day of 10-30 eggs. Egg production also increases with increasing temperature to an optimum temperature above which production decreases (Mauchline, 1998b). Optimal temperature for maximum egg production by *A. tonsa* was reported as 15 °C and 20 °C and production was estimated at 34 eggs/day/female by Kiørbe *et al.* (1985) and 40-50 eggs/day/female by Saiz *et al.* (1992). Lemus (2004) demonstrated that production of eggs for 100 individuals/ liter densities was in the order of 10 to 30 eggs/female at temperatures up to 25 °C. Production of eggs in all three experiments in this study testing copepod fecundity individually was satisfactory at 22 °C.

Copepods fed a mixed diet produced more eggs both individually and as a group than copepods fed *C. mulleri,* but significantly fewer copepods fed T-Iso in experiment 4 (Tables 4-5; Figures 9 and 12). In experiment 5, total fecundities (Figure 10) and individual fecundities of copepods fed T-Iso, *C. mulleri* or a mixed diet were not significantly different (Table 5, Figure 13). The unfed copepods in both experiments produced significantly fewer eggs both individually and as a group (Tables 4-5; Figures 9-13). Experiments 4 and 5 are more representative of a mass culture system. Copepods in these experiments produced eggs at the same levels as the copepods in experiments 1-3. This is a clear indication that the change in container from an individual 10-ml cell to a 500-ml cage suspended in a 1-L beaker had no effect on the performance of the

copepods. Production in these experiments also was comparable to that documented by Lemus (2004) who found production of eggs for 100 individuals/liter densities was in the order of 10 to 30 eggs/female at temperatures up to 25 °C. Production also fell within the previously demonstrated range of 20-150 eggs/female/day (Parrish and Wilson, 1978 Sullivan and Ritacco, 1985; Mauchline, 1998b; McManus and Foster, 1998).

Based on the results of experiments 1-3 in which the total and mean production in the mixed diet treatments were 28% to 40% higher than the next highest production from a single diet treatment, it may be suggested that the average production per female from the mixed diets would produce twice as many eggs from our existing mass culture system assuming equal stocking and algal feed densities. Lemus (2004) reported a significant, positive linear relationship between stocking density and eggs and nauplii production per 12 hours for two separate experiments. However, production per female decreased as the stocking density increased and seemed to plateau at around 200-300 adults per liter where production per female is in the order of 10-20 eggs/female/12 hours. Even though at the individual level in experiments 1-3, a 34% average better production per female appeared insignificant. In a large scale set up, a 34% increase in per female production can be substantial. With the 500 liters per tank of working volume currently utilized in our mass culture system and a copepod stocking density of 100-150 adults/L females producing 30 eggs/female/day an estimated total daily production of approximately 1.125 million eggs can be achieved assuming a 1:1 sex ratio in a culture fed 50,000 to 100,000 cells/ml of an algal diet and held at a water temperature of 25°C. Females producing only 66% of 30 eggs/day would produce only a little over 700,000 eggs out of the same system under the same conditions. Since copepods are so much better than traditional live foods such as rotifers of various sizes and *Artemia* because of their fatty acid content, a 300,000 egg per

day reduction in production per tank makes the system less efficient than the system that produces 1.125 million eggs utilizing the same volume of culture water. Therefore, more water and space than is typically used in our culture system would have to be utilized to produce enough nauplii to feed larval marine finfish. However, the larger scale experiments (Experiments 4 and 5) are more representative of our current mass culture system. The fecundity of female *A. tonsa* from Experiments 4 and 5 was within a current production range from both the mixed diet treatments and the T-iso fed treatments indicating that a mixed diet could replace our current T-Iso diet without compromising production.

An advantage to a mixed diet is the possibility of providing copepods more complete nutrition that will translate into increased egg production making more nauplii available to feed *L. campechanus* larvae. Algae, though easy to produce, is sometimes inconsistent in quality among batches. Poor quality algae will result in poor production regardless of cell density due to the effect of diet quality on copepod egg production (Kleppel and Burkart, 1995; Kleppel, 1992; Støttrup and Jensen, 1990; Donaghay, 1985; Parrish and Wilson, 1978). The T-Iso diet for Experiments 2 and 3 produced fewer eggs as a group and individually than the *C. mulleri* diet, but Lemus (2004, 2006), my unpublished data and data from the existing mass culture suggest that the opposite should be true (i.e. T-Iso fed copepods should produce more eggs than *C. mulleri* fed copepods). Batch production of algae and time between experiments may have resulted in the use of algae with inconsistent quality which may have contributed to some of the variability in the results among experiments. The levels of egg production in all the mixed diet treatments in all 5 experiments were within the documented values on individual production (Parrish and Wilson, 1978; Sullivan and Ritacco, 1985; Mauchline, 1998b;

McManus and Foster, 1998; Lemus 2006, 2004). Total production in the mixed diet treatments was the least variable in all 5 experiments. This may be an indication that using two species of algae will result in more complete nutrition (i.e., variability in the quality of a single algal species may not be as important in a diet composed of two species), thus resulting in consistent and predictable production. There was higher variability in single diet fed treatments in all 5 experiments. Since algae used to feed the copepods in the different experiments originated from different batches with variable time between experiments, variability between the different algal batches may be the cause of the variability in single diet treatments in all experiments. A mixed diet may, therefore, provide nutrition that is not only complete but of consistent quality if nutritionally the two species complement each other. An algal diet of consistent quality will result in a consistent and predictable supply of copepod nauplii for the challenging and tedious task of rearing larvae of the "difficult to rear" marine finfish species.

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