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Use of RNA:DNA Ratios for Assessing Secondary Production of Planktonic Food Webs: Effects of Temperature, Salinity, Food and Heavy Metals

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Dedication

I dedicate this work to my mother, Judi Speekmann. Though my studies have taken me away from home, you are always in my heart and I appreciate your love, encouragement and support over the years.

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Use of RNA:DNA Ratios for Assessing Secondary Production of Planktonic Food Webs: Effects of Temperature, Salinity, Food and Heavy Metals

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Copepods, the dominant component of the zooplankton community, function as a vital ecological link within marine food webs. This dissertation evaluates using RNA:DNA analysis of individual copepods (*Acartia tonsa*) as an indicator for secondary production under a variety of environmental conditions (temperature, salinity, food and heavy metals) in the laboratory and field. A method was developed to measure RNA and DNA of individual *A. tonsa* using a nucleic acid fluorescent dye. RNA:DNA and egg production (EP) were all significantly higher for fed copepods compared to starved copepods after 48 hrs. The relationship between RNA:DNA and EP was measured for *A. tonsa* cultured at three different temperatures and salinities to determine if RNA:DNA analysis is a useful indicator of secondary production under a wide range of environmental conditions. Wild *A. tonsa* were also sampled bi-monthly from Nueces Bay and their RNA:DNA measured as an index of secondary production in the field. RNA:DNA of wild *A. tonsa*, corresponded with high RNA:DNA and EP of well fed

laboratory copepods and were inversely related to temperature. Secondary production of copepods is reduced in the presence of the dinoflagellate *Karenia brevis*, but it is unclear whether the effects are due to brevetoxin or poor nutritional quality. Fecundity, fecal production and RNA:DNA of wild *A. tonsa* were measured after being fed mono-algal and mixed-algal culture diets of *K. brevis* and *Peridinium foliaceum* for 48 hrs. Similar low values in all measured variables between 100 % *K. brevis* diet and starved copepods suggests *A. tonsa* does not graze *K. brevis* when offered as its sole food source. Significant differences in EP were found between mixed diets, but not RNA:DNA, suggesting EP is a more sensitive indicator of nutritional quality. Anthropogenic introductions of heavy metals also reduce zooplankton production. Cultured copepods were fed *Thalassiosira* spp. contaminated with either Cd or Cu, to determine if RNA:DNA and EP were affected by heavy metals. The present study found metals had no affect on EP or RNA:DNA. RNA:DNA analysis can be used as a proxy for zooplankton condition under known environmental parameters with strongest responses to change in food and temperature.

Table of Contents

List of Tables

List of Figures

Chapter 1: A method for measuring individual variability in RNA:DNA ratios of *Acartia tonsa*

ABSTRACT

Acartia tonsa is a dominant copepod in coastal waters and is an important link in the food web between microplankton and higher trophic levels. RNA:DNA ratios have been used to describe growth and nutritional condition of field collected copepods and to show strong correlation between group egg production and RNA:DNA ratios. A method was developed using a sensitive, nucleic acid fluorescent dye and automated microplate fluorometer to measure RNA, DNA and RNA:DNA ratio of individual *A. tonsa*. RNA, DNA, RNA:DNA ratios and egg production were all significantly higher in copepods fed *Thalassiosira* spp. compared to starved copepods. There was a general trend toward an increase in RNA:DNA ratios with increase in egg production, but due to the high degree of variation in both RNA:DNA ratios and egg production of individual copepods no significant correlation between RNA:DNA ratios and egg production was found. Significant differences in the RNA:DNA ratios between fed (7.2) and starved (3.4) copepods were found after 2 days. In the future this assay may be applied to other species of copepods sampled directly from the field, to provide an index of the health of planktonic food webs in nature.

INTRODUCTION

Zooplankton are an important food source for numerous marine organisms and their productivity affects food availability to higher trophic levels, such as larval fish. Numerous studies have shown that the nutritional condition of zooplankton changes with diet quantity and quality, which affects their secondary productivity in terms of growth

rates and egg production (Checkley 1980; Durbin et al. 1983; Kiørboe et al. 1985; Verity and Smayda 1989; Kleppel and Burkart 1995; Peterson et al. 2002; Hazzard and Kleppel 2003). Several methods have been used for estimating zooplankton secondary productivity, including measuring the increase in dry weight or carbon of individuals (i.e. growth rates) and/or measuring egg production (Durbin et al. 1983; Kimmerer and MacKinnon 1987; Richardson et al. 1999). However, these methods are labor intensive and require lengthy incubations (3-5 days) during which ambient environmental conditions must be maintained. Applying molecular assays, such as RNA:DNA ratio analysis, directly to field collected organisms, provides a direct approach to assessing nutritional condition and potential secondary production, because organisms can be collected from nature and their condition measured without further manipulation.

RNA is necessary for protein synthesis and the concentration of RNA in tissue is an index of the rate of protein synthesis. Because DNA per somatic cell is assumed to be constant in sexually mature adults, the RNA:DNA ratio is an estimate of the magnitude of protein synthesis within each cell (Bulow 1987). RNA:DNA ratios are positively correlated with growth rates and egg production of calanoid copepods (Nakata et al. 1994; Saiz et al. 1998; Bersano 2000; Wagner et al. 1998, 2001; Gorokhova 2003). Saiz et al. (1998) showed that egg production of female *Acartia grani* was significantly higher in well fed animals and positively correlated with RNA values and RNA:DNA ratios. *A. tonsa*'s egg production and RNA:DNA ratios were low when fed the non-nutritious brown tide alga *Aureoumbra lagunensis* compared to diets of more nutritious algae species, such as *Isochrysis galbana* or *Thalassiosira weissflogii* (Bersano 2000). *Calanus finmarchicus* also displayed high RNA:DNA ratios with increased food concentrations

(Wagner et al. 1998, 2001). Nakata et al. (1994) showed an increase in RNA:DNA ratios and egg production in *Paracalanus* sp. taken from the phytoplankton rich frontal waters of Kurshio current.

The aforementioned studies examining changes in nucleic acid values of *Acartia* spp. pooled between 3 - 20 individual *Acartia* spp. to obtain nucleic acid values (Saiz et al. 1998; Bersano 2000; Gorokhova 2003). Pooling organisms to obtain nucleic acid values eliminates the possibility of measuring individual variability and requires a larger number of organisms per experimental condition or location. Wagner et al. (1998, 2001), using ethidium bromide and a microplate reader, were able to measure RNA:DNA ratios of individual *Calanus finmarchicus* stages C1 - C6. Adult *C. finmarchicus* prosome length is approximately 3 times larger than *A. tonsa*, with the C1 stage of *C. finmarchicus* being roughly the same length (0.77 mm) as adult *A. tonsa* (Wagner et al. 1998). RiboGreen (Molecular Probes, Inc) is a fluorescent dye that binds both RNA and DNA and fluoresces (520 nm) when excited by blue light (480 nm) when bound to nucleic acids. RiboGreen can be used to measure extremely small concentrations (1.0 ng ml^{-1}) of RNA and DNA and is 200-fold more sensitive than ethidium bromide, another commonly used fluorescent dye (Jones et al. 1998). RiboGreen has been used to measure nucleic acid concentrations in relation to somatic growth of individual *Daphnia* (Vrede et al. 2002; Gorokhova and Kyle 2002). The increased sensitivity of the fluorescent dye and the use of a microplate fluorometer allow for the analysis of small individual copepods (~1.0 mm), such as *A. tonsa*.

This paper develops a method and discusses the application of RiboGreen to measure nucleic acid concentrations and RNA:DNA ratios of individual *A. tonsa* as an

estimate of nutritional condition and a proxy for secondary production. To determine the usefulness of RNA:DNA ratio analysis as an indicator of nutritional condition *A. tonsa,* egg production and nucleic acid content was compared for fed and starved copepods. Nucleic acid values were always measured individually, but estimates of egg production were investigated at both the group and individual level for fed and starved copepods to investigate individual egg production variability. In order to determine the most appropriate time to sample copepods, fed and starved *A. tonsa* were sampled every 24 hr for a 96 hr period for nucleic acid analysis.

METHODS

Zooplankton Collections

Zooplankton were collected at The University of Texas at Austin, Marine Science Institute pier located on the southern shore of the Aransas Ship Channel (27°50.3'N; 97°03.1'W) the evening prior to the initiation of an experiment. Evening tows were taken to increase capture of the target species because *A. tonsa* vertically migrates into surface waters at night (Stearns and Forward 1984). Collections were made using a 0.25 m diameter net with 153 μm mesh, which was deployed for 5 - 10 minutes depending on current speed. Large organisms were removed by pouring the sample through a 1000 μm mesh sieve. The remainder of the sample was taken back to the laboratory where it was aerated and kept in the dark until the sample was sorted the following morning. Adult *A. tonsa* from the plankton sample were sorted using a dissecting microscope.

Nucleic Acid Analysis

The protocol for nucleic acid analysis was modified from Westerman and Holt (1988) and Vrede et al. (2002). RNA and DNA were measured using the cyanine base fluorescent dye RiboGreen, which binds non-specifically to nitrogenous bases of nucleic acids (Jones et al. 1998). RiboGreen and 20 X TE buffer (200 mM Tris-HCL, 20 mM EDTA, pH 7.5, RNase/DNase-free water) were purchased from Molecular Probes. RNA standard (Type III baker's yeast), DNA standard (Type I calf thymus), RNase (Type III-A bovine pancreas), protease (Type VIII bacterial), Triton X-100, Tris[hydroxymethyl]aminomethane acetate, bovine albumin serum (BSA) and diethyl pyrocarbonate were purchased from Sigma. RNase/DNase-free water was made by treating ultra-pure water (Barnstead nanopure) with diethyl pyrocarbonate according to Sambrook and Russell (2001). Extraction buffer was prepared by diluting (20:1) 20 X TE buffer to 1 X TE buffer with RNase/DNase-free water and adding Triton X-100 (0.1% final concentration) and protease (0.1 mg ml^{-1} final concentration). Extraction buffer solution was made on the day of the analysis and kept in the refrigerator (4 ºC). RiboGreen reagent was prepared in the dark within an hour of analysis by diluting stock reagent 200-fold with 1 X TE buffer (i.e. reagent buffer) and held at 4 ºC in the dark until used in the analysis. Nucleic acid standards (RNA= 0.15 mg ml^{-1}; DNA = 0.10 mg ml^{-1} final concentration) were prepared by mixing the respective standard stocks in 25 ml of 1 X TE buffer on ice for 1 hr. Nucleic acid standards were separated into 1 ml aliquots and frozen at –80 ºC for up to 6 months. RNase (5.0 mg ml^{-1} final concentration) was prepared by dissolving RNase A and Tris acetate in 20 ml of RNase/DNase-free water,

then covered and boiled for 10 min. After cooling to room temperature, BSA at 10.0 mg ml^{-1} was added and mixed with a stir bar for 1 hr. The RNase was separated into 1 ml aliquots and kept frozen at –80 ºC for up to 6 months.

The day of the analysis the concentration of RNA and DNA standards were determined by measuring absorbance at 260 nm using a Spectra Max 190 microplate spectrometer and SOFTmax Pro 3.1.2 software (Molecular Devices). Concentrated standards were then diluted using the extraction buffer and diluted concentrations were measured by reading absorbance at 260 nm using the microplate spectrometer. RNA standards ranged from approximately 0.0 (blank) to 1500 ng ml^{-1} and DNA standards ranged from ca. 0.0 (blank) to 500 ng ml^{-1}. Two 100 µl aliquots of each RNA and DNA standard were placed into individual wells of a black 96-well plate. Four wells containing only extraction buffer were used as blanks. Individual copepods in microcentrifuge tubes were homogenized in extraction buffer (65 µl) using a 60 Sonic Dismembrator (6 pulses at level 4 or 2-3 Watts; Fisher Scientific) while on ice. The dismembrator probe was then rinsed with 50 µl of extraction buffer, which was added to the copepod homogentate. The homogenized copepod solution was incubated at room temperature on a shaker table (50 RPM; Daigger Orbital Shaker) for 1 hr. After incubation, 100 µl of copepod homogenate was placed into a black 96-well plate. Approximately 60 copepods were individually analyzed at a time.

RiboGreen reagent (100 µl) was automatically injected into each well using the FLUOstar Optima microplate spectrofluorometer (version 1.10-0 BMG Labtechnologies), the plate was shaken for 5 sec, incubated in the dark for 5 min and initial fluorescence readings (FL_1) taken. RNase A (25 µl) was then automatically

injected into one set of standards, half of the blank wells and all the copepods samples, which were allowed to incubate at 25 ºC for 30 min. RNase A was added to the RNA standards to insure that all RNA was digested and did not contribute to residual fluorescence. The enzyme was also added to the DNA standard to insure it did not alter DNA fluorescence. The final fluorescence value (FL_2) was then measured. The first fluorescent reading, FL_1 was a measure of all the nucleic acid present; FL_2 was a measure of DNA present, so that FL_1-FL_2 equals the amount of RNA present. Concentrations of copepod RNA and DNA were determined based RNA and DNA standard curves. RNA:DNA ratios were directly calculated form estimates of copepod RNA and DNA.

Egg Production / Hatching Success Experiments

Experiments to measure egg production (EP), hatching success and nucleic acid concentrations of *A. tonsa* were conducted on both groups of copepods and individuals. Copepods were acclimated under high food conditions (*Thalassiosira* spp. at 1.0 mg C L^{-1}) for 24 hr prior to the initiation of the experiment. Egg production experiments on groups or individual copepods were performed in which the two treatments were food (fed *Thalassiosira* spp. at 1.0 mg C L^{-1}) and no food (starved) over a 48 hr period. Group egg production experiments had three replicates per treatment, while individual egg production experiments had approximately 30 replicates per treatment. Group egg production experiments were carried out with 10 - 15 adult females (C6) in Nalgene bottles with 1 L of filtered (0.2 μm porosity) seawater with or without food. Individual egg production experiments were carried out with 1 adult female per glass scintillation vial in 15 ml of filtered seawater with or without food. Salinity was maintained at the

ambient conditions when copepods were collected (25 – 31 ppt). The experiment was conducted in a temperature controlled room (22.5 $\pm$ 0.5 ºC) under a 16:8 hr light-dark cycle and copepod incubations were slowly mixed using a rotating plankton wheel (1.5 rpm). After 24 hrs. the treatments were gently passed through nested 153 and 40 μm mesh sieves to remove eggs and nauplii produced during the initial 24 hr incubation period. The copepods were captured on the 153 μm mesh and returned to their original container in the environmental room with filtered seawater (with or without food) for an additional 24 hr incubation.

Eggs collected on the 40 μm mesh sieve from the initial 24 hrs were discarded. Previous work with *A. tonsa* concluded that there is a lag time of 17 - 24 hr before new food conditions were incorporated into egg production (Tester 1986; Tester and Turner 1990). After 48 hrs, the treatments were again gently passed through nested 153 and 40 μm mesh sieves. Copepods were examined under a dissecting scope to determine female survival. Individual live females were placed into a RNase/DNase-free microcentrifuge tube and flash frozen on dry ice for RNA:DNA analysis. Eggs collected on the 40 μm mesh sieve were placed in scintillation vials with 15 ml of clean, filtered seawater and returned to the environmental room to incubate for another 24 hr to determine hatching success rate. At the end of the incubation vial contents were fixed with 10 % formalin. Counts of preserved eggs and nauplii were made using a dissecting microscope. It was assumed that clear eggs were empty egg membranes, many of which may have decomposed before the final count, and they were not included in the count. For group egg production experiments, egg production rates were calculated for the second 24 hr

period as the number of eggs produced divided by the number of live females at the end of the experiment. Percent hatching was the number of nauplii divided by the total number of offspring (dark eggs + nauplii) multiplied by 100.

Time Series Experiment

An experiment was conducted to determine the optimal time to sample *A. tonsa* to observe differences in nucleic acids caused by changes in nutrition. Approximately 10 adult females were isolated into 1 L containers of filtered (0.2 µm porosity) seawater with or without food (*Thalassiosira* spp. at 1.0 mg C L^{-1}). The experimental treatments were placed on a rotating plankton wheel (1.5 rpm) in a temperature controlled room (22.5 $\pm$ 0.5 ºC) with a 16:8 hr light-dark cycle. Every 24 hr over a 96 hr period 6 - 10 copepods were collected on a 153 µm sieve and individually frozen for RNA:DNA analysis.

S*tatistical Analysis*

The statistical computer program Systat version 11.0 was used to perform parametric two-sample t-test using the Dunn-Sidak adjustment (to control for Type 1 error) or nonparametric Mann-Whitney U rank test on data sets with unequal normality or variance. In all cases, tests were performed at an experiment-wide alpha of 0.05. Statistical differences between the means of nucleic acid values for food and no food treatments were compared for DNA, RNA and RNA:DNA ratio. Statistical differences of egg production and hatching success for food and no food treatments were examined by comparing means of the total offspring (nauplii + viable eggs) per female and mean of percent hatching after arcsin (square root) transformation (Zarr 1999). Data are presented as means $\pm$ SE.

RESULTS

Preliminary experiments were run to determine the most efficient way to homogenize copepods and to evaluate the ability of this assay to measure DNA and RNA. An experiment comparing copepod homogentate made using the sonic dismembrator versus pestle and mortar showed no statistical difference in DNA and RNA between the two methods (DNA $t = -2.159$, $df = 13.7$, $p = 0.140$; RNA $t = -2.162$, $df = 13.7$, $p = 0.139$). Known quantities of DNA and RNA were added (i.e. spiked) to DNA and RNA standards. Recovery of the DNA spike (200 and 400 ng ml^{-1}) averaged 99 $\pm$ 2% and recovery of RNA spike (400 and 800 ng ml^{-1}) averaged 107 $\pm$ 5%. Standard curves from all experiments measuring RNA and DNA were linear with average r^2 values of 0.989 for DNA and 0.999 for RNA (Fig. 1.1). In all experiments, $\leq$ 1.0% of RNA standards remained after RNase digestion. To insure there was no residual fluorescence not associated with nucleic acids, reagent buffer minus RiboGreen was added to DNA and RNA standards and homogenized copepod and no fluorescence was observed.

DNA, RNA, RNA:DNA ratio, egg production and percent hatching of *A. tonsa* in the food treatment with group estimates of egg production were significantly higher in the food vs. no food experiment (Fig. 1.2; Table 1.1). DNA values for the food treatment averaged 107.9 $\pm$ 5.3 ng ind^{-1} and were significantly greater than the no food treatment with an average DNA concentration of 91.9 $\pm$ 4.4 ng ind^{-1} (Mann-Whitney $U = 1546.000$, $p = 0.029$). RNA values for the food treatment copepods averaged 1402.0 $\pm$ 70.3 ng ind^{-1}, while RNA values for the no food treatment copepods were 798.4 $\pm$ 32.5 ng ind^{-1} (Mann-Whitney $U = 2181.000$, $p < 0.001$). The average RNA:DNA ratio of 10.5 $\pm$ 0.8 for no food treatment individuals was significantly less than the average RNA:DNA ratio of 14.3 $\pm$ 1.0 for the food treatment individuals (Mann-Whitney $U = 1752.000$, $p < 0.001$). Egg production for fed copepods averaged about 82 $\pm$ 7 eggs $ind^{-1}day^{-1}$

compared to 8 $\pm$ 2 eggs $ind^{-1}day^{-1}$ for starved copepods (Fig. 1.2 D, E). There was significantly higher egg production in the food treatment than the no food treatment (Mann-Whitney U = 36.000, $p < 0.004$). There was also a significant difference in percent hatching between food (94% $\pm$ 2%) and no food (85% $\pm$ 2%) treatments (Mann-Whitney U = 33.000, $p < 0.016$).

There was no significant correlation between RNA:DNA ratios and egg production in individual copepods, however RNA:DNA ratios and egg production for the food treatment were significantly higher than no food treatments (Fig. 1.3; Mann-Whitney U: RNA:DNA ratio U = 715.000, $p < 0.001$; EP U = 732.500, $p < 0.001$). The variance of RNA:DNA ratios and egg production was high and is significantly higher for the food treatment copepods (RNA:DNA ratio $F_{30,26} = 2.641$, $p = 0.014$; EP $F_{30,26} = 36.204$, $p < 0.001$). In general, copepods from the no food group had RNA:DNA ratios below 3 and produced less than 10 eggs ind^{-1} day^{-1}, while *A. tonsa* with food had RNA:DNA ratios above 4 and egg production of greater than 15 eggs ind^{-1} day^{-1}.

The time series data trend was minimal change in DNA and maximal change in RNA and RNA:DNA after 2 days (Fig. 1.4). Significant differences between food and no food treatments were found in DNA and RNA per copepod on days 1, 2 and 3 (Table 1.2). RNA:DNA ratio values were significantly different between treatments on day 2 (Table 1.2). Copepods in the no food treatment did not survive past day 3, so no comparisons of nucleic acid values could be made after that time.

DISCUSSION

The RiboGreen RNA:DNA ratio protocol used in this study allows for the analysis of nucleic acid in individuals of small copepod species, such as *A. tonsa*. This allows for examination of variability between individual copepods, which is lost when

multiple individual must be pooled to produce adequate material for analysis. The present study has also shown RNA:DNA ratios and egg production at the individual level is far more variable than previously suspected. However, RNA:DNA ratio measurements may provide a direct estimate of zooplankton nutritional condition with minimal laboratory manipulations. Because *A. tonsa* is a dominant copepod species in coastal and estuarine systems throughout the northern hemisphere, this method may be applied broadly to assess the condition of marine zooplankton. In addition, this method can be applied to other zooplankton species and should show similar relationship with RNA:DNA ratios, egg production and food conditions, though absolute values of nucleic acids and productivity will be species specific.

The increase in RNA, RNA:DNA ratios and egg production of *Acartia* spp. with food found in the present study is in agreement with previous studies looking at the relationship between nucleic acid values and egg production with respect to nutritional condition (Saiz et al. 1998; Bersano 2000; Gorokhova 2003). However, direct comparison of nucleic acid values with these studies is difficult because the target species and/or methods used vary. Bersano (2000) used ethidium bromide to measure RNA:DNA ratios of pooled adult *A. tonsa* and showed that RNA:DNA ratios and egg production were highest for copepods feeding on *T. weissflogii* compared to other algae or no food. RNA:DNA ratio values ranged from 5 on a nutritionally poor diet of Texas brown tide *A. lagunensis,* to 17 on a diet of *T. weissflogii* and egg production ranged from 1 - 40 eggs $ind^{-1}d^{-1}$ between the diets (Bersano 2000). Saiz et al. (1998) worked with pooled sample of *A. grani* and showed a linear increase in egg production of 5 - 65 eggs $ind^{-1}day^{-1}$ with an increase in RNA (0.35 - 1.00 μg ind^{-1}) and RNA:DNA ratios (10 - 29)

that was temperature dependant. Studies with *Acartia bifilosa* showed RNA (50-100 μg) and RNA:DNA ratios (2 - 4.5) that were dependant on diet (low, medium or high diets based on particulate organic carbon), but not temperature dependant (Gorokhova 2003). Based on the body carbon-length regression analysis for *A. tonsa* under similar temperature (20 °C) and food conditions (*T. weissflogii*) from Thompson et al. (1994), the present study estimates the carbon content (μg C ind^{-1}) of *A. tonsa* to be between 3 - 6 μg C ind^{-1} depending on food condition and prosome length. Using this range of carbon estimates for the fed copepods in the present study, the range of DNA concentration is 18.8 - 37.0 μg mg C^{-1} and of RNA range is 233.7 – 473.3 μg mg C^{-1}. This translates into a range of RNA:DNA ratios of 12.4 – 12.8. These calculated values for RNA and RNA:DNA ratios are two to three times higher than those presented for *A. bifilosa*, but within the range of values previous published for *A. tonsa* and *A. grani* (Sazi et al. 1998; Bersano 2000) indicating that the RiboGreen RNA:DNA ratio analysis method used in the present study is reasonable.

Strong correlations between RNA:DNA ratios and egg production have been noted for other calanoid species. A positive relationship between nucleic acid concentrations and growth rate in response to food quantity was shown for individual *C. finmarchicus* at several different developmental stages using ethidium bromide (Wagner et al. 1998, 2001). Nakata et al. (1994) concluded that RNA:DNA ratios accounted for 70% of the variation in egg production of *Paracalanus* sp. from frontal waters of the Kurshio. Differences in species life history, metabolism, cell biochemistry, as well as different methodology, such as extraction buffers, binding of fluorescent dyes and/or fluorometric equipment can all be factors contributing to the wide range of nucleic acid

values between these studies, but the positive relationship between nucleic acid and egg production with food holds true for all three studies.

Fed copepods have a significant difference in all nucleic values compared to starved after 2 days in the time series experiment, but RNA:DNA ratios were not statistically different at day 1 and day 3 (Fig. 1.4). Furthermore, day 2 had maximum difference in RNA:DNA ratios and minimal difference in DNA. After one day there was little change in RNA, suggesting that the copepods did not have sufficient time to utilize the new food resulting no change in RNA:DNA ratio. Day 3 had the maximum decline in DNA per copepod, most likely due to cell death in the starved copepods; this lower DNA content contributed to the lack of significant difference in RNA:DNA ratio. In contrast to these results with *A. tonsa*, Verde et al. (2002) measured RNA:DNA ratios of *Daphnia* on diets of different food quality and found significant differences in RNA:DNA ratios after only 5 hr.

The present study found that DNA values of fed *A. tonsa* were higher than for the starved copepods (Fig. 1.2 A and 1.4 A). A difference in DNA with food was also shown for *Pseudocalanus elongates, Calanus glacilais*, *C. finmarchicus* and *A. tonsa* (Escribano et al. 1992; Wanger et al. 1998, 2001; Gorokhova 2003). The assumption that the amount of nuclear and DNA within cells is constant and can be used as an indicator of cell number (Bulow 1987) was based on vertebrate work and may not apply directly to invertebrates. There is evidence that the number of cell nuclei per individual at several developmental stages for crustaceans (three calanoid species and *Artemia*) remains the same (McLaren and Marcogliese 1983; Olson and Clegg 1978). It is not known if the change in total DNA content of *C. finmarchicus* is due to difference in cell number or

cellular DNA. Wagner et al. (2001) showed RNA:DNA ratios were strongly related to food concentration despite a difference in DNA, but cautioned that RNA:DNA ratios may not represent the true RNA content per cell and could be underestimating the difference between treatments.

There are two basic ways in which intra-individual DNA can vary: the expansion of somatic DNA through endopolypoidy and reducing DNA of the somatic genome via chromatin diminution; both processes have been documented in arthropods (Gregory and Hebert 1999 and references therein; Wyngaard and Rasch 2000). Wyngaard and Rasch (2000) working with several cyclopoid species found increased DNA in gonadal nuclei due to chromatin diminution and suggested the change occurred in germ cells during gametogensis. In cyclopoid copepod *Acanthocyclops,* mitochondrial DNA increases due to increased demand for mRNA during oogenesis (Standiford 1988). The author suggests that loss of DNA in starved copepods may be due to cell death. In the time series data set there was a decline in DNA over the 3 days of starvation followed by death at day 4, but there was little change DNA of the fed copepods over 4 days (Fig. 1.4 A). There is some evidence in other invertebrates systems, such as cnidarians and bivalves, that exposure to stressors may induce programmed cell death (Bosch and David 1984; Steinert 1996).

Gorokhova (2003) suggests that RNA per carbon weight is a more reliable indicator of growth because of the variability in DNA. The author agrees that the response range of RNA is larger than that of DNA, but it is difficult to calculate RNA per carbon weight at the individual level without estimating weights based on group measurements or regression correlation, both of which introduce uncertainty. Regardless of the reason for the change in DNA values, the amount of DNA variation is much less

than that for RNA and the overall trend in RNA:DNA ratios were as expected under extreme nutritional conditions, thus providing a reasonable approximation of potential productivity under well defined conditions.

Egg production values of 80 eggs ind^{-1} day^{-1} for groups of fed copepods are high, but within the range of previously published values (Ambler 1986; McManus and Foster 1998). Individual egg production values for the fed copepods are lower (between 0 - 40 egg ind^{-1} day^{-1}), but also within the range of reported values of 1.6-51.6 egg ind^{-1} day^{-1} (Durbin et al. 1983), 9.6-57.6 egg ind^{-1} day^{-1} (Stearns et al. 1989) and 1.5-42 egg ind^{-1} day^{-1} (Kleppel et al. 1998). Natural changes in environmental history (temperature, salinity and food quantity and quality) of the copepods may account for the differences in egg production between experiments. Although the current study attempted to hold temperature and salinity conditions within the ambient collecting range, no attempt was made to feed copepods a natural food assemblage. *Thalassiosira* spp. was chosen as our food source because it has shown to be an adequate food source in previous studies (Checkley 1980; Tester and Turner 1990; Tiselius et al. 1995; Bersano 2000). However, recent studies have suggested that indicators of food quality, such as protein, lipid, fatty acid, amino acids, and carbon and nitrogen content are critical for maximum egg production (Jónasdóttir 1994; Kleppel et al. 1998; Kleppel and Hazzard 2000; Jones et al. 2002; Hazzard and Kleppel 2003). Egg production of *A. tonsa* can be greater than or equal to 40 eggs ind^{-1} day^{-1}, but can drop to less than 16 eggs ind^{-1} day^{-1} when fed a diet deplete of essential fatty acids (Kleppel and Hazzard 2000; Hazzard and Kleppel 2003). The present study made no effort to quantify food quality in the environment from which zooplankton samples were taken and prior food history could affect egg production.

Alternatively, the type of sample bottles (plastic vs. glass) and the volume 1000 ml to 15 ml were different between treatments, which could have contributed to the difference in egg production between group and individual egg production. It has been suggested that adult calanoid copepods may cannibalize nauplii and copepoidites when copepod abundance is high and/or food availability is low (Dong et al. 1999; Turner et al. 1999).

An increase in individual egg production variability is expected compared to egg production measured from a group of copepods. Because of the inherent difficulties of monitoring individual egg production, there are only a few published values. Stearns et al. (1989) monitored seasonal egg production of individual *A. tonsa* and presented mean values ranging from 0 - 57 eggs ind^{-1} day^{-1}, with egg production values correlating strongly with gut fullness. Egg production of individual *Calanus helgolandicus, Temora longicornis* and *Pseudocalunas elongates* was measured over a two year period and production of *C. helgolandicus* had the greatest range of 10 - 40 eggs ind^{-1} day^{-1} (Bautista et al. 1994). Estimates of egg production are usually based on experiments monitoring 5 - 10 copepods L^{-1}, so any measure of individual variability will be lost (Calbet and Alcaraz 1996; McManus and Foster 1998; Saiz et al. 1998; Bersano 2000; Hazzard and Kleppel 2003). It is likely that RNA:DNA ratios would vary when comparing individual specimens because of the high degree of variation in individual egg production values. The high variation in individual egg production and RNA:DNA ratios estimates helps explain the lack of correlation between these two variables compared to findings of Saiz et al. (1998) and Gorokhova (2003) in which estimates were made on pooled samples of 3 - 10 individuals.

The timing at which nucleic acid samples were taken may also explain the lack of correlation between egg production and RNA:DNA ratios in *Acartia*. Diel patterns of egg production have been well documented in several cyclopoid copepod species (Hopcroft and Roff 1996; Ambler et al. 1999), as well as a few calanoid copepod species including *A. tonsa* (Stearns et al. 1989). Egg production of *A. tonsa* is higher at night and correlated to gut content 24 - 27 hr earlier (Stearns et al. 1989). The author is not aware of any studies that have estimated the timing of RNA production with egg production. Biegala et al. (1999), working with *C. helgolandicus* concluded that aspartate transcarbamoylase (ATCase) activity was correlated with egg production and that ATCase activity increased instantly with a change in food, but food was not utilized in egg production for about 24 hr. ATCase is an enzyme used in the biosynthesis of pyrimidine nucleotides (Jones 1980), so it is necessary for synthesis of nucleic acids. It would be useful to do a fine scale (hourly) study of nucleic acid values and egg production of *A. tonsa* to determine the time between peak nucleic acid concentration and egg production. All of the copepods in the present study were sampled within the same time frame, but it is possible that the peak period of nucleic acid production was missed if there is a cycle of production. However, changes in egg cycle may make little difference in DNA values because the relatively few haploid gametic cells contribute nominal DNA compared to the much more numerous diploid somatic cells. For example, assuming optimal egg production of 80 eggs day^{-1} (equaling only 40 gametic cells), the contribution from gametic cells would equal only 0.42% of the total nuclei count for *Acartia hudsonica* (averaging 9,595) (McLaren and Marcogliese 1983).

The present protocol has been modified slightly from that of Westerman and Holt (1988) and Vrede et al. (2002). The same protocol for standards and enzymes in Weterman and Holt (1988) were used in combination with the same fluorescent dye (RiboGreen), extraction buffer and similar microplate spectroflourmeter technique as in Vrede et al. (2002). Unlike the previous studies, the current study used a sonic dismembrator to homogenize copepod samples. Vrede et al. (2002) suggested that it was difficult to obtain accurate volume measurements using the dismembrator due to 'production of lather'. In the present study, samples were sonicated in a slightly higher volume (115 µl) of extraction buffer than used for analysis (100 µl) to compensate for the lather. In addition, a preliminary test showed no difference in quantity of DNA and RNA recovered using the sonic dismembrator versus a pestle with mortar, but samples could be processed more quickly and easily using the sonic dismembrator. RNA and DNA spike recovery from standards was excellent and usually greater than 99%. RNase digested greater than 99% of RNA from the highest RNA standard and did not affect DNA standards after a 30 min digestion. DNase was not used because optimal incubation time and buffers were not compatible with RiboGreen reagent and buffers. The single fluorescent reagent (either ethidium bromide or RiboGreen) and single enzyme (RNase) method was used successfully to investigate somatic growth of calanoid copepod species and *Daphina* (Wagner et al. 1998; 2001; Vrede et al. 2000; Gorokhova and Kyle 2002; Gorokhova 2003). RNase controls added to RNA standards revealed no measurable residual fluorescence due to incomplete digestion of RNA. In addition, reagent buffer minus RiboGreen additions to DNA and RNA standards and copepod homogenate did

not fluoresce confirming that there is no residual fluorescence not associated with nucleic acids.

RNA:DNA ratios of individual *A. tonsa* can be consistently measured and these values increase in the presence of food. The positive trend in RNA:DNA ratios with food were also seen with egg production. Using this method it is possible to obtain nearly instantaneous estimates of zooplankton nutritional condition and use it as a proxy for secondary production under specified conditions. Further laboratory studies examining nucleic acids in other zooplankton species and under a variety of environmental conditions (temperature, salinity and food quality) need to be conducted before this method can be used in the study of other zooplankton species under natural environmental conditions. RNA:DNA ratio analysis is a useful tool for determining the nutritional condition of zooplankton in specific situations in which the species, stage of development and temperature are known. In the future this assay may be applied to wild populations to determine nutritional condition and estimate egg production when lengthy field manipulations are not practical.

Table 1.1. Mann-Whitney U rank test results of *Acartia tonsa* nucleic acid measurements and egg production for food or no food treatments; bold value are significant at $\alpha \leq 0.05$.

Variable	U	p
DNA	1546.000	**0.029**
RNA	2181.000	**0.001**
Ratio	1752.000	**0.001**
Egg Production	36.000	**0.004**
% Hatch	33.000	**0.016**

Table 1.2. Mann-Whitney U rank test results of *Acartia tonsa* nucleic acid measurements for food or no food treatments on day 1, 2 and 3; bold value are significant at $\alpha \leq 0.05$.

Day	Variable	U	p
1	**DNA**	104.000	**0.001**
1	**RNA**	85.000	**0.017**
1	**Ratio**	60.000	0.562
2	**DNA**	67.000	**0.016**
2	**RNA**	80.000	**0.001**
2	**Ratio**	79.000	**0.001**
3	**DNA**	59.000	**0.004**
3	**RNA**	63.000	**0.001**
3	**Ratio**	45.000	0.153

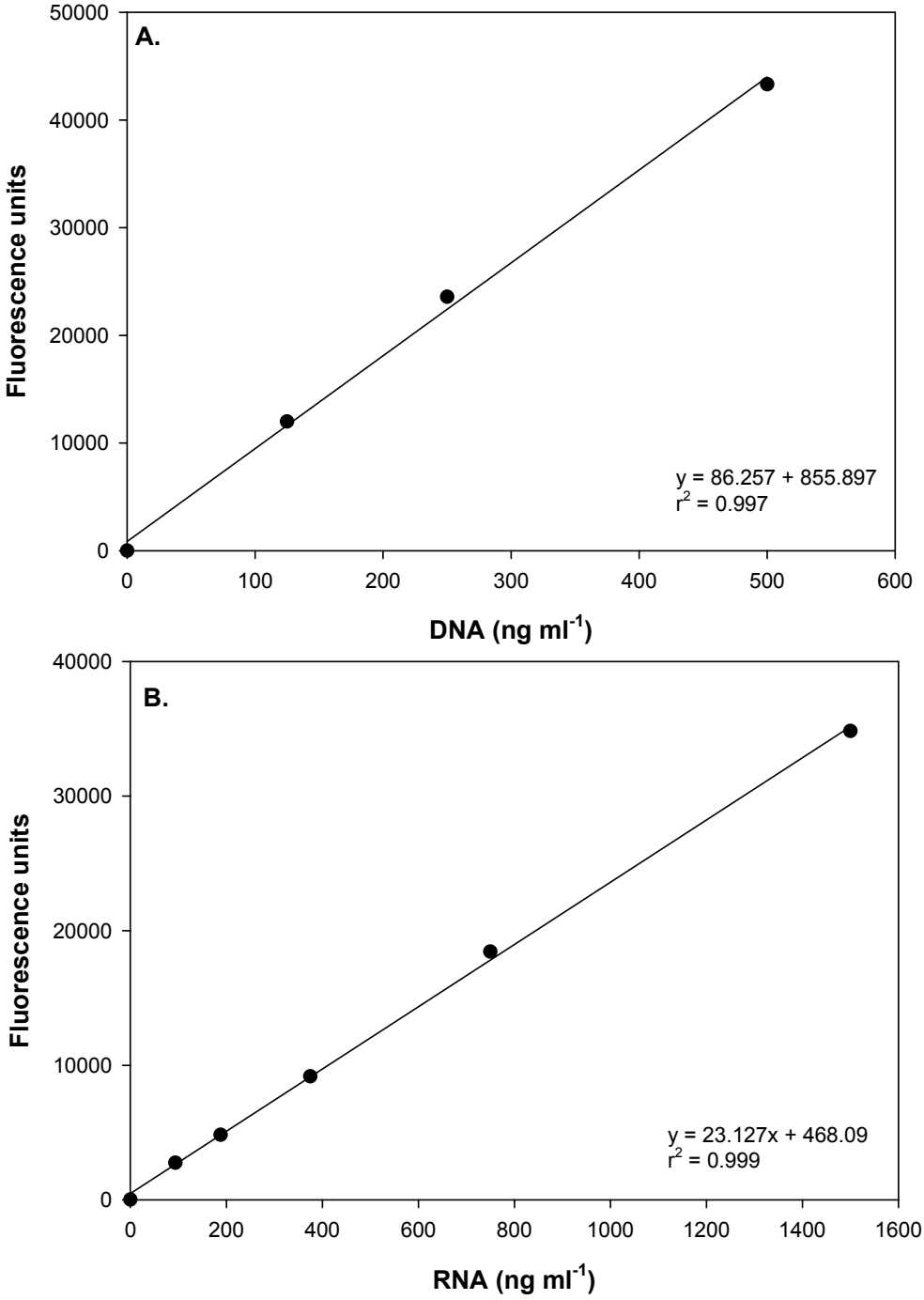


Figure 1.1. Representative standard curves run for nucleic acid assay; A. DNA and B. RNA.

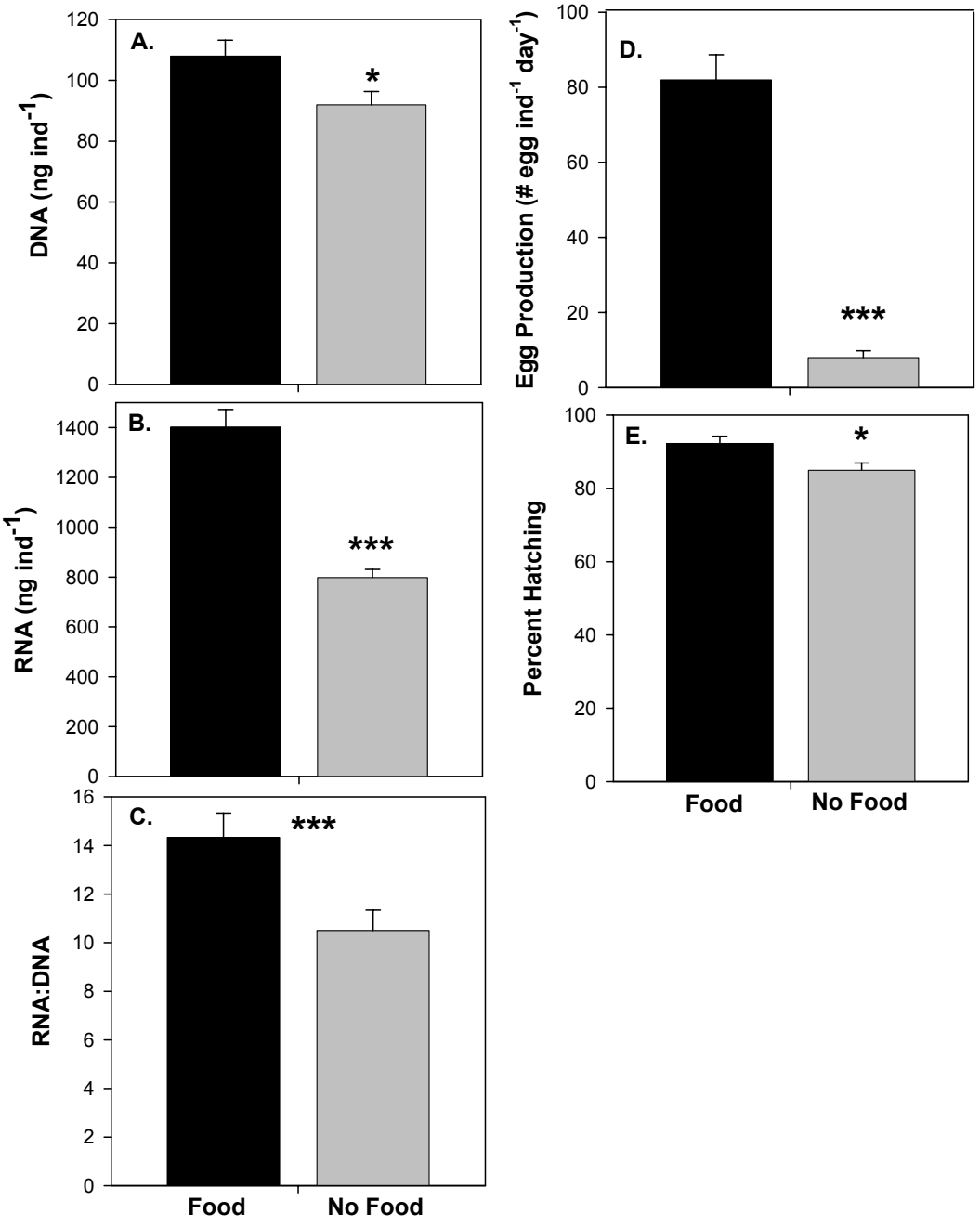


Figure 1.2. Changes of nucleic acid content of *Acartia tonsa* under food (dark bars) and no food (gray bars) treatment conditions after 48 hr; A. individual DNA estimates; B. individual RNA estimates; C. individual RNA:DNA ratio; D. group estimates of egg production (EP); E. group estimates of percent hatching; * = significant at $\alpha < 0.05$; *** = significant at $\alpha < 0.001$; error bars represent standard error of the mean.

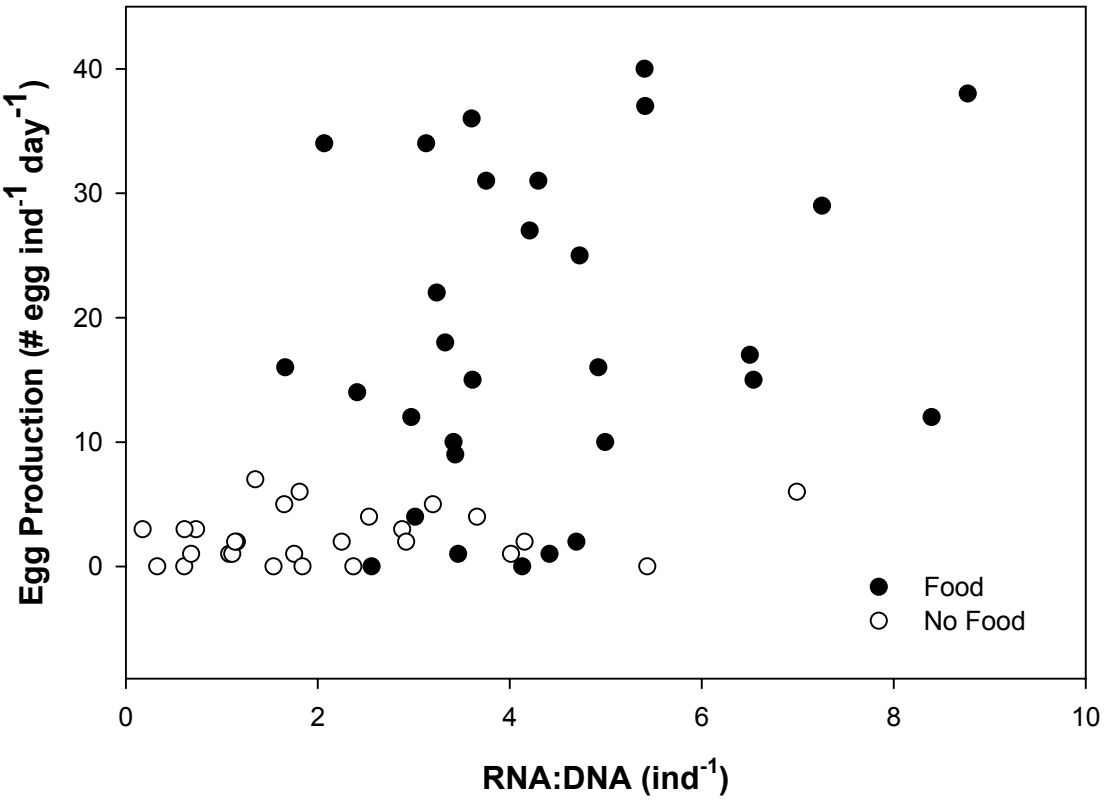


Figure 1.3. Relationship between individual egg production and RNA:DNA ratios in *Acartia tonsa* under food (dark circles) or no food (open circles) treatment conditions; food mean RNA:DNA = 4.7 $\pm$ 0.5 std error, mean EP = 18.7 $\pm$ 2.3 std error; no food mean RNA:DNA = 2.2 $\pm$ 2.2 std error, mean EP = 2.4 $\pm$ 0.4 std error.

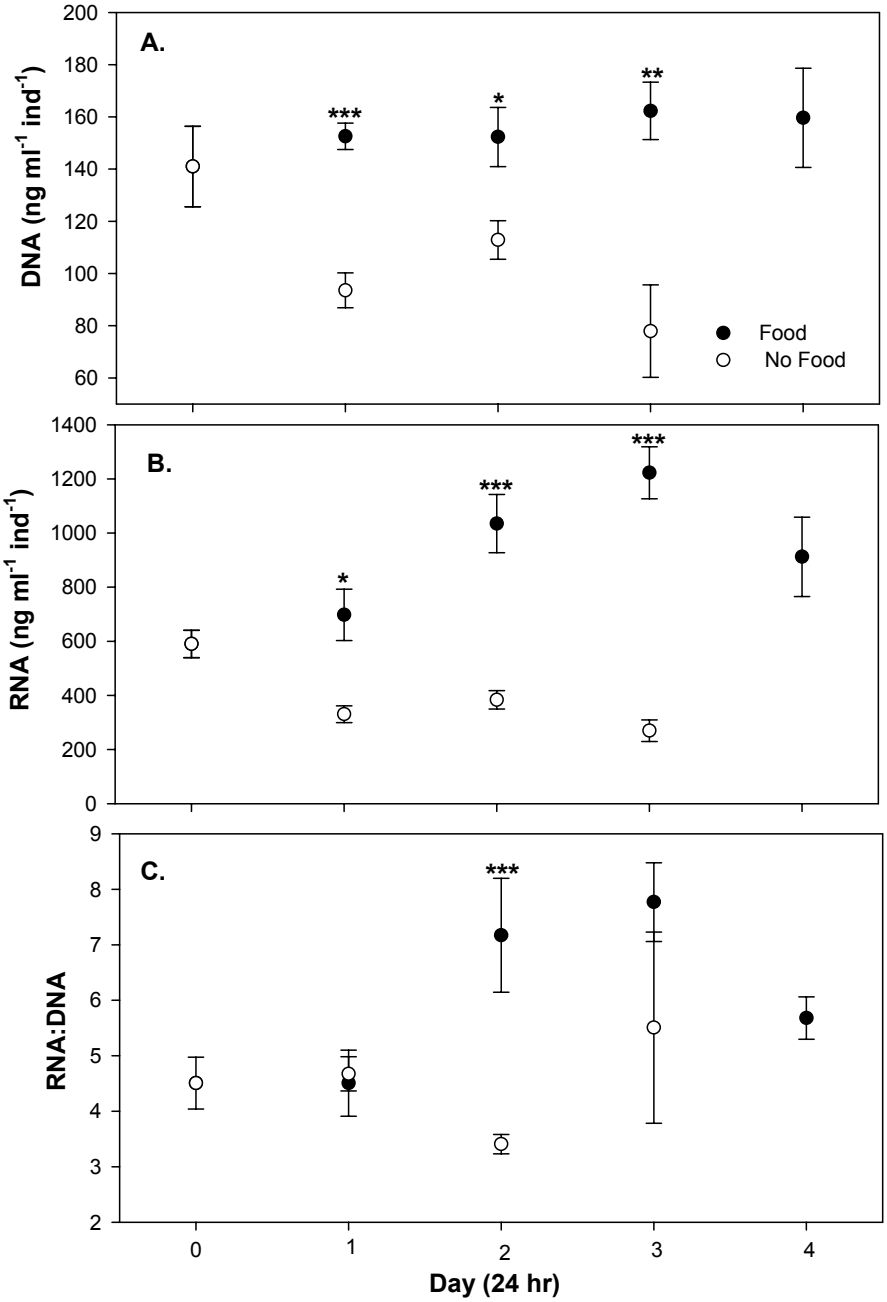


Figure 1.4. Time series changes in nucleic acid content of *Acartia tonsa* under food (dark circles) or no food (open circles) treatment conditions; A. individual DNA estimates; B. individual RNA estimates; C. individual RNA:DNA ratio; * = significant at $\alpha < 0.05$; ** = significant at $\alpha < 0.01$; *** = significant at $\alpha < 0.001$; error bars represent standard error of mean.

Chapter 2: RNA:DNA ratios as an index of zooplankton condition under different temperate, salinity and food parameters: laboratory and field comparison

ABSTRACT

RNA:DNA ratios are correlated with growth rate and/or egg production and serve as an estimate of nutritional condition for several copepod species. A sensitive nucleic acid fluorescent dye in combination with an automated microplate fluorometer was used to measure nucleic acids of individual adult female *Acartia tonsa*. Egg production experiments followed by RNA:DNA ratio analysis were conducted with and without food (*Thalassiosira* spp.) over a 48 hr period under a variety of environmental conditions. *A. tonsa* was cultured at three different temperatures (15, 23 and 30 °C) and three different salinities (15, 25 and 30 ppt) to determine how these environmental factors may affect egg production, egg viability and RNA:DNA ratios. In addition, samples were taken bi-monthly from the Nueces River estuary to examine changes in RNA:DNA ratios of *A. tonsa* under natural conditions. In laboratory experiments, food and temperature significantly affected both egg production and RNA:DNA ratios of *A. tonsa*, as did temperature-salinity interactions. Egg production was greatest (66 egg ind^{-1} day^{-1}) at high temperature and intermediate salinity, while RNA:DNA ratios were greatest (14) at intermediate temperature and salinity. *A. tonsa* from Nueces River estuary displayed high RNA:DNA ratios (>10) throughout the year, which were weakly correlated with temperature and microzooplankton abundance, but not chlorophyll *a* or salinity. Egg production is a more sensitive indicator of secondary production of copepods than RNA:DNA ratios over a broad range of environmental conditions in the laboratory.

However, initial field studies suggest RNA:DNA ratios do provide a reasonable index of copepod condition of samples collected directly from the field with minimal manipulation.

INTRODUCTION

Acartia spp. is a dominant copepod genus in coastal waters throughout the northern hemisphere (review by Mauchline 1998). Its broad distribution has led to numerous studies examining the effects of environmental conditions, such as food, temperature and salinity, on its survival and productivity (Checkley 1980; Durbin et al. 1983; Ambler 1986; Kimmerer and MacKinnon 1987; Paffenhöfer and Stearns 1988; Jónasdóttir 1994; Kleppel and Burkart 1995). Changes in secondary production are typically estimated by egg production and/or molting rate experiments, or by measuring increases in biomass (Durbin et al. 1983; Miller et al. 1984; Kimmerer and MacKinnon 1987; Richardson and Verheye 1999). Unfortunately, these techniques are labor intensive and require lengthy incubations during which it can be difficult to maintain ambient environmental conditions. In the last decade, RNA:DNA ratio analysis has been used as a proxy to estimate secondary production under specified environmental conditions (Saiz et al. 1998; Gorokhova 2003).

RNA:DNA ratios have shown a positive relationship with growth rates and egg production of calanoid copepods (Nakata et al. 1994; Saiz et al. 1998; Wagner et al. 1998, 2001; Bersano 2000; Gorokhova 2003; Chapter 1). RNA is necessary for protein synthesis and its concentration in tissue is an indicator of the rate of protein synthesis, while DNA per somatic cell is assumed to be constant in sexually mature adults. The resulting RNA:DNA ratio is an estimate of protein synthesis ability within each cell

(Bulow 1987). Saiz et al. (1998) showed that egg production and RNA:DNA ratios of *A. grani* were significantly higher in well fed copepods and egg production was positively correlated with temperature, while RNA:DNA ratios were negatively correlated with temperature. *Calanus finmarchicus* also displayed high RNA:DNA ratios with increased food concentrations, but had a reduction in RNA:DNA ratios with an increase in temperature (Wagner et al. 2001). However, nucleic acid values of *Acartia bifilosa* were not temperature dependant, but did change in response to food concentration (Gorokhova 2003). There has been little published work examining the effect of salinity changes on RNA:DNA ratios and none with copepods. RNA:DNA ratios of the freshwater prawn *Macrobrachium nipponense* did increase over a salinity change from 0 to 14 ‰, with a small decrease at 20 ‰ (Wang et al. 2004). Conversely, higher RNA:DNA ratios were associated with lower salinity of 15 ‰ compared to 33.5 ‰ for juvenile turbot *Scophthalmus maximus* (Imsland et al. 2002).

Survival, growth and reproduction are strongly influenced by temperature, salinity and food conditions, which are all variables that fluctuate seasonally as well as annually in estuarine systems. *A. tonsa* has a broad geographical distribution and is found in North and South America, Australia, Indian Ocean and European waters (González and Bowman 1965, references within; Brylinski 1981). This broad distribution is thought to be due largely to its ability to adapt to a wide range of temperatures (-1 - 32 °C) (González 1974) and salinities (1 - 72 ‰) (Cervetto et al. 1999). Tolerance to salinity differences is thought to control the distribution of *A. tonsa* in Berre Lagoon, France (Cervetto et al. 1999) and Dunkirk, France (Brylinski 1981). Because *A. tonsa's* regional abundance and its adaptation to a wide range of thermal and salinity conditions, it is an

ideal species to further examine the relationship between RNA:DNA ratios and egg production over a large range of environmental conditions.

Nueces Estuary is representative of a South Texas estuary system, which experiences low annual rainfall, hot dry summers and diversion of fresh water to support municipal, agricultural and industrial requirements. Unpredictable flooding events occur in association with tropical storms that can cause significant seasonal changes in freshwater inflow and associated changes in temperature, salinity and nutrients within the estuary. In Nueces Bay, average temperature is 23 °C, fluctuating from less than 10 °C to over 30 °C (Buskey 1993) and salinity ranges of 0.6 - 36 ‰ (Odum et al. 1963). Estimates of phytoplankton biomass from chlorophyll *a* values varied throughout the year with mean of 8.8 μg L^{-1} ranging from ca. 5 to 20 μg L^{-1} (Buskey 1993). *A. tonsa* is the dominant copepod in this estuary comprising greater than 50 % of the mesozooplankton population and is an important food source for larval and juvenile fish (Armstrong 1987; Buskey 1993). Changes in *A. tonsa*'s productivity (i.e. secondary production) in response to fluctuating temperature, salinity and food are critical to higher trophic levels that depend on them for food. Controlled egg production experiments in Nueces Estuary are logistically difficult because of the remote location.

The goal of the present study is to examine changes in egg production, egg viability and RNA:DNA ratios of *A. tonsa* raised under varying environmental conditions (food, temperature and salinity) to determine the usefulness of RNA:DNA ratio analysis as an indicator of secondary production under a wider range of environmental factors than previously examined. Laboratory experiments were conducted with cultured *A. tonsa*, to control temperature, salinity and food regimes that copepods will be exposed

throughout development thereby reducing the natural variability in these environmental parameters. Tiselius et al. (1995) concluded that egg production of laboratory reared *A. tonsa* were very similar to that of wild *A. tonsa* with the only difference being the lack of diel feeding rhythm in the cultured copepods. In addition, seasonal changes in RNA:DNA ratios of wild *A. tonsa* from Rincon Bayou in Nueces Estuary are compared to laboratory values as an estimate of the zooplankton condition in the field.

METHODS

Zooplankton and Phytoplankton Culturing

Copepod cultures were isolated from zooplankton samples collected at The University of Texas at Austin, Marine Science Institute pier located on the southern edge of the Aransas Ship Channel (27°50.3'N; 97°03.1'W) using a 0.25 m diameter plankton net with 153 μm mesh, which was allowed to drift in the tidal current for ca. 10 minutes. The plankton samples were taken back to the laboratory where adult female and male *A. tonsa* were sorted using a dissecting microscope. Each culture was started with 40 females and 5 males that were fed *Isochrysis galbana* and *Thalassiosira* spp. (0.5 mg C L^{-1} each) in a 2 L container with clean filtered (0.2 μm porosity) seawater at salinity of 25 ppt or 5 ppt either higher or lower than ambient collecting salinity (ca. 26 ppt). Salinity was adjusted using reverse osmosis (R.O.) water to reduce salinity or evaporation to increase salinity. Over the course of 2 - 3 days copepods were gradually acclimated to final salinity conditions of 10, 25 and 35 ppt. Once adults were acclimated (24 hr), eggs (ca. 250) were collected and transferred to a 2 L bottle with filtered seawater at 10, 25 or 35 ppt and food (*I. galbana* 1.0 mg C L^{-1}). Once eggs hatched and copepodites were present (after 5 - 7 days) they were transferred to aerated 10 L carboys filled with filtered

seawater at one of the 3 salinities. Food levels (*I. galbana* and *Thalassiosira* spp. 0.5 mg C L^{-1} each) were monitored on alternate days based on chlorophyll fluorescence using a hand-held Aquafluor fluorometer (Turner Design 8000-001) and food concentrations were adjusted accordingly. Cultures were initiated and maintained in a temperature controlled environmental chamber held at 15, 23 or 30 $\pm$ 0.2 °C. *A. tonsa* were exposed to 16:8 hr light-dark cycle under fluorescent and incandescent light. Adult copepods of generation two or higher raised under specific temperature and salinity conditions were used in all experiments.

Phytoplankton cultures used to feed the copepod cultures were grown at 10, 25 and 35 ppt seawater in f/2-Si media for *I. galbana* and f/2 for *Thalassiosira* spp. at 20 or 25 °C under fluorescent lights on a 12:12 hr light/dark cycle (Guillard 1975). The 10 ppt phytoplankton cultures were maintained at 25 °C because their growth rate was slower than 25 and 35 ppt cultures. There were 2 - 3 replicate cultures at each set of conditions. All cultures were diluted with fresh culture media at the appropriate salinity once a week, or more frequently if cultures were growing rapidly. Aliquots of phytoplankton cultures were fixed with 1 % acid Lugol's iodine solution to determine cell densities once a week and on the day prior to each experiment. A Sedgewick-Rafter cell was used to count a 1 ml sample of *Thalassiosira* spp. at 100x magnification and a hemacytometer was used to count a 0.1 ml sample of *I. galbana* at 200x magnification. Cell density of each culture was used to estimate carbon content based on volume conversion equations for marine phytoplankton (Strathmann 1967).

Egg Production / Hatching Success Experiments

A factorial design experiment was conducted with the three factors; temperature at three levels (10, 23 and 30 °C), salinity at three levels (10, 25 and 35 ppt) and food (food and no food over 48 hr period). Egg production experiments were conducted with salinity and food treatments at each of the three temperatures separately due to equipment limitations and logistics. Salinity and food treatments were run in triplicate. The food treatments consisted of *Thalassiosira* spp. and *I. galbana* each at 0.5 mg C L^{-1}. Ten to fifteen adult females (C6) were placed in Nalgene bottles with 1 L of filtered (0.2 μm) seawater at one of three salinities with and without food. All treatment bottles were incubated on a plankton wheel (1.5 rpm) in a temperature controlled room at one of the three temperatures (15, 23, 30 $\pm$ 0.2 ºC) under a 16:8 hr light-dark cycle. After 24 hrs, the treatments were gently passed through nested 153 and 40 μm mesh sieves. The copepods were captured on the 153 μm mesh and returned to their original containers in the environmental room with filtered seawater (with or without food) for an additional 24 hr. Eggs collected on the 40 μm mesh sieve from the initial 24 hrs were discarded as explained previously. Previous work with *A. tonsa* concluded that there is a lag time of 17 - 24 hr before new food conditions were incorporated into egg production (Tester 1986; Tester and Turner 1990). After 48 hours, the treatments were again gently passed through nested 153 and 40 μm mesh sieves. Copepods were examined under a dissecting microscope to determine the number of survivors. Individual live females were rinsed in RNase/DNase-free water and placed into RNase/DNase-free microcentrifuge tube and then flash frozen on dry ice for RNA:DNA ratio analysis.

Eggs collected on the 40 μm mesh sieve at 48 hr were placed in scintillation vials with 15 ml of filtered seawater and returned to the environmental room for another 24 hrs to determine hatching success. Vial contents were fixed with 10 % formalin at the end of the incubation for later counting of eggs and nauplii using a dissecting microscope. Egg production rates were calculated for the last 24 hr period as the total number of offspring produced (eggs + nauplii) divided by the number of live females at the end of the experiment. Percent hatching was the number of nauplii divided by the total number of eggs, multiplied by 100.

Seasonal Sampling

Zooplankton samples were taken at approximately bi-monthly intervals during 2003 in the Rincon Bayou within the Nueces Estuary at stations 450 (27°52.26'N; 97°31.17'W) and 451 (27°53.00'N; 97°31.17'W) (Fig. 2.1). Station 450 is located at the boundary between the Nueces River delta and the bay, while station 451 is in the interior of the delta. Zooplankton were collected using a 153 μm mesh and 0.25 m diameter net towed for 5 minutes from a boat. The samples were taken back to the laboratory where adult female *A. tonsa* were sorted under a dissecting microscope within 3 - 4 hr of collection. Individual, sexually mature females were rinsed in RNase/DNase-free water and placed into RNase/DNase-free microcentrifuge tube and then flash frozen on dry ice for later RNA:DNA analysis.

Temperature and salinity were measured at each station using a multi-parameter, hand-held meter (YSI 600 XL). Phytoplankton biomass was estimated from chlorophyll *a* (Chl *a*) measurements. Surface water samples were collected and filtered onto glass fiber filters (Whatman 25 mm diameter) from which Chl *a* was extracted overnight in

methanol using a non-acidification technique and measured on a fluorometer (Turner Model 10-AU), calibrated to a pure Chl *a* (Sigma) and solid secondary standard (Turner Designs) (Welschmeyer 1994; Arar and Collins 1997). Microzooplankton (20-200 μm) samples were also taken at stations 450 and 451. At each site, a 100 ml plastic bottle was filled with surface water and fixed with ca. 10 ml of 1 % acid Lugol's iodine solution. To enumerate microzooplankton, the sample bottle was gently inverted several times and a 1 ml aliquot was placed in a settling chamber for 24 hr. All organisms were counted, measured using an ocular micrometer and classified into broad taxonomic groups.

Nucleic Acid Analysis

Detailed description of nucleic acid analysis is presented in Chapter 1. Briefly, RNA and DNA were measured using the fluorescent dye RiboGreen (Molecular Probes) in combination with RNase A. Extraction buffer (1 X TE + protease + Triton X-100), reagent buffer (1 X TE) and diluted RNA and DNA standards were made fresh on the day of the analysis. The concentration of RNA and DNA standards were determined using a Spectra Max 190 microplate spectrometer and SOFTmax Pro 3.1.2 software (Molecular Devices) from which dilutions were prepared using the extraction buffer. RNA standards ranged from approximately 0 (blank) to 1500 ng ml^{-1} and DNA standards ranged from approximately 0 (blank) to 500 ng ml^{-1}. Two replicates of the RNA and DNA standard (100 μl each) were placed into individual wells of a black 96-well plate. RiboGreen reagent and RNase was automatically injected and fluorescence intensities (excitation at 480 nm and emission at 520 nm) were measured using the FLUOstar Optima microplate spectrofluorometer with computer software package version 1.10-0

(BMG Labtechnologies). Approximately 60 individual copepods were analyzed per analytical run.

Statistical Analysis

The statistical program Systat version 10.0 was used to perform a general linear models (GLM) 3-way factorial analysis of variance (ANOVA) to compare results at an experiment-wide alpha of 0.05. Statistical differences in RNA:DNA ratios of *A. tonsa* due to temperature, salinity and food treatments were compared. Statistical differences of egg production and hatching success after arcsin (square root) transformation (Zarr 1999) due to temperature, salinity and foods treatments were also examined. Response surface analysis using a quadratic smoothing function was used to visualize the effect of temperature and salinity together on both egg production, hatching success and RNA:DNA ratios.

RESULTS

Egg production of fed *A. tonsa* was quite variable, ranging from 4.3 to 101.5 eggs ind^{-1} day^{-1} with a mean of 42.6 $\pm$ 5.2 averaged across temperature and salinity. Starved *A. tonsa* had less variable egg production, ranging from 0.0 to 17.1 eggs ind^{-1} day^{-1} with a mean of 4.6 $\pm$ 0.9 averaged across temperature and salinity. Statistical results reveal significant main effects of food and temperature and significant interactions of food-temperature and temperature-salinity (Table 2.1). Because egg production increase ca. 5 times due to food, egg production as a function of temperature and salinity were graphed separately for food and no food treatments (Fig. 2.2). Quadratic surface analysis of food treatment indicate egg production was greatest (66.2 $\pm$ 6.8 eggs ind^{-1} day^{-1}) at the warmest temperature (30 °C) and the same analysis of the no food treatment had greatest

egg production (7.7 $\pm$ 1.7 eggs ind^{-1} day^{-1}) at the intermediate temperature (23 °C) (Fig. 2.2). The temperature-salinity interaction is manifested as a slight increase in egg production at the intermediate salinity (25 ppt) under both food treatments at 30 and 23 °C, but not at 15 °C.

Hatching success ranged from 0-100 % for both fed and starved *A. tonsa* with mean hatching success being 49.4 $\pm$ 7.7 % for food treatment and 47.3 $\pm$ 7.3 % for no food treatment averaged across temperature and salinity. The only significant main effect was of temperature, which also contributed to significant interactions of food-temperature and temperature-salinity (Table 2.2). Quadratic surface plots showed the highest percent hatching (>52.3 %) at the warmest temperature (30 °C) (Fig. 2.3). Hatching success was extremely low (< 16.5 %) at 15 °C in the food treatment and 0 % in the no food treatment. The temperature-salinity interaction indicate slightly higher hatching success at intermediate salinity (25 ppt) and the intermediate temperature (23 °C), but higher hatching success at the high salinity (35 ppt) and the warmest temperature (30 °C). There was no difference in percent hatching at the coldest temperature (15 °C).

The change in RNA:DNA ratios of *A. tonsa* was not as large as that of egg production and hatching success. RNA:DNA ratios of the food treatment ranged between 2.3 – 51.8 with a mean of 8.9 $\pm$ 0.4, while RNA:DNA ratios of the no food treatment ranged from 1.1 - 44.3 with a mean of 7.9 $\pm$ 0.4 averaged across temperature and salinity (Fig 2.4). There were significant main effects of food, temperature and salinity as well as a significant interaction of temperature-salinity and food-temperature-salinity (Table 2.3). Surface analysis plots reveal that temperature has the largest affect on RNA:DNA ratios with the highest ratios at the intermediate temperature (23 °C) in both the food (averaging

13.7 ± 0.9) and no food (averaging 13.8 ± 0.8) treatment averaged over salinity (Fig. 2.4). The intermediate salinity (25 ppt) had the highest RNA:DNA ratios for food (averaging 9.6 ± 0.4) and no food (averaging 9.9 ± 0.6) when averaged over temperature. RNA:DNA ratios differed by 1-2 units between food and no food treatments at extreme high and low temperatures and salinities. The interactions of salinity-temperature and salinity-temperature-food confirm that ratios are dependant on all factors.

Field samples of *A. tonsa* from the Rincon Bayou in Nueces Bay had no seasonal patterns in RNA:DNA ratios ($F_{5,182} = 1.229$, $p = 0.297$) and no significant difference in RNA:DNA ratios between stations 450 and 451 ($F_{1,186} = 0.281$, $p = 0.597$; Fig. 2.5). RNA:DNA ratios averaged within bays (450 and 451) were high, ranging from 11.1 - 16.5 (Fig. 2.5). There were no correlations between RNA:DNA ratios and Chl *a* (2.8 - 24.6 μg L^{-1}) or salinity (2.0 - 22.0 ppt) (Fig. 2.6 A, B). A weak negative correlation was found between RNA:DNA ratios and temperature (10.6 – 28.6 ˚C) and a weak positive correlation was found between RNA:DNA ratios and microzooplankton abundance (Fig. 2.6 C, D).

DISCUSSION

Zooplankton are a critical link between microplankton and higher trophic levels, such as larval or juvenile fish, so an estimate of their productivity may serve as an indicator of trophic function. Traditional methods for estimating zooplankton secondary production such as egg production, growth and/or molting rates require lengthy incubations over which ambient environmental conditions must be maintained. The objective of the present study was to determine if RNA:DNA ratio analysis is an appropriate indicator of copepod secondary production under a broad range of

environmentally realistic parameters by comparing RNA:DNA ratios and egg production of cultured *A. tonsa* over a 48 hr period. In addition, RNA:DNA ratios of *A. tonsa* from Nueces Bay were also estimated from monthly samples and compared to data from cultured copepods. Egg production is a more sensitive indicator over a broad range of conditions (Fig. 2.2, 2.4), but results indicate that RNA:DNA ratios did provide a directly measured index of *A. tonsa*'s potential reproductive growth. Using RNA:DNA ratios as a proxy for zooplankton condition is particularly useful in field studies where conducting egg production incubation experiments is logistically challenging.

The present study found egg production of fed copepods to be greatest at the warmest temperature (30 °C) and intermediate salinities (25 ppt) (Fig. 2.2). Previous studies of *A. tonsa* from East Lagoon, Galveston, Texas showed a strong positive correlation of egg production with temperature and a weak inverse correlation with salinity down to 10 ‰ (Ambler 1985). Seasonally, mean egg production of *A. tonsa* fed a diet of natural particles was 56 $\pm$ 2 eggs ind^{-1} 24 hr^{-1}, ranging from 23-105 eggs ind^{-1} 24 hr^{-1} with the greatest egg production in summer and early fall with collecting conditions of temperature $\geq$ 29 °C and salinity between 12 to 26 ‰ (Ambler 1985). A high degree of variation in egg production (4.2-101.5 eggs ind^{-1} day^{-1}) was seen in the current study for fed copepods averaged across temperatures and salinities similar to those in East Lagoon (Fig. 2.2 A). Egg production of starved copepods was very low and much less variable, ranging from 0.0-17.1 eggs ind^{-1} day^{-1} across differences in temperature and salinity (Fig. 2.2 B). The greatest increase in egg production of *A. tonsa* from East Lagoon, on an enriched (nutrients added) diet of natural particles compared to a non-enriched diet occurred in November, suggesting food was only limiting during

winter (Ambler 1985). Similar to *A. tonsa* populations in Galveston, Texas, populations of *A. tonsa* from Laguna Madre, Texas showed positive increases with temperature and salinity with greatest increase in summer months corresponding to temperature of 30 $\pm$ 1 °C and salinities between 47-75 ‰ (Simmons 1957). Both Texas populations have high productivity at temperatures greater than 26 °C, but productivity of the Laguna Madre is greater at significantly higher salinities than that of Galveston. Egg production of *A. tonsa* at low temperature (15 °C) was general depressed ($\leq$ 20 eggs ind^{-1} day^{-1}) regardless of salinity in this study as well as that of Ambler (1985).

The present study found hatching success was not a function of food, but was influenced significantly by temperature, which interacted with salinity (Fig. 2.4). The percent of eggs hatching at the intermediate (23 °C) and warmest (30 °C) temperatures was usually > 50 %, but at the coldest (15 °C) temperature was < 20 % irrespective of food or salinity conditions. Both Ambler (1985) and Parrish and Wilson (1978) concluded that egg viability was not a function of diet. Ambler (1985) found no difference in egg viability for *A. tonsa* fed diets of *T. weissflogii*, natural particles or modified natural particles, while Parrish and Wilson (1978) showed that egg viability was not different between fed and starved *A. tonsa*. Ambler (1985) found egg viability to vary seasonally, being the highest (near 100 %) in spring and lower ($\leq$ 70 ‰) in fall. She reported spring collecting conditions were temperatures of 22 - 26 °C and salinities of 29 – 31 ‰, while fall collecting conditions were temperatures of 20 - 29 °C and salinities of 18 – 23 ‰. Conditions under which eggs are spawned also influences hatching success. *A. tonsa* eggs spawned at 6.5 °C had a much higher hatching success at 4.8 °C than at 17.5 °C (Uye and Fleminger 1976). Hatching success of *A. tonsa* from southern

California was dependant on temperature and salinity, with hatching success being roughly 50 % within 20 – 50 ‰ salinity at temperatures of 9.5, 17.5 and 25.6 °C (Uye and Fleminger 1976). The response of egg viability, like egg production of *Acartia* spp. to changes in temperature and salinity are largely affected by environmental history or acclimation, which is why culturing copepods for several generations was important in the present study.

The response of RNA:DNA ratios to food and temperature presented here (Fig. 2.4) are consistent with results of previous studies with calanoid copepod species. Increase in algal concentration contributed to a slight, but significant increase in RNA:DNA ratios, which was also observed in other studies with *A. tonsa* (Bersano 2000), *A. grani* (Saiz et al. 1998), *A. bifilosa* (Gorokhova 2003), *Paracalanus* sp. (Nakata et al. 1994) and *C. finmarchicus* (Wagner et a. 1998, 2001). Of these studies, several also examined the relationship of temperature to RNA:DNA ratios. Saiz et al. (1998) found a strong positive linear relationship with RNA:DNA ratios and egg production in which the slope was dependent on temperature. Egg production was found to be higher at 23.3 °C compared to 17.8 °C, while RNA:DNA ratios were lower (Saiz et al. 1998). Wagner et al. (2001) also concluded that RNA:DNA ratios of *C. finmarchicus* were inversely related to temperature at 4, 8 and 12 °C. The present study found egg production of fed copepods increased between 15, 23 and 30 °C, while RNA:DNA ratios at the intermediate temperature were greatest, but egg production per RNA:DNA unit effort increased with temperature. The inverse relationship with temperature and RNA:DNA ratios is the result of increased metabolic reaction rates at higher temperatures, which allows more eggs to be produced with a smaller amount of genetic material. Contrary to

the findings in the current study and previously discussed papers, growth rates and RNA content of *A. bifilosa* were not significantly correlated with temperatures of 9 and 16 °C (Gorkhova 2003). The difference in response of RNA:DNA ratios to temperature between species and the relatively small number of studies examining changes in nucleic acid concentration indicates that this topic needs further investigation.

The response of RNA:DNA ratios to salinity changes in the present study was significantly influenced by temperature, with RNA:DNA ratios being higher in the 25 ppt treatments averaged across temperature and food (Fig. 2.4). There is only one other published study examining the effect of salinity changes on RNA:DNA ratios of crustaceans and they found RNA:DNA ratios and growth rates of the freshwater prawn *Macrobrachium nipponense* were the highest at salinities of 14 ‰ compared to 0, 7 and 20 ‰ over a 14 day period (Wang et al. 2004). They also found an increase in protein concentration of the haemolymph at 14 and 20 ‰, which further suggest increased synthesis activities.

Salinity changes alter metabolism by affecting glycolytic pathways and the pentose-phosphate shunt or changing rates of metabolism of amino acids and consequent excretion of ammonia, which likely affects the exchange of sodium in hypo-osmotic environments (Heinle 1981 and references within). Heinle (1981) presents data from several other researchers, which concludes that growth and respiration rates of *A. tonsa* are greater at lower salinities when corrected to a temperature of 20 °C. The metabolic responses (change in free amino acid pool and ammonium excretion) of a Florida population of *A. tonsa* to changes in salinity was greater when salinity was reduced (21 ‰ from 34 ‰) compared to increased (34 ‰ to 39 ‰) over a 3 day period (Farmer

and Reeve 1978). Farmer (1980) suggests *A. tonsa*'s ability of hyporegulation above salinities of 34 ‰ is "an adaptive energy conserving strategy". Respiration rates of a population of *A. tonsa* from the Mediterranean coast, were the greatest at salinity of 35 ‰ compared to 15 and 25 ‰, but ammonia excretion was highest at 25 ‰ independent of temperature (10, 15 and 20 °C) after 24 hr incubation (Gaudy et al. 2000). The differences in metabolic response of *A. tonsa* to salinity depend largely on the ambient salinity conditions it has adapted to, so RNA:DNA ratios should be highest under those conditions. More research is needed to explain the relationship between RNA:DNA ratios and salinity, but because *A. tonsa* is a euryhaline copepod, changes in RNA:DNA ratios may be difficult to detect. A more reasonable approach to examining salinity effects at the molecular level may to investigate mRNA specific to osmotic regulation genes, as has been done with Atlantic salmon, *Salmo salar* (Martin et al. 1999) and tilapia, *Oreochromis mossambicus* (Feng et al. 2002).

Field data from Nueces Estuary indicated a negative correlation between temperature and RNA:DNA ratios of wild *A. tonsa*, in that higher ratios were associated with colder temperatures (Fig. 2.6). A quadratic relationship with temperature and RNA:DNA ratios was found with cultured *A. tonsa* in the present study. The smaller RNA:DNA ratios at higher temperatures was indicative of greater egg production in the laboratory experiments in the present study, as well as for studies with *A. grani* (Saiz et al. 1998) and *C. finmarchicus* (Wagner et al. 2001). Over all the samples, RNA:DNA ratios of *A. tonsa* in Nueces Bay were always >10 over a temperature range of 10 - 29 °C and salinity range of 2.0 - 22.0 PSU. In laboratory experiments with fed copepods averaged across temperature (15 - 30 °C) and salinity (10 - 35 ppt) mean RNA:DNA

ratios were 8.9 and mean egg production were 42.6 eggs ind^{-1} day^{-1}, while for starved copepods mean RNA:DNA ratios were 7.9 and mean egg production was 4.2 eggs ind^{-1} day^{-1} (Fig. 2.2 and 2.4). RNA:DNA ratios in the field at temperatures below 15 °C were higher than copepods from the laboratory at 15 °C, but this may be because of difference in food conditions. Comparison of field and laboratory data indicate that food was available and egg production was greater than that of starved copepods.

Changes in RNA:DNA ratios of wild copepods have been shown to correlate with egg production and food. A recent study in winter 1999 and 2000 found high RNA:DNA ratios for *C. finmarchicus* correlated with high egg production and surface phytoplankton biomass (estimated as Chl *a* concentrations) in the Gulf of Maine (Durbin et al. 2003). Relative RNA:DNA ratios and egg production rates were found to be higher in 2000 due to the presence of an usually dense phytoplankton bloom, with concentrations of Chl *a* > 2 μg L^{-1} (Durbin et al. 2003). Earlier field work in the Kurshio current found higher RNA:DNA ratios of *Paracalanus* sp. in the frontal zone, which was attributed to higher phytoplankton biomass near the front compared to away from the front (Nakata et al. 1994).

Interestingly, RNA:DNA ratios of *A. tonsa* in Nueces Bay did not correlate with phytoplankton biomass estimated by Chl *a* levels, but did correlate with microzooplankton abundance. Uriarte et al. (1998) did not find a strong relationship between egg production and phytoplankton biomass, which they suggested was because phytoplankton appeared to be at concentrations that would not limit grazing. Poor correlations between egg production and chlorophyll concentration have been found for *A. tonsa* from East Lagoon, Texas (Ambler 1986) and Chesapeake Bay (White and

Roman 1992). High Chl *a* levels may be associated with phytoplankton that are unpalatable due to toxins or size limitations, or copepods may prefer microzooplankton making chlorophyll abundance non-limiting to grazing resulting in no correlation between chlorophyll concentration and egg production (White and Roman 1992). High productivity of *Acartia* spp. has been linked to food sources other than phytoplankton, including microzooplankton, specifically ciliates and rotifers (Stoecker and Egloff 1978; Lonsdale et al. 1996) and particulate organic matter and particulate organic carbon depending on the estuarine system and time of year (Burdloff et al. 2002). The increase in egg production with protozoan diets is attributed to higher food quality of protozoa, which often contain essential fatty acids and high protein content (Jønasdøttir 1994). White and Roman (1992) suggest that in Chesapeake Bay the best indicator of *A. tonsa*'s egg production is temperature and the carbon concentration of microzooplankton, which may be a function of temperature. The author is not aware of any published studies examining the relationship between microzooplankton diet on RNA:DNA ratios of copepods nor were direct comparisons made in the present study. However, it is hypothesized that RNA:DNA ratios will respond to a variety of dietary sources, which support copepod growth and reproduction.

Changes in the environment, including food, temperature and salinity, play important roles determining the productivity of *A. tonsa*. Egg production and RNA:DNA ratios both respond to differences in these three factors, but the response range for egg production (0 - 101 eggs ind^{-1} day^{-1}) is greater than that of RNA:DNA ratios (7.9-8.9). Egg production experiments are more sensitive indicators over a wide range of environmental conditions, compared to RNA:DNA ratios. However, as seen from the

Nueces Bay data set RNA:DNA ratios do provide a nearly immediate estimation of zooplankton nutrient and growth potential with minimal experimental manipulations. Very limited field collections were made in this study, so to confirm the usefulness of RNA:DNA ratios as a field indicator of zooplankton productivity will require more extensive field collections on a finer time scale with increased sampling sites covering a broader ranger of environmental conditions. It is also important to examine the extent of change in RNA:DNA ratios between zooplankton populations from different geographical regions, because temperature and salinity effects on egg production may vary regionally. Laboratory egg production and RNA:DNA ratio experiments using algal and microzooplankton food sources will also validate the significance of microzooplankton in the planktonic food web and the change in RNA:DNA ratios with mixed diet.

Table 2.1. The result of a General Linear Model (GLM)**,** 3-way analysis of variance (ANOVA) of *Acartia tonsa* for egg production response to food (food, no food), temperature (Temp = 15, 23, 30 °C) and salinity (Salt = 10, 25, 35 ppt); bold values are significant at alpha ≤ 0.05**.**

Variable	df	SS	MS	F	p
Food	1	19525.944	19525.944	207.525	**0.001**
Temp	2	7989.052	3994.526	42.454	**0.001**
Salt	2	544.708	272.354	2.895	0.065
Food*Temp	2	5505.636	2752.818	29.257	**0.001**
Food*Salt	2	109.095	54.548	0.580	0.565
Temp*Salt	4	1378.278	344.569	3.662	**0.013**
Food*Temp*Salt	4	790.690	197.672	2.101	0.101
Error	35	3387.225	94.090		

Table 2.2. The result of a General Linear Model (GLM)**,** 3-way analysis of variance (ANOVA) of *Acartia tonsa* for percent hatch after arcsin (sqrt) transformation response to food (food, no food), temperature (Temp = 15, 23, 30 °C) and salinity (Salt = 10, 25, 35 ppt); bold values are significant at alpha ≤ 0.05**.**

Variable	df	SS	MS	F	p
Food	1	0.041	0.041	1.904	0.176
Temp	2	12.944	6.472	300.236	**0.001**
Salt	2	0.079	0.040	1.842	0.173
Food*Temp	2	0.140	0.070	3.237	**0.051**
Food*Salt	2	0.045	0.022	1.036	0.365
Temp*Salt	4	1.011	0.253	11.730	**0.001**
Food*Temp*Salt	4	0.046	0.011	0.531	0.714
Error	35	0.776	0.022		

Table 2.3. The result of a General Linear Model (GLM)**,** 3-way analysis of variance (ANOVA) of *Acartia tonsa* for RNA:DNA ratios response to food (food, no food), temperature (Temp = 15, 23, 30 °C) and salinity (Salt = 10, 25, 35 ppt); bold values are significant at alpha ≤ 0.05**.**

Variable	df	SS	MS	F	p
Food	1	111.910	111.910	5.000	**0.026**
Temp	2	6271.167	3135.583	140.098	**0.001**
Salt	2	203.789	101.895	4.553	**0.011**
Food*Temp	2	116.729	58.365	2.608	0.075
Food*Salt	2	100.836	50.418	2.253	0.106
Temp*Salt	4	375.792	93.948	4.198	**0.002**
Food*Temp*Salt	4	231.039	57.760	2.581	**0.037**
Error	446	9982.108	22.381		

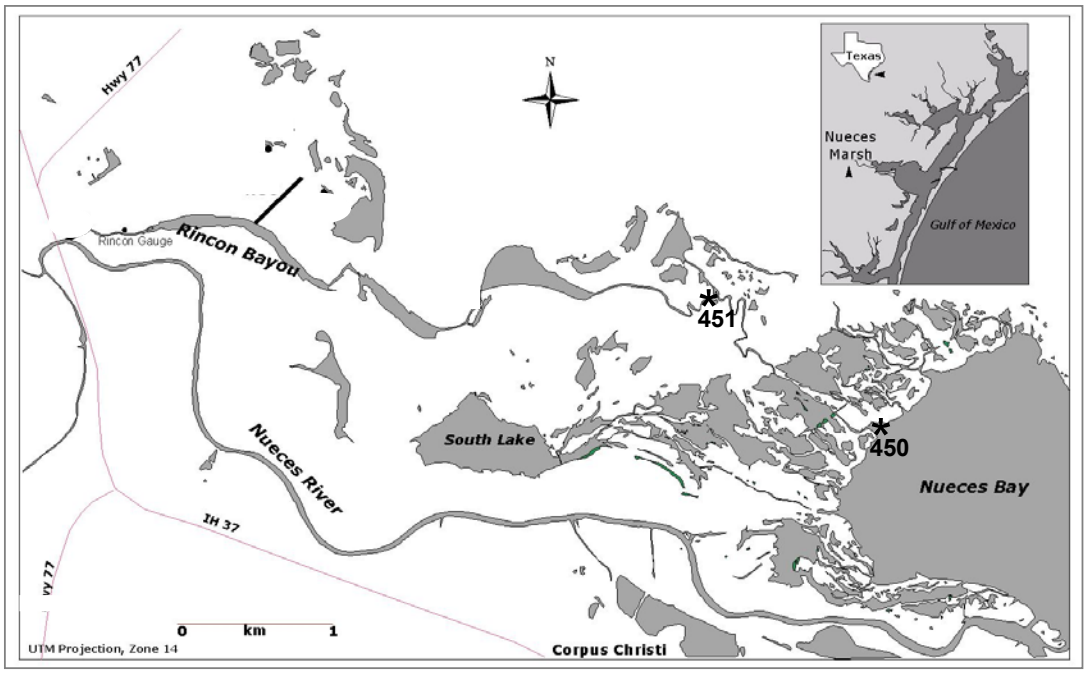
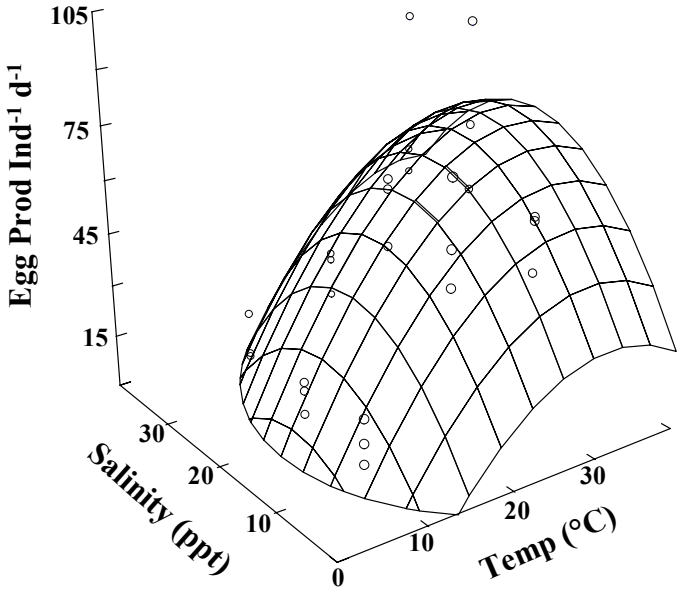


Figure 2.1. Map of Nueces Bay with location of sampling stations 450 and 451 denoted by stars (modified from Alexander 2000).

A.



B.

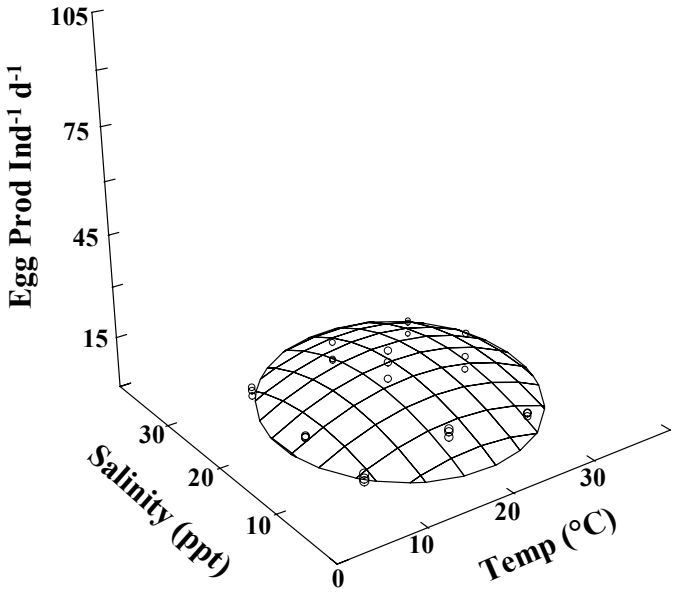


Figure 2.2. 3D-Response surface of *Acartia tonsa* mean egg production as a function of 9 combinations of temperature (15, 23, 30 °C) and salinity (10, 25, 35 ppt); A. represents fed copepods; B. represents starved copepods.

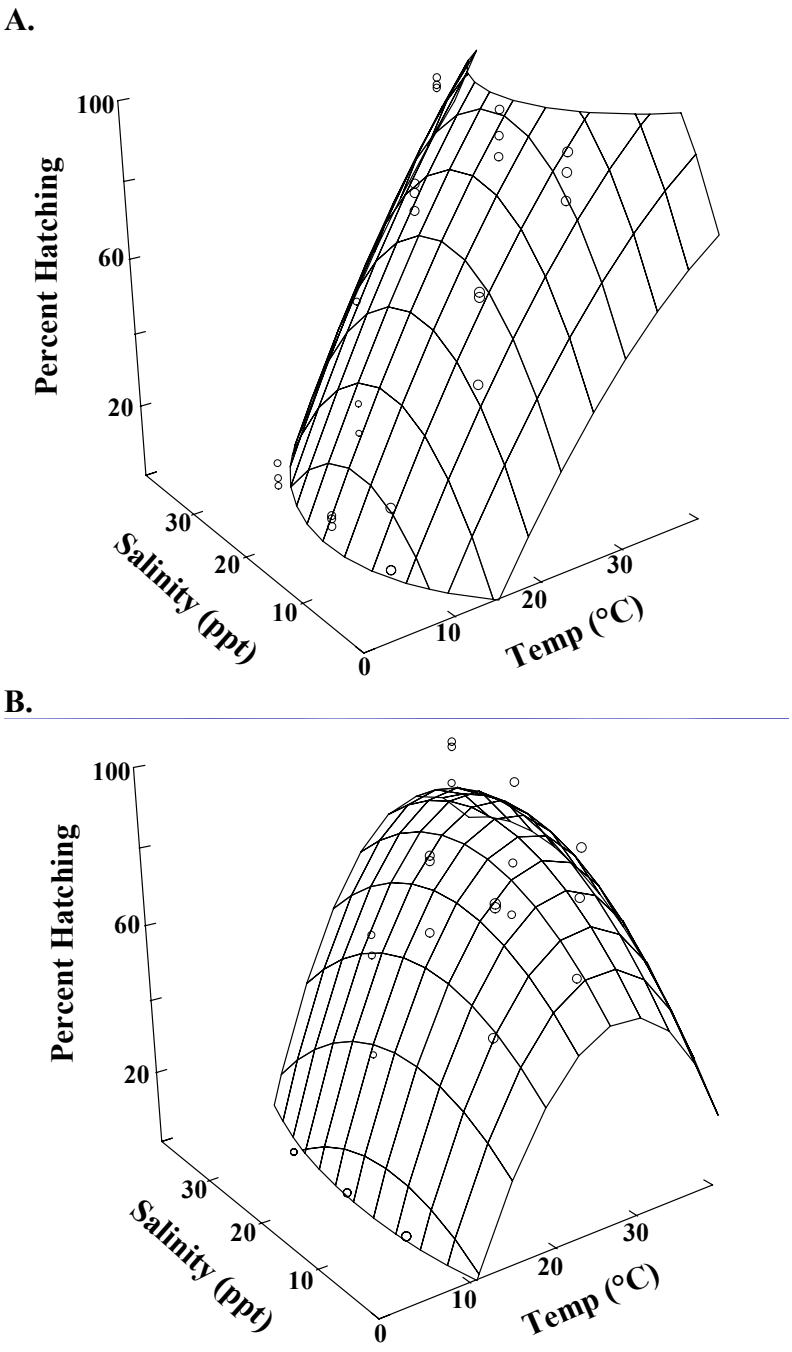


Figure 2.3. 3D-Response surface of *Acartia tonsa* mean percent hatching as a function of 9 combinations of temperature (15, 23, 30 °C) and salinity (10, 25, 35 ppt); A. represents fed copepods; B. represents starved copepods.

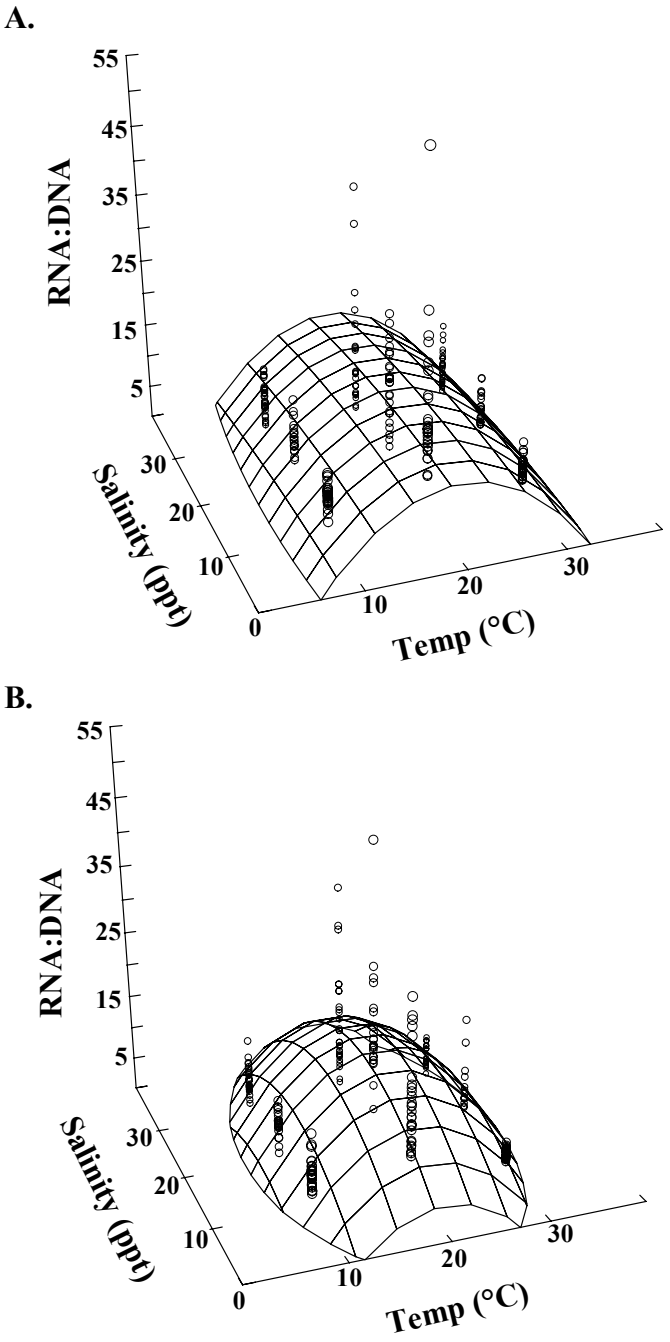


Figure 2.4. 3D-Response surface of *Acartia tonsa* mean RNA:DNA ratios as a function of 9 combinations of temperature (15, 23, 30 °C) and salinity (10, 25, 35 ppt); A. represents fed copepods; B. represents starved copepods.

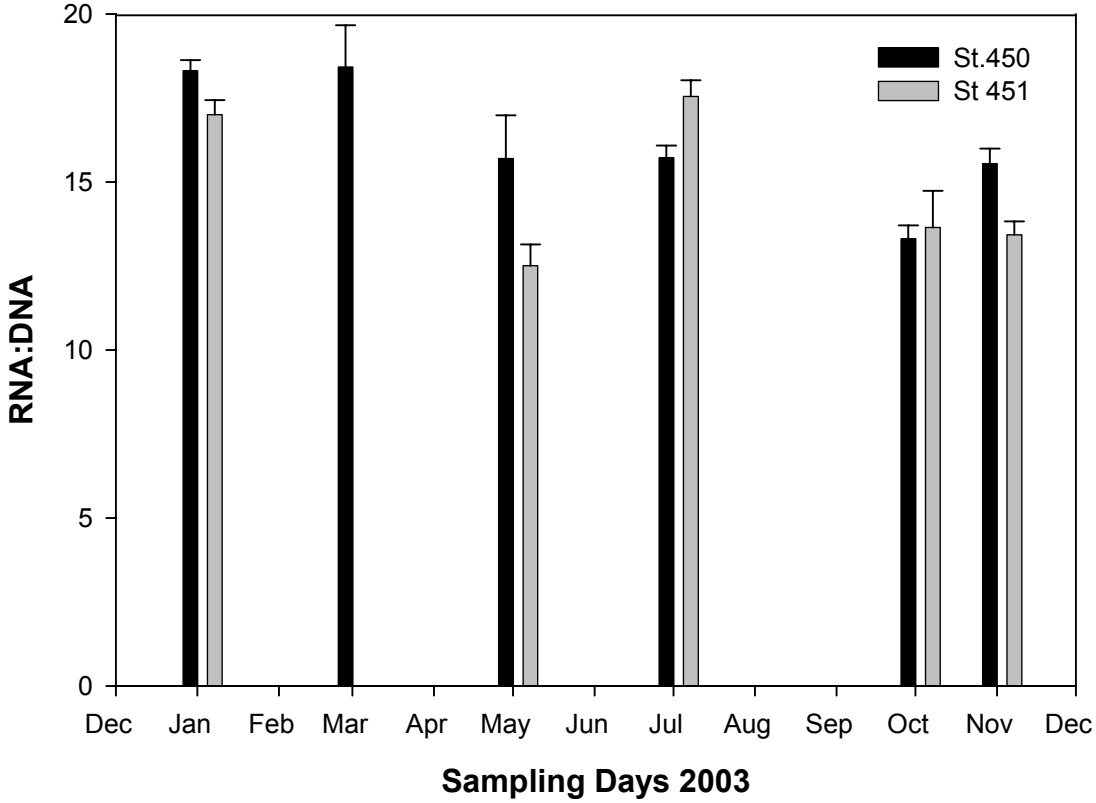


Figure 2.5. Seasonal changes in RNA:DNA ratios of wild *Acartia tonsa* from Rincon Bayou in Nueces Bay, Texas at two stations (450 and 451); vertical bars represent standard error.

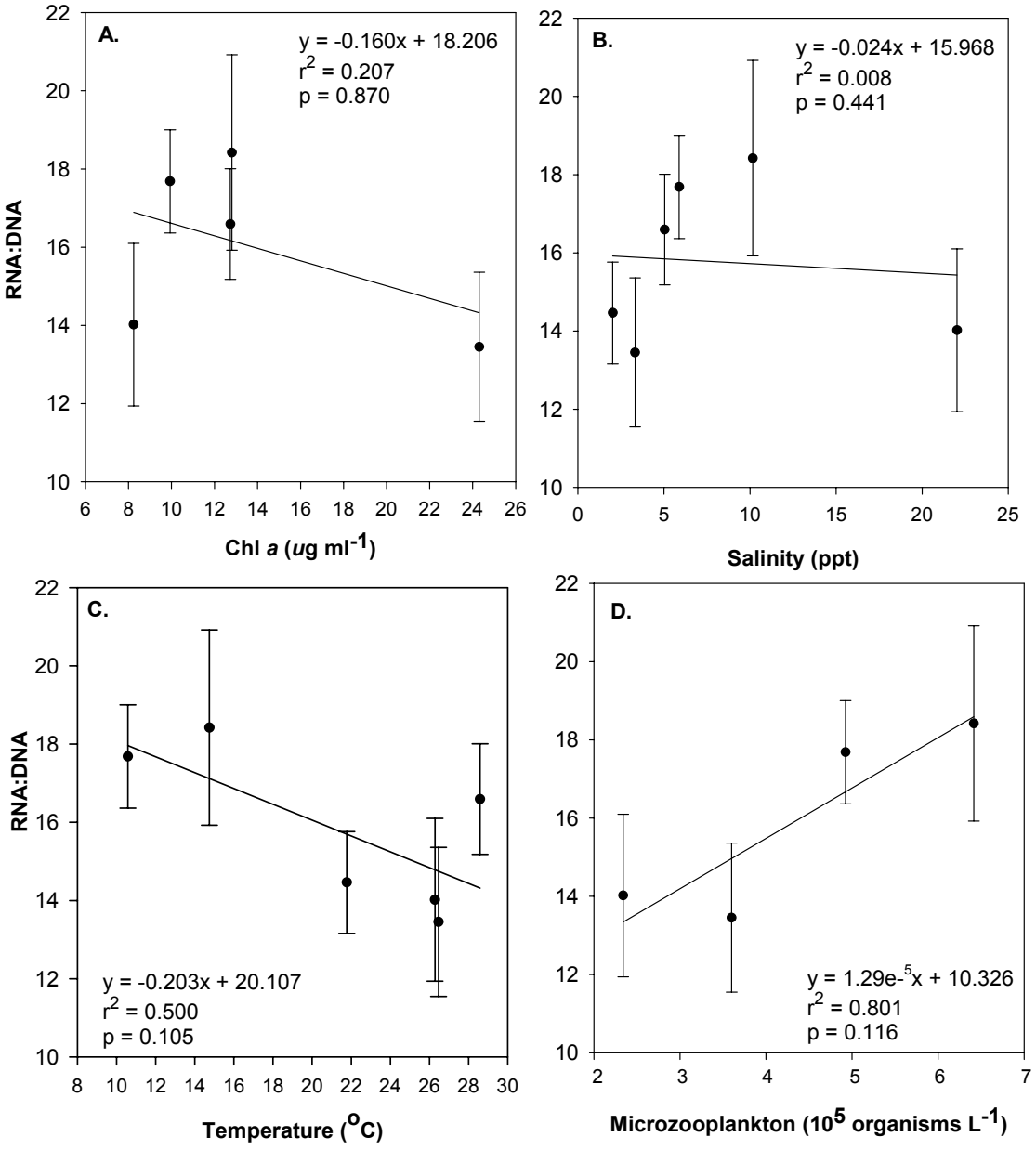


Figure. 2.6. Correlation of *Acartia tonsa* RNA:DNA ratios with physical parameters in Nueces Bay, Texas 2003; A. phytoplankton biomass, B. salinity, C. temperature and D. microzooplankton abundance; vertical bars represent std error.

Chapter 3: Effects of two different strains of *Karenia brevis* diet on RNA:DNA ratios and egg production of *Acartia tonsa*

ABSTRACT

Karenia brevis is a harmful alga associated with deleterious affects on zooplankton, but the exact cause (e.g. toxin, nutritionally inadequate or starvation) of these adverse effects is not clear. RNA:DNA ratios, fecundity and fecal pellet production of *Acartia tonsa* were measured on mono-algal and mixed-algal culture diets of *K. brevis* and *Peridinium foliaceum* to examine the usefulness of RNA:DNA ratios as an indicator of nutrition and to determine if adverse effects of *K. brevis* are due to the presence of toxins, poor nutritional quality or starvation. RNA:DNA ratios and egg production values were significantly higher for 100 % *P. foliaceum* diet compared to 100 % *K. brevis* diet. Significant differences in egg production, but not RNA:DNA ratios, were found between the various mixed diets, suggesting egg production is a more sensitive indicator of nutritional quality than RNA:DNA ratios. Changes in RNA:DNA ratios, fecundity and fecal pellet production of copepods fed toxic or non-toxic diets were nearly identical, indicating the presence of brevetoxins has little effect on *A. tonsa*. The similarity in RNA:DNA ratios, egg production, percent hatching and fecal production between the 100 % *K. brevis* diet and starved copepods suggest that *A. tonsa* does not consume *K. brevis* when offered as its sole food source.

INTRODUCTION

Karenia brevis [formerly *Gymnodinium breve* (Daugbjerg et al. 2000) and *Ptychodiscus brevis* (Steidinger 1979)] is a toxic dinoflagellate that commonly forms 'red tide' blooms in the Gulf of Mexico (Anderson 1989; Tester et al. 1991; Buskey et al.

1996; Tester and Steidinger 1997; Örnólfsdóttir et al. 2003). In the Gulf of Mexico, non-bloom concentrations of *K. brevis* range from 10 - 100 cells L^{-1} (Geesey and Tester 1993), but harmful blooms concentrations exceeding a million cells L^{-1} (Roberts 1979; Trebatoski 1988; Buskey et al. 1996) occurs when winds and currents transport and concentrate cells inshore. Blooms of *K. brevis* usually last 1 - 2 months, but have been observed to persist for up to 5 months (Tester et al. 1991; Buskey et al. 1996). Although algal blooms are naturally occurring events that are critical to maintain zooplankton populations and the higher trophic levels that feed on them, blooms can be considered harmful if the algal bloom reduces the survival, fecundity or behavior of marine organisms, changes trophic dynamics and/or poses health hazards to humans. There has been a global increase in the occurrence of harmful algal blooms (HABs) in the last two decades (Anderson 1989; Smayda 1990; Hallegraeff 1993; Magaña et al. 2003) due in part to anthropogenic influence on coastal waters and increased awareness of HABs.

Several different brevotoxins (PbTx) have been identified in *K. brevis* cultures (Chou et al. 1985; Baden et al. 1989; Roszell et al. 1990), which interfere with sodium channels causing depolarization of the nerve leading to muscle paralysis (Huang and Wu 1989). Larval red drum showed erratic swimming behavior prior to paralysis and death when exposed to as little as 40 cells ml^{-1} of *K. brevis* (Riley et al. 1989). The presence of *K. brevis* blooms has been associated with mass fish mortality (Trebatoski 1988; Anderson and White 1992; Buskey et al. 1996; Magaña et al. 2003). Tester et al. (2000) showed copepods fed *K. brevis* transferred brevetoxins to fish, which could possibly be transferred to higher trophic levels. Evidence of *K. brevis* was found in the stomachs of beached dolphins (Anderson and White 1992) and liver and lungs of manatees (Bossart et

al. 1998), which is thought to have contributed to their death. The toxins produced by *K. brevis* are also accumulated in shellfish tissue and are responsible for Neurotoxic Shellfish Poisoning (NSP) when consumed by humans (Yasumoto 1985; Plakas et al. 2002). In addition, exposure to brevetoxin aerosols is linked to respiratory distress in humans and in some instances to the death of marine mammals (Gunter et al. 1947; Myerson and Krzyanowski 1985; Pierce et al. 1990; Cheng et al. 2005). Blooms of *K. brevis* often result in the closure of shellfish fisheries and massive fish kills resulting in economic losses to fisheries and tourism (Trebatoski 1988; Tester et al. 1991; Buskey et al. 1996; Hay and Kubanek 2002).

There have been conflicting reports in the scientific literature as to how HAB's will affect the growth, fecundity and survival of zooplankton (Turner and Tester 1997). Studies of the copepod *Calanus pacificus* feeding on *K. brevis* demonstrated a loss of motor control and an increased heart rate (Huntley et al. 1986; Sykes and Huntley 1987). These effects are likely caused by the brevetoxins, which affect the nervous system by altering sodium ion fluxes and causing repeat firing of nerve impulses (Baden and Trainer 1993). In another study, Turner and Tester (1989) found *K. brevis* ingested by three copepod species (*Acartia tonsa*, *Oncaea venusta* and *Labidocera aestiva*) to have no adverse affect, but Turner et al. (1998) reported *A. tonsa* to be 'lethargic' after grazing on *K. brevis*. Collumb and Buskey (in press) showed that *A. tonsa* would not feed on *K. brevis* even when it was the sole food source, suggesting the copepods were not exposed to the toxin. Egg production of *A. tonsa* was also reduced on a diet of *K. brevis*, which was suggested to be an effect of starvation rather than toxin (Collumb and Buskey in press). In addition, *A. tonsa* did not show any change in swimming behavior after

exposure to *K. brevis* (Collumb and Buskey in press). Similarly, Firth (2004) showed reduced grazing, fecundity and clearance of *A. tonsa* when fed high concentrations of a *K. brevis* mono-algal culture.

It is uncertain if the reported adverse effects on copepods are due to *K. brevis* toxin content or to poor nutritional quality, particularly when the effects are not lethal, but sublethal causing effects on zooplankton fecundity. Jónasdóttir et al. (1998) suggest that egg production and/or hatching experiments using a mixture of potentially toxic alga (treatment) with non-toxic alga (control) mixed in different proportions can determine if the algae in question is beneficial, toxic or just nutritionally insufficient (Fig. 3.1). In general, if the treatment alga is nutritionally inadequate mixing it with the control would diminish the adverse effects, but if the treatment alga is toxic, mixing with control alga would not remove the adverse effects. Based on this method, a reference line is drawn connecting the egg production (or hatching success) rates of copepods on a diet of 100% treatment diet and 100% control diet. If the treatment alga is toxic, egg production on mixed diet would be lower than the reference line, that is the effects of the toxin would not be eliminated as the fraction of the non-toxic food increased. However, if the treatment alga is not toxic, but of lower nutritional value, egg production should increase rapidly with the increasing proportion of good food in the diet, and the measured values would fall on or above the reference line.

In the above scenarios, it is assumed copepods are offered a constant and limited total food concentration with no feeding selectivity. Feeding selectivity between food of different quality may also play a significant role in fecundity. In a situation where egg production is reduced with increasing concentration of the treatment alga, the change

may be because the treatment alga is not consumed, thus indirectly altering egg production. Copepods have been shown to feed non-selectively when food concentrations are low, but may feed selectively on higher quality food when food concentrations are high (Ives 1985; Turner and Tester 1989). Grazing experiments using a mixed diet of control and treatment algae are also needed to determine if copepods selectively feed on different algae in mixtures and to decide if selectivity was a function of toxicity or nutritional inadequacy (Jónasdóttir et al. 1998).

The current study addresses the issue of toxicity versus poor nutritional value of *K. brevis* using several indices of copepod condition including RNA:DNA ratios, egg production and hatching success. Although grazing was not directly measured, fecal pellet production was measured as an index of grazing rate. RNA:DNA ratio analysis has proven to be a useful indicator of the effect of nutrition on growth rates with lower RNA:DNA ratios generally corresponding to low egg production at lower food concentration (Saiz et al. 1998; Bersano 2000; Wagner et al. 1998, 2001; Gorokhova 2003; Chapters 1, 2). *A. tonsa* was fed mono-algal or mixed-algal culture diets of *K. brevis* (treatment) and *P. foliacium* algae (control) and the above indices were measured. *A. tonsa* was chosen to study because it is a dominant copepod in coastal waters of the Northern hemisphere and plays a critical role in the planktonic food web (Conover 1956; Buskey 1993). *K. brevis* and *P. foliacium* are both dinoflagellates of similar size (21 and 20.3 μM, respectively) and carbon content (690 and 822 pg C $cell^{-1}$, respectively). Toxic and non-toxic strains of *K. brevis* were studied because toxin levels within an algal clone can vary due to culture age, culturing condition, metabolic capacity and geographical origin of clone (Baden and Tomas 1988; Baden et al. 1989; Roszell et al. 1990; Loret et

al. 2002). The present study aims to determine if RNA:DNA ratios are a useful indicator of exposure to *K. brevis* and if adverse effects of *K. brevis* on *A. tonsa* are due to the presence of brevetoxin or poor nutritional quality.

METHODS

Phytoplankton Culturing

Cultures of *P. foliacium* (stock from UTEX Culture Collection) and toxic clone 2281 (stock from Center for Culture of Marine Phytoplankton- CCMP) and non-toxic clone SP-3 (stock from T. Villareal) *K. brevis* were grown at 30 ppt salinity in L1 media (Guillard and Hargraves 1993) at 20 °C under fluorescent lights on a 12:12 hr light-dark cycle. All cultures were diluted with fresh culture media every 2 - 4 week depending on growth rate of cultures. To determine culture densities the day of each experiment, samples of phytoplankton cultures were fixed with 1% acid Lugol's iodine solution. A sample (1 ml) of each culture was counted under a microscope at 40x magnification using a Sedgewick-Rafter slide. Cell density of each culture was used to estimate carbon content based on volume conversion equations for marine phytoplankton (Strathmann 1967). Two, 25 ml samples of *K. brevis* culture were filtered on a GF/F glass fiber filter and frozen for each experiment to determine the toxin concentration. Toxin analysis was performed by S. Morton at the National Oceanic and Atmospheric Administration (NOAA) Marie Biotoxin lab in Charleston, SC using a method developed by Van Dolah et al. (1994).

Zooplankton Collections

Zooplankton were collected at The University of Texas at Austin, Marine Science Institute pier located on the southern edge of the Aransas Ship Channel (27°50.3'N;

97°03.1'W) the evening prior to the initiation of an experiment. Evening tows were taken to increase capture of the target species because *A. tonsa* vertically migrates toward the surface at night (Stearns and Forward 1984). Collections were made using a 0.25 m diameter net with 153 μm mesh, which was deployed for 5 - 10 minutes depending on current speed. Large organisms were removed by gently pouring the sample through a 1000 μm mesh sieve. The remainder of the sample was taken back to the laboratory where it was aerated and kept in the dark until the sample was sorted the following morning. Adult *A. tonsa* from the plankton sample were sorted using a dissection microscope.

Egg Production, Hatching Success and Fecal Production Experiments

A. tonsa were exposed to seven diets consisting of 100 % *K. brevis* (Kb) (i.e. treatment alga), 100 % *P. foliacium* (Pf) (i.e. control alga), no food (NF) or mixed diet with both species 75:25 Kb:Pf, 50:50 Kb:Pf, 25:75 Kb:Pf. Experiments with toxic and non-toxic clones of *K. brevis* were conducted separately. Ten to twelve adult female copepods (C6) were placed in Nalgene bottles with 1 L of filtered (0.2 μm porosity) seawater at 27 ppt salinity with one of the six algal diets (0.1 mg C L^{-1}) or without food. Each treatment was run in triplicate. All experiments were incubated on a plankton wheel (1.5 rpm) in a temperature controlled room (26 $\pm$ 0.2 ºC) under a 16:8 hr light-dark cycle. After 24 hrs the treatments were gently passed through nested 153 and 40 μm mesh sieves. The copepods were captured on the 153 μm mesh and returned to their original containers in the environmental room with filtered seawater (with or without food) for an additional 24 hr. Eggs collected on the 40 μm mesh sieve produced during

the initial 24 hr incubation period were discarded because previous work concluded there is a lag time of 17 - 24 hr before new food conditions were incorporated into egg production (Tester 1986; Tester and Turner 1990). After 48 hr the treatments were again gently passed through nested 153 and 40 μm mesh sieves. Copepods were examined under a dissecting microscope to determine the number of survivors. Individual live females were rinsed in RNase/DNase-free water and placed into RNase/DNase-free microcentrifuge tubes and flash frozen on dry ice for RNA:DNA ratio analysis.

Eggs collected on the 40 μm mesh sieve were placed in scintillation vials with 15 ml of filtered seawater and returned to the environmental room to incubate for another 24 hr to determine hatching success. Vial contents were preserved with 1 % acid Lugol's iodine at the end of the incubation for later counting of eggs and fecal pellets using a dissecting microscope. Egg production rates were calculated for the last 24 hr period as the total number of offspring (eggs + nauplii) divided by the number of live females at the end of the experiment. Hatching success was determined using 10 eggs from each treatment. Eggs were placed in a tissue culture plate with 15 ml of filtered seawater and incubated for 24 hr under experimental conditions. After 24 hr they were fixed with 1 % acid Lugol's iodine and percent hatching was calculated based on the number of nauplii observed. Fecal production rate was determined from these samples by counting all the fecal pellets and dividing by the number of live females at the end of the experiment.

Nucleic Acid Analysis

Detailed description of nucleic acid analysis is presented in Chapter 1. Briefly, RNA and DNA were measured using the fluorescent dye RiboGreen (Molecular Probes)

in combination with RNase A. Extraction buffer (1 X TE + protease + Triton X-100), reagent buffer (1 X TE) and diluted RNA and DNA standards were made fresh the day of the analysis. The concentration of RNA and DNA standards were determined using a Spectra Max 190 microplate spectrometer and SOFTmax Pro 3.1.2 software (Molecular Devices) from which dilutions were prepared using the extraction buffer. RNA standards ranged from approximately 0 (blank) to 1500 ng ml^{-1} and DNA standards ranged from approximately 0 (blank) to 500 ng ml^{-1}. Two replicates of the RNA (0 - 1500 ng ml^{-1}) and DNA (0 - 500 ng ml^{-1}) standard (100 µl each) were placed into individual wells of a black 96-well plate. RiboGreen reagent and RNase was automatically injected and fluorescence intensities (excitation at 480 nm and emission at 520 nm) were measured using the FLUOstar Optima microplate spectrofluorometer with computer software package version 1.10-0 (BMG Labtechnologies). Approximately 60 copepods were individually analyzed at a time.

Statistical Analysis

The statistical program Systat version 11.0 was used to perform 1-way factorial analysis of variance (ANOVA) at an experiment-wide alpha of 0.05. Statistical differences of RNA:DNA ratios, egg production, percent hatching [after arcsin (square root) transformation (Zarr 1999)] and fecal production due to diet treatment were compared. Post-hoc Tukey multiple comparisons were made for significant main effects to determine which treatment levels were different.

RESULTS

Biotoxin analysis for the present experiments has not been completed, so the results of the most recent set of analysis are reported. The toxic *K. brevis* clone 2281 had 0.008 nM PbTx3 $cell^{-1}$ and the non-toxic SP-3 clone had undetectable toxin levels.

Toxic K. brevis Clone Experiment

Observed RNA:DNA ratios, egg production and percent hatching of *A. tonsa* were higher than the reference line between 100 % *P. foliacium* and 100 % *K. brevis* diet indicating the adverse effects of toxic *K. brevis* is not due to the brevetoxin, but nutritional inadequacy of the alga (Fig. 3.2). *A. tonsa* showed a significant change in RNA:DNA ratios ($F_{5,129} = 8.593$, $p < 0.001$), egg production ($F_{5,129} = 27.894$, $p < 0.001$) and fecal production ($F_{5,129} = 9.623$, $p < 0.001$), but not hatching success when exposed to a mixed diet of toxic *K. brevis* and *P. foliacium* algae (Table 3.1). Tukey multiple comparisons of RNA:DNA ratios revealed that starved copepods had significantly lower ratios (ca. 3.1) than those fed 100 % Pf, or the mixed diets (ca. 7.7), but was not significantly different from 100 % Kb (ca 5.6) (Fig. 3.2 A). Results of the Tukey multiple comparisons on egg production are more complex, revealing egg production on 100 % Pf and 25:75 Kb:Pf diets to be the highest (> 110 eggs $ind^{-1}day^{-1}$), intermediate egg production (avg. 85 eggs $ind^{-1}day^{-1}$) on 50:50 Kb:Pf and 75:25 Kb:Pf diets and lowest egg production (< 10 eggs $ind^{-1}day^{-1}$) on 100 % Kb and NF diets (Fig. 3.2 B). Tukey multiple comparisons on fecal pellet production showed highest fecal production (ca. 40 pellets $ind^{-1}day^{-1}$) on 25:75 Kb:Pf diet, which decreased significantly with increased Kb in diet (Fig. 3.2 D). Fecal production on 100 % Pf diet was lower than for mixed diets, but higher than the 100 % Kb and NF diets (Fig. 3.2 D).

Non-toxic K. brevis Clone Experiment

The observed RNA:DNA ratios and egg production on all diets were above the reference line connecting 100 % *P. foliacium* and 100 % *K. brevis* diets (Fig. 3.3 A, B). Percent hatching of *A. tonsa* on 25:75 Kb:Pf and 50:50 Kb:Pf diets, but not the 75:25 Kb:Pf, were also higher than the reference line (Fig.3.3 C). These results indicate *K. brevis* is nutritionally insufficient and not toxic to *A. tonsa*. *A. tonsa* showed a significant change in RNA:DNA ratios ($F_{5,127} = 13.335$, $p < 0.001$), egg production ($F_{5,127} = 48.147$, $p < 0.001$), hatching success ($F_{5,127} = 10.010$, $p < 0.001$) and fecal production ($F_{5,127} = 71.817$, $p < 0.001$), when exposed to a mixed diet of non-toxic *K. brevis* and *P. foliacium* algae (Table 3.2). Results of Tukey multiple comparison for RNA:DNA ratios showed that ratios were significantly lower (ca. 5.5) for 100 % Kb and NF than the other diets (ca. 10.3) (Fig. 3.3 A). Tukey multiple comparisons of egg production results are complex, with egg production on 100 % Pf and 25:75 Kb:Pf diets the highest (> 110 eggs ind^{-1} day^{-1}), intermediate egg production (avg. 90 eggs $ind^{-1}day^{-1}$) on 50:50 Kb:Pf and 75:25 Kb:Pf diets and lowest egg production (< 10 eggs $ind^{-1}day^{-1}$) on 100 % Kb and NF diets (Fig. 3.3 B). Tukey multiple comparisons of hatching success revealed that percent hatching of the NF diet was significantly lower at 43 % compared to the other diets (Fig. 3.3 C). Tukey multiple comparisons of fecal pellet production are also complex, showing significantly lower fecal production (< 5 pellets $ind^{-1}day^{-1}$) on the 100 % Kb and NF diet than the other diets and 25:75 Kb:Pf to have significantly higher fecal production (ca. 40 pellets $ind^{-1}day^{-1}$) than the other diets (Fig. 3.3 D).

DISCUSSION

Egg production and RNA:DNA ratios of *A. tonsa* fed a mono-algal culture of *K. brevis* were reduced compared to copepods fed a mono-algal culture of *P. foliacium.* In mixed diets of *K. brevis* and *P. foliacium*, egg production was reduced with increased concentration of *K. brevis*, but RNA:DNA ratios were not affected. Adverse effects of *K. brevis* exposure on *A. tonsa* appear to be caused by lower nutritional quality and not the presence of brevetoxin using the interpretation presented by Jónasdóttir et al. (1998). Observed egg production and RNA:DNA ratios are always above the reference line connecting production of copepods on the 100 % Pf (control) diet to the 100 % Kb (treatment) diet, thus inferring that the reduction in egg production with increased *K. brevis* is due to its poor nutritional quality and not the presence of toxins (Fig. 3.2 and 3.3). The similarity of RNA:DNA ratios, fecundity and fecal production of *A. tonsa* fed diets containing toxic or non-toxic *K. brevis* also suggests that adverse effects of the algal diet are not due to the presence of the measured brevetoxin.

A similar methodology was recently utilized by Colin and Dam (2002) with several phytoplankton species to determine their potential toxicity to *A. tonsa*. Colin and Dam (2002) concluded that only the high-toxin strain *Alexandrium* sp. was directly toxic to *A. tonsa* due to reduced ingestion and egg production when mixed with control alga. Two other species, *Heterosigma carterae* and *Phaeodactylum tricornutum*, were not considered toxic because reduced egg production was only observed on 100 % treatment diets where grazing was suppressed. In the present study, the similarity in RNA:DNA ratio, egg production and fecal pellet production of the 100 % Kb and no food diet suggest *K. brevis* is not consumed when offered as the sole food source. Increased

exposure to *K. brevis* caused a step-wise reduction in egg production and fecal pellet production, but only caused a slight reduction in RNA:DNA ratio values and percent hatching for the 100 % Kb and no food treatments. This suggest that RNA:DNA ratios are a less sensitive indicator of nutritional condition than egg production.

RNA:DNA ratios have a positive relationship with egg production under different food concentrations (Saiz et al. 1998; Wagner et al. 1998, 2001; Gorokhova 2003; Chapter 1, 2), but seldom different food types. RNA:DNA ratio analysis has not been used to examine the effects of harmful algal species, such as *K. brevis*, but it was used in a grazing study of the potentially toxic alga *Aureoumbra langunensis* (Bersano 2000), a pelagophyte species responsible for the 'Texas brown tide' found in Laguna Madre of Texas (DeYoe et al. 1995). Bersano (2000) examined RNA:DNA ratios changes of *A. tonsa* fed monocultures of *A. languensis, Thalassiosira weissflogii* and *Isochrysis galbana* and found RNA:DNA ratios of the copepods fed the *A. languensis* diet to be the lowest. Previously, Buskey and Hyatt (1995) demonstrated that copepod egg production rate decreased when grazing on *A. lagunensis*. In the present study significantly lower RNA:DNA ratios were only seen with the no food or 100 % *K. brevis* diets compared to the 100 % *P. foliaceum* and mixed diets (Fig. 3.2 and 3.3). However, egg production was significantly different between mixed diets of *K. brevis* and *P. foliaceum*, as well as between mono-algal culture diets of either species and no food. Egg production appears to be a more sensitive indicator of nutritional condition of copepods over a broader range of diets. The overall range in egg production is between 5-115 eggs ind^{-1} day^{-1}, while the range in RNA:DNA ratios is only between 3.2 and 10.8. In addition, there is a high degree of variation between individual RNA:DNA ratios (Chapter 1). The combination

of the small range of RNA:DNA ratio values and the high degree of individual variation makes RNA:DNA ratios a less responsive indicator of copepod nutritional condition than egg production.

The deleterious effects of *K. brevis* on *A. tonsa* appear to be due to its poor nutritional value. This is indicated by the step-wise decrease in egg production with increase in *K. brevis* concentration in the diet that is above the reference egg production line connecting production on mono-alga culture of *P. foliaceum* and 100% *K. brevis* diets. In addition, the similarity in the response of *A. tonsa* to both toxic and non-toxic strains of *K. brevis* suggest that the reduced fecundity is not the result of the presence of brevetoxins (Fig. 3.2 and 3.3). The reduction of zooplankton fecundity as a function of the nutritional quality (protein, lipid, carbohydrate, fatty acid and/or amino acid) of algae has been documented for *Acartia* spp. (Jónasdóttir 1994; Jónasdóttir and Kiørboe 1996; Kleppel et al. 1998; Hazzard and Kleppel 2003). Polyunsaturated fatty acids (PUFAs), specifically 20:5(ω-3) (eicosapentaenoic acid, EPA) and 22:6(ω-3) (docosahexaenioc acid, DHA), are essential to high zooplankton egg production because crustaceans cannot biosynthesize PUFAs ω-3 and ω-6 (Jónasdóttir 1994; Müller-Navarra et al. 2000; Anderson and Pond 2000). Study of the nutrient requirements of *A. tonsa* showed that egg production is positively correlated to protein and fatty acid 20:5ω3, 22:6ω3 and 18:0 ratios, but negatively correlated to fatty acids 16:1ω7 ratio on diets of *Thalassiosira weissflogii*, *Rhodomonas lens* and *Prorocentrum minimum* (Jónasdóttir 1994). The fatty acid 18:3(ω-3) has also been shown to be positively correlated with egg production and it has been suggested that some copepods may be able to convert 18:3(ω-3) to 20:5(ω-3) (Jónasdóttir 1994; Kleppel et al. 1998; Hazzard and Kleppel 2003).

Little work has been done on the nutritional composition of *K. brevis*, but recently two unique sterols gymnodinosterol ((24R)-4ω-methylergosta-8(14)) and brevesterol ((24R)-4ω-methyl-27-norergosta-8 (14)) have been identified in *K. brevis* (Giner et al. 2003). Gymnodinosterol and brevesterol are the dominate sterols (44 % and 41 % respectively) in *K. brevis* and Giner et al. (2003) suggested that structural modifications of these sterols make them non-nutritious to marine invertebrates. The dominant sterol in most animals is cholesterol, but many invertebrates depend on dietary sterols. A few arthropods, including *Artemia salina* and *Penaeus japonicus,* have been shown to convert ergosterol into cholesterol via the dealkylation process (Teshima and Kanazwa 1971; Teshima et al. 1983). However, the novel sterols of *K. brevis* have modified side chains, which cannot be transformed into cholesterol by invertebrates (Giner et al. 2003 reference therein; Harvey et al. 1989). These novel sterols and the lack of needed cholesterols can interfere with hormone production resulting in reduced growth and reproduction of invertebrates (Grieneisen 1994; Giner et al. 2003). Several fatty acids in different fractions of *K. brevis* have been identified; octadecapentaenoic acid (18:5(ω-3)) unsaturated fatty acid octacosaoctaenoic acid (28:8(ω-3)) and octacosaheptaenoic acid (28:7(ω-6)) (Leblond et al. 2003). The goal of Leblond et al. (2003) was to identify fatty acids in wild and cultured *K. brevis* and compare those to other dinoflagellates, but not to determine the nutritional quality of these components. A study of the nutritional value of *K. brevis* in conjunction with a fecundity study of *A. tonsa* feeding on *K. brevis* is needed to resolve the food quality issue.

Suppression of fecundity with little change in hatching success has been reported in copepods fed various algae (Fig. 3.2 B, C and Fig. 3.3 B, C). Turner et al. (1998)

working with *A. tonsa* showed reduced egg production, but high hatching success for copepods on a diet of *K. brevis*. Verity and Smayda (1989) found hatching success was only slightly reduced in one out of three *Acartia* spp. fed *Phaeocystis pouchettii*, while for all species egg production was very low and similar to that of starved copepods. *P. pouchettii* is a bloom forming alga, but it is unclear if the adverse effects are due to toxins (aldehydes or dimethylsulfide) or the difficulty of grazing on small single cells (5 μm) versus large colonies (200 μm) (Guillard and Hellebust 1971; Liss et al. 1994; Verity and Smayda 1989). Other studies have shown large reductions in hatching success of copepods fed certain species of diatoms. *Acartia clausi* egg production increased on a diet of the diatom *Thalassiosira rotula*, but hatching success decreased due to the presence of aldehydes, which act as a mitotic inhibitor by stopping cell proliferation and inducing apoptosis (i.e. programmed cell death) (Miralto et al. 1999). The difference in the effects of diet on egg production and hatching success suggest that each process is controlled by different nutritional factors (Jónasdóttir and Kiørboe 1996; Arendt et al. in press). Arendt et al. (in press) suggest that food quantity is more important for high egg production, while food quality, specifically fatty acids (DHA/EPA ratio) are critical for successful hatching. In contrast, in the present study it appears that as long as enough food is available and ingested viable eggs will be produced, but the number of eggs will be reduced as a function of food quality.

Algal ingestion rates were not measured directly in the present study, but fecal production was used as an indirect estimate of ingestion of copepods (Gaudy 1974; Gamble 1978; Ayukai and Nishizawa 1986; Haney and Trout 1990; Besiktepe and Dam 2002). Fecal pellet production generally decreased with increased proportion of *K. brevis*

in mixed diets, suggesting grazing decreased with increased concentrations of *K. brevis* (Fig. 3.2 D and 3.3 D). Collumb and Buskey (in press) also found fecal pellet production of *A. tonsa* on a mono-algal culture diet of *K. brevis* to be near zero. Interestingly in this study, fecal production in the 100 % *P. foliaceum* diet was somewhat lower than mixed 25:75 Kb:Pf diet. It seems possible that on the 100 % *P. foliaceum* diet food was more efficiently assimilated than the other diets, resulting in less fecal pellet production. The number, size and shape of zooplankton fecal pellets have been shown to change depending on diet (Marshall and Orr 1955; Haberyan 1985; Haney and Trout 1990; Besiktepe and Dam 2002), but the author is unaware of a study measuring digestion in relation to fecal pellet production for *K. brevis*. Besiktepe and Dam (2002) did show the best curvilinear relationship between ingestion and fecal pellet production for *A. tonsa* on a diet of an autotrophic dinoflagellate (*Prorocentrum minimum*) or diatom (*Thalassiosira weissflogii*). Alternatively, fecal pellets produced on a *P. foliaceum* diet may have been recycled (i.e. consumed) more readily, assuming *P. foliacium* fecal pellets had a higher nutritional value. Nutrient composition of fecal matter was not measured, but there is some evidence the zooplankton fecal pellets may be consumed by zooplankton themselves (review Turner 2002).

Reduced grazing of both toxic and non-toxic clones of *K. brevis* by *A. tonsa* when presented as the sole food source was recently confirmed by Firth (2004). Collumb and Buskey (in press) also reported zero grazing on mono-algal culture of toxic *K. brevis*. However, there is evidence that *A. tonsa* fed a natural assemblage of plankton including ciliates and diatoms, spiked with cultured *K. brevis*, exhibited increased grazing at higher *K. brevis* treatments (Firth 2004). She suggested that the increase in uptake of *K. brevis*

was incidental due to chemical cues produced by other plankton in the water sample which stimulated grazing. Amino acids released by phytoplankton have been shown to stimulate feeding in calanoid copepods (Poulet and Ouelett 1982; Gill and Poulet 1988). Chemical exudates produced by either algae species were not examined in that study. The similarity in RNA:DNA ratios, egg production and fecal pellet production of the no food and 100 % *K. brevis* diet implies that *A. tonsa* does not consume *K. brevis* when it is the sole food source. The findings in the present study seem to support those of Firth (2004) and Collumb and Buskey (in press) that *K. brevis* ingestion is reduced in the presence of mono-algal cultures of *K. brevis*, though grazing was not directly measured.

Similarity in values of the RNA:DNA ratios, egg production and fecal production for *A. tonsa* with 100 % Kb and no food treatments support the notion that grazing of *K. brevis* is suppressed. Response of *A. tonsa* to toxic and non-toxic strains of *K. brevis* to all variables (RNA:DNA ratios, egg production, percent hatch and fecal production) was nearly identical and in both experiments egg production increased step-wise with the addition of *P. foliacium*, which suggest that the presence of brevitoxin is not responsible for the deleterious effects on copepods sometime seen with *K. brevis* exposure. RNA:DNA ratios do not appear to be as sensitive an indicator of *K. brevis* exposure as egg production when copepods are exposed to mixed diet of *K. brevis* and *P. foliacium*. Additional fecundity and grazing experiments with copepods fed *K. brevis* that focus on its nutritional component (protein, lipids, fatty acid ect.) are needed to conclude what specific aspect of *K. brevis* are responsible for the reduction in egg production, without reducing hatching success. A comparison of nutrition, grazing and fecundity studies of *A. tonsa* fed mono-algal and mixed-algal diets of *K. brevis* and *Thalassiosira* spp. might

help determine what nutritional aspect of these algal species is responsible for the differences in reproductive success on these algal diets.

Table 3.1. Analysis of variance (ANOVA) results of RNA:DNA ratio analysis, egg production (EP), hatching success (% hatch) and fecal pellet production (FP) of *Acartia tonsa* on diet of toxic (2281) *Karenia brevis* (Kb) and *Peridinium foliacium* (Pf); diets of 100 % Kb, 75:25 Kb:Pf, 50:50 Kb:Pf, 25:75 Kb:Pf , 100 % Pf and no food (NF); bold values are significant at $p \leq 0.05$.

Variable	df	SS	MS	F	p
Ratio	5	364.120	72.824	8.593	**0.001**
Error	129	1093.304	8.475		
EP	5	40381.342	8076.268	27.894	**0.001**
Error	12	3474.366	289.531		
% Hatch	5	0.610	0.122	2.698	0.074
Error	12	0.542	0.045		
FP	5	3266.856	653.371	9.623	**0.001**
Error	12	814.727	67.894		

Table 3.2. Analysis of variance (ANOVA) results of RNA:DNA ratio analysis, egg production (EP), hatching success (% hatch) and fecal pellet production (FP) of *Acartia tonsa* on diet of non-toxic (SP-3) *Karenia brevis* (Kb) and *Peridinium foliacium* (Pf); diets of 100 % Kb, 75:25 Kb:Pf, 50:50 Kb:Pf, 25:75 Kb:Pf , 100 % Pf and no food (NF);bold values are significant at $p \leq 0.05$.

Variable	df	SS	MS	F	p
Ratio	5	693.374	138.675	13.335	**0.001**
Error	127	1320.666	10.399		
EP	5	40685.321	8137.064	48.147	**0.001**
Error	12	2028.052	169.004		
% Hatch	5	1.360	0.272	10.010	**0.001**
Error	12	0.326	0.027		
FP	5	3631.926	726.385	71.817	**0.001**
Error	12	121.373	10.114		

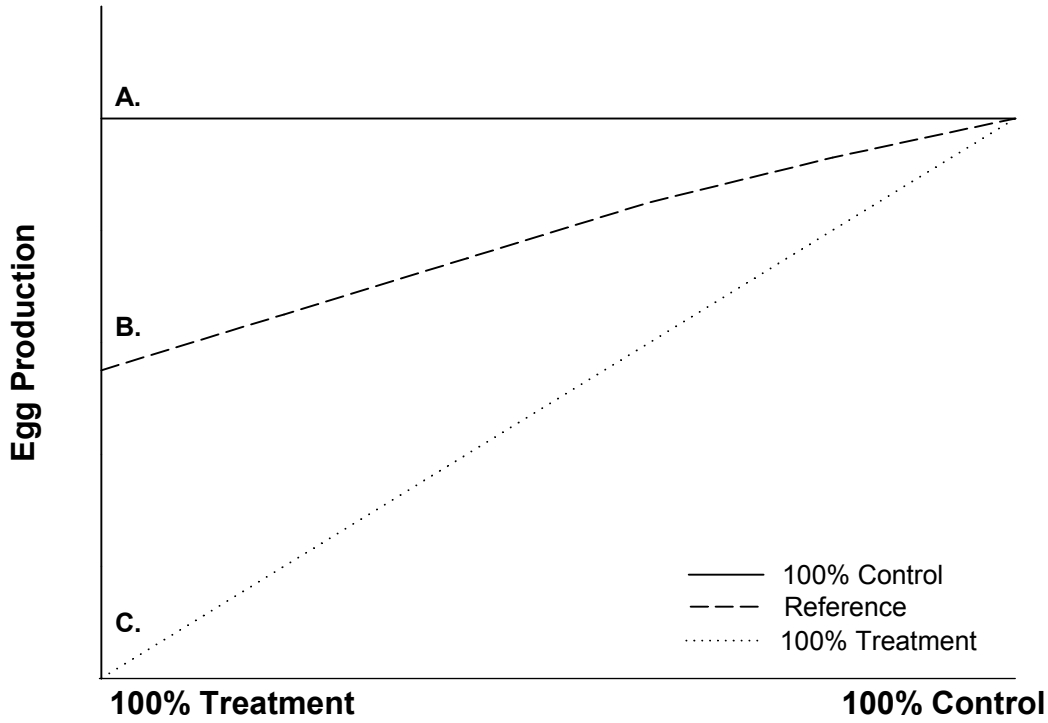


Figure 3.1. Theoretical egg production patterns of copepods on mixed algal diets; A. represent egg production on a diet of 100 % control (i.e. nutritional alga); B. represents observed or 'reference' egg production between the two extreme diets of 100 % treatment and 100 % control; C. represents egg production on a diet of 100 % treatment (i.e. questionable toxic alga). If egg production falls below the reference line deleterious effects are thought to be caused by toxins or poor nutritional value, but if egg production falls on or above the reference line the alga in question is though to be nutritional inadequate, but not toxic (modified from Jónasdóttir et al. 1998).

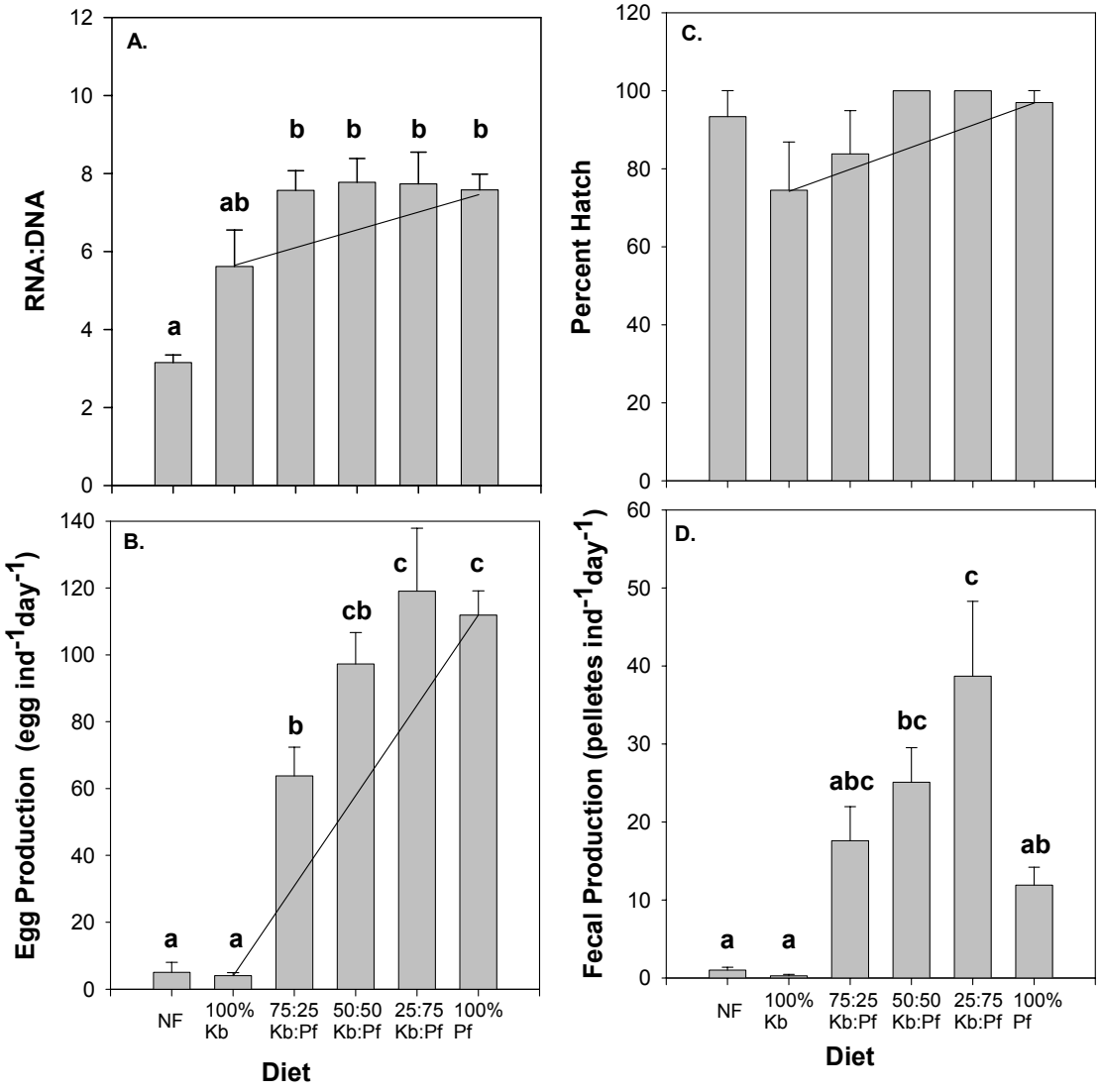


Figure 3.2. Response of *Acartia tonsa* to diet of toxic (2281) *Karenia brevis* (Kb) and *Peridinium foliacium* (Pf); A. RNA:DNA ratio, B. egg production, C. hatching success and D. fecal production; black line is the reference line connecting productivity between 100 % Kb and 100 % Pf diet; Tukey multiple comparisons $\alpha < 0.05$ and bars with the same letters are not significantly different from each other; error bars represent standard error.

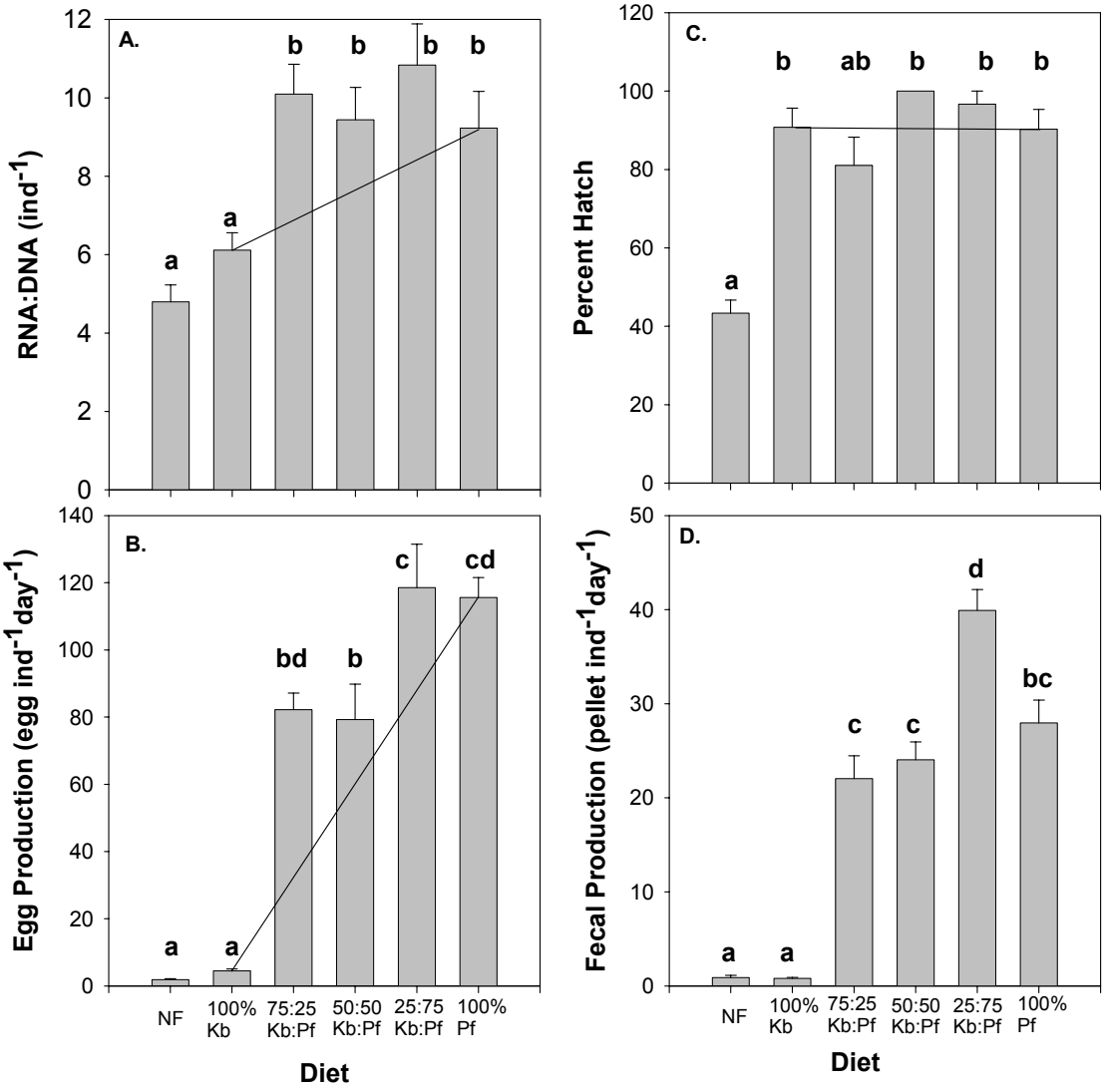


Figure 3.3. Response of *Acartia tonsa* to diet of non-toxic (SP-3) *Karenia brevis* (Kb) and *Peridinium foliacium* (Pf); A. RNA:DNA ratio, B. egg production, C. hatching success and D. fecal production; black line is the reference line connecting productivity between 100 % Kb and 100 % Pf diet; Tukey multiple comparisons $\alpha < 0.05$ and bars with the same letters are not significantly different from each other; error bars represent standard error.

Chapter 4: Assessing the effects of sublethal exposure to heavy metals (Cd, Cu) on planktonic copepods using RNA:DNA ratio analysis

ABSTRACT

Relatively little is known about sublethal effects of toxic heavy metals on zooplankton such as copepods. The use of RNA:DNA ratio analysis as an indicator of sublethal exposure to Cd (0 - 20 nM) and Cu (0 - 20 nM) was tested using *Acartia tonsa*, a dominant coastal copepod species. *A. tonsa* was fed either a diet of Cd or Cu contaminated diatoms (*Thalassiosira* spp.) or starved for 48 hr. Egg production was measured in incubation experiments and RNA:DNA ratios of individual copepods were measured using a fluorescent dye that binds to nucleic acids. Egg production, but not RNA:DNA ratios, of the starved individuals were significantly different from fed individuals. Hatching success was also not affected by starvation or the presence of heavy metals. Even though similar contamination levels have caused adverse effects on copepod reproduction in prior studies, there was no difference in reproduction or RNA:DNA ratios between Cd or Cu contaminated diets. Only levels of toxic metals that cause change in growth rate or reproduction of copepods would be expected to cause a change in RNA:DNA ratio, so this assay may still be useful at higher metal concentrations.

INTRODUCTION

Urbanization and industrialization have contributed to an increase in heavy metals in coastal bay and estuaries throughout the United States (O'Connor and Ehler 1991). Several Texas bays have shown elevated levels of cadmium (Cd) and copper (Cu) compared to background levels in sediments and/or water (Trefry and Presley 1976;

Benoit et al. 1994; Saleh and Wilson 1999; Carr et al. 2000, 2001; Tang et al. 2001). Cadmium and copper are two metals of biological concern because they have been linked to impaired reproductive development, reduced reproductive output, reduction in growth and increased adult mortality of zooplankton (Sonsowski and Gentile 1978; Sonsowski et al. 1979; Sullivan et al. 1983; Sunda et al. 1987; Toudal and Riisgård 1987; Sunda et al. 1990; Hook and Fisher 2001, 2002). Copepods are the dominant component of marine zooplankton communities and form a critical trophic link between phytoplankton and juvenile fish. Since copepods are so abundant and important in marine food webs, they could serve as a useful indicator of the ecological effects of increased heavy metals in coastal waters (i.e. impaired water quality). Biological toxicity tests of contaminated sites are often very time consuming, taking days to weeks to complete over which period constant environmental conditions must be maintained (ASTM 1995; U.S. EPA 2002). The majority of toxicology studies of heavy metals focus on lethal, rather than sublethal levels. In addition, many toxicology tests are focused on sediment quality and thus, conduct studies on benthic organisms, such as amphipods, mysids and sea urchins (Carr et al. 2000, 2001). It is critical to develop an indicator, that is sensitive enough to detect changes with exposure to sublethal levels of trace metals in water on a shorter time scale and minimal experimental manipulation. In this study, the suitability of RNA:DNA ratio analysis as an indicator of sublethal effects of heavy metals on zooplankton is tested.

RNA:DNA ratios have a positive relationship with growth rates and egg production of calanoid copepods (Saiz et al. 1998; Wagner et al. 1998, 2001; Bersano 2000; Gorokhova 2003; Chapter 1, 2). No studies have been reported on the effect of sublethal exposure to toxic heavy metals on the RNA:DNA ratios of marine copepods.

However, egg production of *A. tonsa* and *Acartia hudsonica* had a significant decline when exposed to the sublethal levels of Cd and Cu (Sonsowski et al. 1979; Sunda et al. 1987; Toudal and Riisgård 1987; Sunda et al. 1990; Hook and Fisher 2001, 2002). Earlier histological work on *A. tonsa* exposed to sublethal levels of 0.8 µM Cd (90 µg Cd L^{-1}) and 0.5 µM Cu (31 µg Cu L^{-1}) showed that females had reduced ova and esophoglands (Sosnowski and Gentile 1978). Sullivan et al. (1983) showed that growth rates of *Eurytemora affinis* exposed to sublethal levels of 0.5 Cu µM (30 µg Cu L^{-1}) were reduced. It is reasonable to expect that exposure to toxins shown to reduce growth and/or reproductive capacity may result in a reduction RNA:DNA ratios. RNA:DNA ratios of *Daphnia magna* were not affected when exposed to 45 nM Cd (5 µg Cd L^{-1}) (Barber et al. 1994). Studies of another crustacean, the blue crab *Callinectes sapidus,* exposed to sublethal levels of crude oil showed reduced growth rate and a reduction in RNA:DNA ratios (Wang and Stickle 1988). RNA:DNA ratio analysis has the potential to be used as a proxy for zooplankton secondary production of organisms taken directly from the field (Nakata et al. 1994; Wagner et al. 1998; Durbin 2003; Chapter 2). If RNA:DNA ratio analysis proves to be a useful indicator of sublethal exposure to toxic heavy metals it could be used in establishing water quality criteria.

The aim of this study is to determine if RNA:DNA ratios, egg production rates and hatching success of *A. tonsa,* a dominant coastal species, will change in response to exposure to food contaminated with either Cd or Cu. Cultured copepods were used in this study to control the exposure of the copepods to metals during their early developmental stages because previous work has concluded that the changes in temperature and salinity can dramatically affect copepods response to heavy metals due

to changes in metal complexing (Sunda et al. 1978; Toudal and Riisgård 1987). Furthermore, Sosnowski and Gentile (1978) determined that cultured and wild *A. tonsa* responses to Cd, Cu and Hg were not statistically different, but variability of responses was less with cultured animals. The ultimate goal of the present study is to determine if RNA:DNA ratios can be used as an indicator of elevated metal exposure at the sublethal level.

METHODS

Zooplankton and Phytoplankton Culturing

Copepod cultures were isolated from zooplankton samples collected at The University of Texas at Austin, Marine Science Institute pier located on the southern edge of the Aransas Ship Channel (27°50.3'N; 97°03.1'W) using a 0.25 m diameter net with 153 μm mesh, which was towed for ca. 10 minutes. The plankton samples were taken back to the laboratory where adult female and male *A. tonsa* were sorted using a dissecting microscope. Each culture was started with 40 females and 5 males that were fed *Isochrysis galbana* and *Thalassiosira* spp. (0.5 mg C L^{-1} each) in a 2 L container with clean filtered (0.2 μm porosity) seawater at 25 ppt. Once adults were acclimated (24 hr), eggs (ca. 250) were collected and transferred to a 2 L bottle with filtered seawater at 25 ppt and food (*I. galbana* 1.0 mg C L^{-1}). Once eggs hatched and copepodites were present (after 5 - 7 days) they were transferred to aerated 10 L carboys filled with filtered seawater and food (*I. galbana* and *Thalassiosira* spp. 0.5 mg C L^{-1} each). Food levels were monitored on alternate days based on chlorophyll fluorescence using a hand-held Aquafluor fluorometer (Turner Design 8000-001) and food concentrations were adjusted accordingly. Cultures were initiated and maintained in a temperature controlled

environmental chamber held at 23 $\pm$ 0.2 °C, with a 16:8 hr light-dark cycle under fluorescent and incandescent light. Adult copepods of generation two or higher were used in all experiments.

Phytoplankton cultures used to feed the copepod cultures were grown at 20 °C under fluorescent lights on a 12:12 hr light-dark cycle in 25 ppt seawater with f/2-Si media for *I. galbana* or f/2 media for *Thalassiosira* spp. (Guillard 1975). All phytoplankton cultures were diluted with fresh culture media once a week, or more frequently if cultures were growing rapidly. Aliquots of phytoplankton cultures were fixed with 1 % acid Lugol's iodine solution to determine cell densities once a week and on the day prior to each experiment. A Sedgewick-Rafter cell was used to count a 1 ml sample of *Thalassiosira* spp. at 100x magnification and a hemacytometer was used to count a 0.1 ml sample of *I. galbana* at 200x magnification. Cell density of each culture was used to estimate carbon content based on volume conversion equations for marine dinoflagellates (Strathmann 1967).

Metal contaminated cultures of the diatom *Thalassiosira* spp. were raised under the same physical conditions with the addition of 0, 1, 2, 5, 10 or 20 nM Cu or Cd according to protocol used in Hook and Fisher (2001). The primary difference in the algal culture media was the removal of Cu, Zn and EDTA, which would reduce metal availability. Phytoplankton densities were calculated weekly as described above.

Sublethal Concentration Experiments

Initial experiments were conducted to determine if all levels of metal (Cd or Cu) exposure used were sublethal to *A. tonsa*. This was done to ensure that subsequent egg production and RNA:DNA ratios analysis was conducted at sublethal metal levels. Five

to ten sexually mature females were placed in 200 ml polycarbonate bottles with 200 ml of 25 ppt filtered seawater and *Thalassiosira* spp. (0.1 mg C L^{-1}) at 0, 1, 2, 5, 10 and 20 nM of either Cd or Cu. *Thalassiosira* spp. was filtered onto a 0.2 μm porosity polycarbonate filter and rinsed into each sample bottle to eliminate metals in the seawater and insure that the primary means of metal exposure was through the diet. There were 3 replicates for each metal concentration. Experiments were conducted in a temperature controlled room (23 $\pm$ 0.2 ºC) under a 16:8 hr light-dark cycle. Every 24 hr, over a 96 hr period samples were gently rinsed through a 153 μm sieve and copepod survival was determined by observing the organism using a dissecting microscope. Samples were also monitored daily to insure that salinity and food levels remained consistent.

Egg Production / Hatching Success Experiments

Experiments were conducted with the 7 levels of metal exposure via metal contaminated *Thalassiosira* spp.; metals levels were 0, 1, 2, 5, 10 and 20 nM and no food (NF) treatment. Experiments with Cd and Cu were conducted separately. Ten to twelve adult females (C6) were placed in Nalgene bottles with 1 L of filtered seawater at 25 ppt salinity with metal contaminated *Thalassiosira* spp. (0.1 mg C L^{-1}) at one of the 6 metal levels or without food. Again *Thalassiosira* spp. was filtered onto a 0.2 μm pore polycarbonate filter and rinsed into each sample bottle. Each treatment was run in triplicate. All treatment bottles were incubated on a plankton wheel (1.5 rpm) in a temperature controlled room (23 $\pm$ 0.2 ºC) under a 16:8 hr light-dark cycle. After 24 hrs the treatments were gently passed through nested 153 and 40 μm mesh sieves. The copepods were captured on the 153 μm mesh and returned to their original containers in

the environmental room with filtered seawater (with or without food) for an additional 24 hr. Eggs collected on the 40 μm mesh sieve produced during the initial 24 hr incubation period were discarded. Previous work with *A. tonsa* concluded that there is a lag time of 17 - 24 hr before new food conditions were incorporated into egg production (Tester 1986; Tester and Turner 1990). After 48 hr, the treatments were again gently passed through nested 153 and 40 μm mesh sieves. Copepods were examined using a dissecting microscope to determine the number of survivors. Individual live females were rinsed in RNase/DNase-free water and placed into RNase/DNase-free microcentrifuge tube and flash frozen on dry ice for RNA:DNA ratio analysis.

To determine hatching success rate eggs collected on the 40 μm mesh sieve were placed in scintillation vials with 15 ml of filtered seawater and returned to the environmental room to incubate for another 24 hr. Vial contents were preserved with 10 % formalin at the end of the incubation for later counting of eggs and nauplii using a dissecting microscope. Egg production rates were calculated for the last 24 hr period as the total number of offspring (eggs + nauplii) divided by the number of live females at the end of the experiment. Percent hatching was the number of nauplii divided by the total number of offspring multiplied by 100.

Nucleic Acid Analysis

Detailed description of nucleic acid analysis is included in Chapter 1. Briefly, RNA and DNA were measured using the fluorescent dye RiboGreen (Molecular Probes) in combination with the digestive enzyme RNase A. Extraction buffer (1 X TE + protease + Triton X-100), reagent buffer (1 X TE) and diluted RNA and DNA standards

were made fresh on the day of the analysis. The concentration of RNA and DNA standards were determined using a Spectra Max 190 microplate spectrometer and SOFTmax Pro 3.1.2 software (Molecular Devices) from which dilutions were prepared using the extraction buffer. RNA standards ranged from approximately 0 (blank) to 1500 ng ml^{-1} and DNA standards ranged from approximately 0 (blank) to 500 ng ml^{-1}. Two replicates of the RNA and DNA standards (100 µl each) were placed into individual wells of a black 96-well plate. RiboGreen reagent and RNase was automatically injected and fluorescence intensities (excitation at 480 nm and emission at 520 nm) were measured using the FLUOstar Optima microplate spectrofluorometer with computer software package version 1.10-0 (BMG Labtechnologies). Approximately 60 copepods were individually analyzed at a time.

Metal Analysis

Metal concentrations were measured in algal culture media, algae and copepods to insure the presence of metals and estimate the concentration of exposure. Media samples were acidified with concentrated trace metal grade nitric acid (HNO_3, 0.17 % final concentration) and diluted 1:10 with 2% HNO_3 on the day of analysis. Cadmium and copper contaminated algae and copepods were processed separately for total metal concentration. All samples for metal analysis were processed using trace metal clean techniques; new polycarbonate bottles were rinsed in HCl followed by three rinses with ultra-pure water (Barnstead Nanopure); Teflon vials were soaked in 1.2 N HCl for 24 hr followed by three rinses with ultra-pure water, then soaked in 50% HNO_3 for 24 hr

followed by three rinses with ultra-pure water. All metal samples were processed in a clean laboratory in a fume hood or laminar flow bench.

Metal contaminated algae were filtered onto a polycarbonate filter (0.2 μm porosity) in a volume containing cells equivalent to 0.1 mg dry weight assuming *Thalassiosira* spp. at 1000 cells ml^{-1} and dry weight of 22 μg L^{-1} (Hook and Fisher 2001). Approximately 100 *A. tonsa* fed contaminated metals for 48 hr were filtered onto a 153 μm sieve and rinsed into an acid cleaned, polycarbonate tube with 10 ml of ultra-pure water. Algae and copepod samples were frozen for at least 24 hr. The copepod samples were then thawed and transferred into an acid cleaned pre-weighed Teflon vial and all the water was removed by evaporating over low heat. Once the copepod samples were dry, 10 - 12 ml of 50% HNO_3 was added to each vial, the samples were capped and heated to reflux. Algae samples were transferred from each filter into 10 - 12 ml of 50% HNO_3 in pre-weighed Teflon vials which were capped and heated to reflux. Once the algae and copepods were completely digested (1 - 4 days), the samples were dried over low heat and then weighed. The samples were taken up in a small amount of concentrated HNO_3 and dried twice, then dissolved in 5 ml of 4% HNO_3. After an hour the samples were diluted with 10 ml of ultra-pure water and allowed to sit for 4 hr before being decanted into acid cleaned polyethylene bottles. Cadmium and copper levels in all f/2 media, all algae samples and copepods exposed to 0, 2 and 5 nM algae were measured by inductively-coupled plasma mass spectrometry (ICP-MS) with internal standard normalization [indium (In) for Cd; yttrium (Y) and holmium (Ho) for Cu] at the Department of Geological Sciences, The University of Texas, Austin.

Statistical Analysis

The statistical program Systat version 11.0 was used to perform a 1-way analysis of variance (ANOVA) at an experiment-wide alpha of 0.05 for Cd and Cu separately. Statistical differences of RNA:DNA ratios, egg production and hatching success after arcsin (square root) transformation of copepods at all treatment levels were tested (Zarr 1999). Post-hoc Tukey multiple comparisons were made for significant main treatment effects to determine which treatment levels were different.

RESULTS

Sublethal Concentration Experiments

Copepod survival was high under all metal treatments and mortality rates were not significantly different at 0, 1, 2, 5, 10 and 20 nM for either Cd or Cu contaminated *Thalassiosira* spp. (Table 4.1). Thus, all 6 levels of metal exposure were considered to be sublethal and used in the egg production and RNA:DNA ratio analysis.

Cadmium Experiment

Cadmium contaminated *Thalassiosira* spp. did not cause significant changes in *A. tonsa's* RNA:DNA ratio, which averaged 6.4 for all treatments (Fig. 4.1 A, Table 4.2). Egg production for *A. tonsa* exposed to Cd contaminated *Thalassiosira* spp. was low ranging from 5.5 to 16.0 eggs ind^{-1} day^{-1} (Fig. 4.1 B). Tukey pairwise comparisons of egg production for the no food treatment was significantly lower (1.5 eggs ind^{-1} day^{-1}) compared to the 0, 1, 10 and 20 nM Cd treatments. There was no difference in percent hatching of *A. tonsa* exposed to Cd contaminated *Thalassiosira* spp. (Fig. 4.1 C, Table 4.2).

Copper Experiment

A. tonsa fed Cu contaminated *Thalassiosira* spp. also had no significant change in RNA:DNA ratios, which averaged 7.8 for all treatments (Fig. 4.2 A; Table 4.3). Starved *A. tonsa's* egg production, but not percent hatching was significantly different with exposure to Cu contaminated diet (Fig. 4.2 B, C, Table 4.3). Tukey pairwise comparisons of egg production found the no food (1.3 eggs ind^{-1} day^{-1}) treatment to be significantly lower than 1 and 2 nM Cu treatment (ca. 16.0 eggs ind^{-1} day^{-1}).

Metal Analysis

Cadmium media had a linear increase in metals from 8 to 148 nM, but the concentrations were approximately 8 times higher than the target values of 1 to 20 nM (Fig. 4.3). The measured increase in metal concentrations was traced back to the original stock solution from which the media was prepared. From this point on actual measured concentration of media will be placed in parenthesis following the expected metal concentration. Cadmium burden in *Thalassiosira* spp. increased sigmoidally with increase in Cd concentration of media and reached a maximum of 1139.4 nmol g^{-1} at 10 (72) nM (Fig. 4.4; Table 4.4). *A. tonsa's* Cd body burden was 1.3 nmol Cd g^{-1} on the 0 nM algae, increased nearly 5 fold to 6.4 nmol Cd g^{-1} with 2 (8) nM algae diet, but increased only slightly to 7.4 nmol Cd g^{-1} on the 5 (38) nM algae diet (Table 4.4). Body burden estimates for copepods were only made at 0, 2 (8) and 5 (38) nM due to the large number of copepods (ca. 100) need per analysis, which was limited supply of metal contaminated algae.

Copper media showed a linear increase in metals from 6 to 19 nM, however measured values were 2 to 5 times higher than the expected concentrations of 1 to 20 nM

(Fig. 4.5). Stock concentrations of copper were correct, so the increase must be due to a dilution error when making the copper solutions. Copper concentrations in *Thalassiosira* spp. were extremely variable and several of the data points were considered contaminated and were not included. Copper concentrations of *Thalassiosira* spp. did not increase in a linear pattern, with minimum contamination at exposure to 2 nM Cu media of 46.3 μmol Cu g^{-1} dry wt and maximum exposure to 20 nM Cu media had 234.8 μmol Cu g^{-1} dry wt (Table 4.5). Unfortunately, the metal measurement for 2 and 20 nM media were considered contaminated. Metal concentration of *Thalassiosira* spp. exposed to 5 (10) nM media increased slightly to 66.6 μmol Cu g^{-1} dry wt, but decreased to 56.6 μmol Cu g^{-1} dry wt at 10 (19) nM media exposure (Table 4.5). *A. tonsa's* Cu body burden was 11.4 nmol Cu g^{-1} on the 2 nM algae and remained relatively unchanged at 11.9 nmol Cu g^{-1} with 5 (10) nM algae diet. Body burden estimates for copepods were only made at 2 and 5 nM due to limited supply of contaminated algae and contamination of 0 nM samples. Due to the lower detection limits of the ICP-MS for measuring Cu, complicated by the salinity of the samples, it was extremely difficult to obtain repeatable and reliable estimates of Cu concentration.

DISCUSSION

A. tonsa was tolerant of biologically relevant levels $\leq$ 20 nM of Cd (147 nM measured) and Cu (19 nM measured). Survival was not affected by the increase in metal concentration, so it is clear that copepods were exposed to sublethal contamination levels. Egg production, but not nucleic acid values or hatching success, changed only slightly in response to nearly a 20 fold increase in either Cd or Cu. There was a significant reduction in egg production in the starved copepods, but egg production and RNA:DNA

ratios were not different between toxic heavy metal diets. Due to the small change in egg production with increased metal exposure, it is reasonable that nucleic acid values showed no significant response between metal treatment levels.

Overall egg production values were reduced for *A. tonsa* throughout these experiments. Egg production of *A. tonsa* has been shown to be quite variable and can range from 40 to 120 eggs $ind^{-1}day^{-1}$ under optimal environmental conditions (Durbin 1983; Ambler 1986; McManus and Foster 1998; Chapter 1). However, when egg production values of *A. tonsa* fall below 20 eggs $ind^{-1}day^{-1}$ as was the case here, it implies inadequate environmental conditions (Fig. 4.1 A and 4.2 A). Hook and Fisher (2001, 2002) reported that *Acartia* spp. egg production at a dietary exposure of 1 and 2 nM Cd dropped from over 100 eggs ind^{-1} day^{-1} to less than 60 eggs ind^{-1} day^{-1} when exposed to dietary Cd of 5 and 10 nM (Hook and Fisher 2001, 2002). Cadmium concentrations of above 89 nM (10 ppb) reduced egg production of *A. tonsa* by 10 % or greater (Toudal and Riisgård 1987). Hazzard and Kleppel (2003) concluded that *A. tonsa* from Florida Bay had reduced egg production (< 20 eggs ind^{-1} day^{-1}) because of the low nutritional value of their diets which lacked essential fatty acids. No measurements of algal quality were made, such as protein, lipids, fatty acids carbon or nitrogen, but cell density in cultures was low and cells often appeared unhealthy (irregular shape) indicating reduced food quality in metal contaminated cultures. Alternatively, cadmium stress may interfere with feeding mechanisms indirectly reducing egg production, which has been shown for *D. magna* (Barber et al. 1994). Algal cell ingestion rate and egg production of *A. tonsa* also showed significant decline with 268 nM (30 ppb) Cd exposure (Toudal and Riisgård 1987). The low egg production in the 0 nM metal treatments is difficult to explain. The

lack of additional trace metal Cu or Zn additions in the f/2 media may have contributed to lower algal quality, but this explanation seems unlikely considering estimates of dissolved Cu in Corpus Christi Bay have been over 1 μM (Benoit et al. 1994). Alternatively, it is possible that cells may have been damaged when filtered onto the 0.2 μm polycarbonate filters, however cell damage appeared minimal upon microscope inspection.

Hatching success was shown to be significantly reduced in *Acartia* spp. exposed to 5 and 10 nM Cd via contaminated diet by Hook and Fisher (2001, 2002), but a reduction in hatching success with Cd or Cu exposure was not found in the present study (Fig. 4.1 C and 4.2 C). Similar to the present findings, Sunda et al. (1987) showed no significant difference in percent hatching with exposure to Cu, but did note that survival of nauplii was significantly lower. Hook and Fisher (2002) suggest that metals bind with enzymes involved with vitellogenesis resulting in reduction of egg production. If this is the means by which metals interfere with egg production it might explain the lack of change in RNA:DNA ratios because RNA and protein are present, but are not being properly utilized by copepods to produce eggs.

A. tonsa in the present study exhibited low RNA:DNA ratios. The RNA:DNA ratios of 5 - 9 measured in this study are somewhat low for *A. tonsa* compared to values of 5 - 17 found by Bersano (2000) and 10 - 15 found in Chapter 1; both studies examining changes in RNA:DNA ratios due to diet (Fig. 4.1 C and 4.2 C). However, RNA:DNA ratios of *Acartia bifilosa* ranging from 2 - 5 were shown by Gorokhova (2003) depending on food conditions. Bersano (2000) fed copepods three different algae types, one of them being *Thalassiosira weissflogii*, which is in the same genus as the

diatom used in Chapter 1. Gorokhova (2003) fed *A. bifilosa* prescreened (60 μm) ambient water at three different dilutions, so copepods were feeding on a natural assemblage of algae and particulate organic carbon. Although temperature and salinity have been shown to cause changes in RNA:DNA ratios, the most dramatic changes in RNA:DNA ratios appear to be caused by differences in diet (Saiz et al. 1998; Bersano 2000; Wagner et al. 1998, 2001; Chapter 2). The increase in metal exposure reducing egg production and RNA:DNA ratios may have also indirectly lowered the food quality of *Thalassiosira* spp.

A few studies have examined the effect of heavy metal exposure on fish nucleic acid concentrations. RNA, RNA:DNA ratios and growth rates were reduced in tilapia (*Oreochromis mossambicus*) exposed to 54 μM Cd (6 ppm), suggesting that RNA:DNA ratios are a good indicator of growth in tilapia (James and Sampath 1999). Fathead minnow larvae (*Pimephales promelas*) also showed reduced RNA and RNA:DNA ratios when exposed to sublethal levels of chromium, hydrogen cyanide, ethyl acetate, *p*-cresol and benzophenone (Barron and Adelman 1984). However, RNA:DNA ratio analysis did not prove to be a useful growth indicator for carp (*Cyprinus carpio*) exposed to 0.8 μM Cu (250 μg Cu L^{-1}) or for juvenile rockfish (*Sebastes schlegeli*) exposed to up to 8 μmol Cu g^{-1} dry weight (500 mg Cu kg^{-1}) through their diet (De Boeck et al. 1997; Kim and Kang 2004). The use of RNA content and RNA:DNA ratios of fish as an indicator of sublethal metal exposure has had mixed results, which may be due to the differences in species, stage of life and type of toxicant exposure used in these studies (Kim and Kang 2004 and references therein).

The number of studies examining the effects of heavy metal exposure on nucleic acid concentrations of invertebrates is small and often focused solely on either DNA or RNA. Increased damage to DNA has been documented in bivalves after exposure to Cd (1 μM $CdCl_2$) or Cu (280 nM and 595 nM $CuCl_2$) (Bolognesi et al. 1999; Pruski and Dixon 2002). Exposure to sublethal levels of Cd and Cu have resulted in increases in mRNA specific for the metallothionein gene in the oyster *Crassostrea virginica,* which is part of their defense against metal contamination (Unger and Roesijadi 1996; Butler and Roesijadi 2001). Metallothionein concentration of *C. virginica* increased with 2.5 μM of Cd and Cu, but concentration of mRNA for Cd and toxicity of Cd were greater than Cu (Butler and Roesijadi 2001). Earlier work reveled *C. virginica's* metallothionein mRNA levels increased significantly when exposed to Cd concentration of only 44 nM Cd (Unger and Roesijadi 1996). Metallothionein is a metal binding protein responsible for metal regulation and detoxification (Di Giulio et al. 1995). Transcription of metallothionein is metal dependant and has been shown to increase in the presence of Cd, Cu, Zn and Hg (Palmiter 1987, 1994; Thiele 1992). The gastropod *Helix pomatia* showed enhanced Cd metallothionein mRNA when exposed to 8.9 μM Cd contaminated food and a slight increase in Cu metallothionein mRNA when exposed to 15.7 μM Cu contaminated food (Chabicovsky et al. 2003). Bulk RNA was measured in the current study and different types of RNA were not distinguished. However, it seems unlikely that changes in mRNA specific to metallothionein would be seen using this assay because total mRNA makes up 3-5% of total RNA (Branhorst and McConkey 1974; Stryer 1988). One study did examine changes in both nucleic acids and found that *D. magna* showed no

change in RNA or RNA:DNA ratios when exposed to 9 or 45 nM Cd (1 and 5 μg Cd L^{-1}) (Barber et al. 1994).

Metal measurements of Cd and Cu in the algal culture media were roughly 2 to 8 fold higher than expected, but still within sublethal levels in these experiments. This increase in overall metal exposure may be responsible for the general suppression in egg production and RNA:DNA ratios of *A. tonsa*. Target levels were chosen based on *Acartia* spp. (*A. tonsa* and *A. hudsonica*) study of Hook and Fisher (2001), who found Cd levels above 5 nM to be lethal and reproduction to be impaired at 5 nM Cd. In the present study, 5 (38 measured) nM media metal was equal to 882.9 nmol Cd g^{-1} dry wt. in *Thalassiosira* spp. and corresponded to a body burden increase of 6.2 nmol Cd g^{-1} dry wt in *A. tonsa*. These increases in Cd body burdens are comparable to increases of 15.3 nmol Cd g^{-1} dry in *Acartia* spp. exposed to 5 nM Cd in Hook and Fisher (2001). Copper levels were found in *Thalassiosira* spp. exposed to 5 (10 measured) nM Cu media to be 66 nmol g^{-1} dry wt. corresponding to copepod body burden of 12 nmol g^{-1} dry wt. The majority of studies examining Cu uptake of phytoplankton and zooplankton have been interested in how differences in free ion will alter metal bioavailability, so it is difficult to directly compare these results. Caution is needed before drawing conclusions regarding uptake of metals (Cd and Cu) by algae or copepods, because of the small number of samples analyzed for metals and lack of sufficient replication. Metal concentrations were measured in the present study to determine their presence, and not to quantify metal transfer in culturing media, algae and copepods.

The target (1 - 20 nM) and as well as the actual measured (8 - 147 nM) metal concentrations used in this study are realistically comparable to coastal bays. Sediment

contamination levels of Cu and Cd in San Antonio-Guadalupe River, TX are 10 – 50 % higher than natural background levels (Trefry and Presley 1976). Galveston Bay, TX has 2.3 to 10.2 nM Cu in dissolved phase, with concentration varying with salinity (Tang et al. 2001). The Houston Ship Channel showed dissolved metals increased (Cu 475 nM to 4,448 nM and Cd 0.733 nM to 8.571 nM) from the river towards the mouth of the channel (Saleh and Wilson 1999). Corpus Christi Bay, Texas has between 3.2 and 17.5 μM dissolved Cu depending on the time of year and salinity of the bay (Benoit et al. 1994). Sediment quality of Corpus Christi Bay, has also been shown to contain elevated levels of Cd and Cu, which have been linked to degraded habitat sites (Carr et al. 2000). Metal contamination is especially problematic in shallow estuarine areas such as those found in South Texas.

In conclusion, *A. tonsa* is tolerant of dietary exposure to Cd and Cu levels of up to 20 expected (148 measured) nM and showed minimal change in egg production and no change in RNA:DNA ratios. RNA:DNA ratios are not a useful indicator of metal exposure at concentrations found in the present study. However, it might be possible to detect changes in RNA:DNA ratios at higher metal levels under periods of acute exposure or lower metal level that may not suppress egg production as greatly as the present study. However, a more practical molecular approach to detecting metals may be to target mRNA specific for metallothionein genes. It is critical to develop a relatively quick and easy means to identify sublethal levels of metal in estuarine systems because current methods of measuring metal level are costly, time consuming and limited to only a few labs with the necessary equipment.

Table 4.1. Analysis of variance (ANOVA) results of mortality test for *Acartia tonsa* after 96 hr exposed to Cd contaminated algae at 0 to 20 nM or Cu contaminated algae at exposure 0 to 20 nM.

Variable	df	SS	MS	F	p
Cd	5	16.667	3.333	1.364	0.304
Error	12	29.333	2.444		
Cu	5	9.611	1.922	2.883	0.069
Error	12	8.000	0.667		

Table 4.2. Analysis of variance (ANOVA) results of RNA:DNA ratio analysis, egg production (EP) and percent hatching (% hatch) of *Acartia tonsa* exposed to no food and Cd contaminated algae at 0 to 20 nM; bold values are significant at $p \leq 0.05$.

Variable	df	SS	MS	F	p
Ratio	6	51.801	8.634	1.096	0.368
Error	140	1103.169	7.880		
EP	6	606.710	101.118	5.197	**0.005**
Error	14	272.415	19.458		
% Hatch	6	0.456	0.076	2.538	0.071
Error	14	0.419	0.030		

Table 4.3. Analysis of variance (ANOVA) results of RNA:DNA ratio analysis, egg production (EP) and percent hatching (% hatch) of *Acartia tonsa* exposed to no food and Cu contaminated algae at 0 to 20 nM; bold values are significant at $p \leq 0.05$.

Variable	df	SS	MS	F	p
Ratio	6	91.304	15.217	0.870	0.519
Error	124	2169.385	17.495		
EP	6	480.009	80.001	6.501	**0.002**
Error	13	159.971	12.305		
% Hatch	6	0.210	0.035	0.998	0.467
Error	13	0.456	0.035		

Table 4.4. Concentration (nmol g^{-1} dry wt) of Cd measured using inductively-coupled plasma mass spectrometry (ICP-MS) in the alga *Thalassiosira* spp. and copepod *Acartia tonsa*; NA = no analysis was conducted.

Expected Cd Exposure (nM)	**0**	**1**	**2**	**5**	**10**	**20**
Concentration in algae (nmol g^{-1})	9.5	265.0	405.1	882.9	1139.7	1028.4
Concentration in copepod (nmol g^{-1})	1.3	NA	6.4	7.4	NA	NA

Table 4.5. Concentration (nmol g^{-1} dry wt) of Cu measured using inductively-coupled plasma mass spectrometry (ICP-MS) in the alga *Thalassiosira* spp. and copepod *Acartia tonsa;* NA = no analysis was conducted, CS = contaminated samples and were not included.

Expected Cu Exposure (nM)	**0**	**1**	**2**	**5**	**10**	**20**
Concentration in algae (nmol g^{-1})	CS	CS	46.3	66.6	56.6	234.8
Concentration in copepod (nmol g^{-1})	CS	NA	11.4	11.9	NA	NA

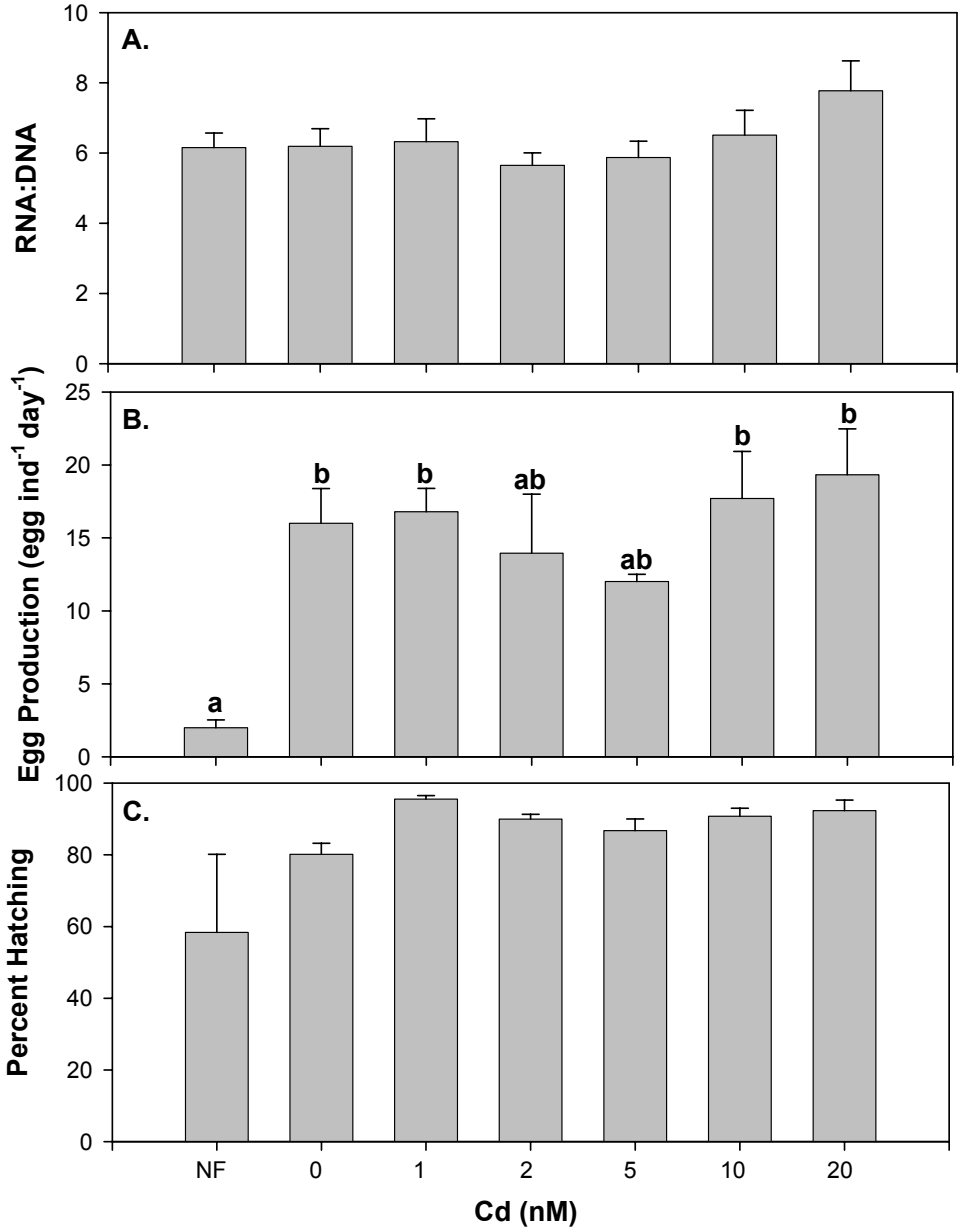


Figure 4.1. Changes in *Acartia tonsa* productivity under no food (NF) and 0 to 20 nM Cd contaminated algal diets; A. individual RNA:DNA ratio, B. group estimates of egg production; C. group estimates of percent hatching; Tukey multiple comparisons $\alpha < 0.05$ and a is significantly different form b, but not ab; error bars represent standard error.

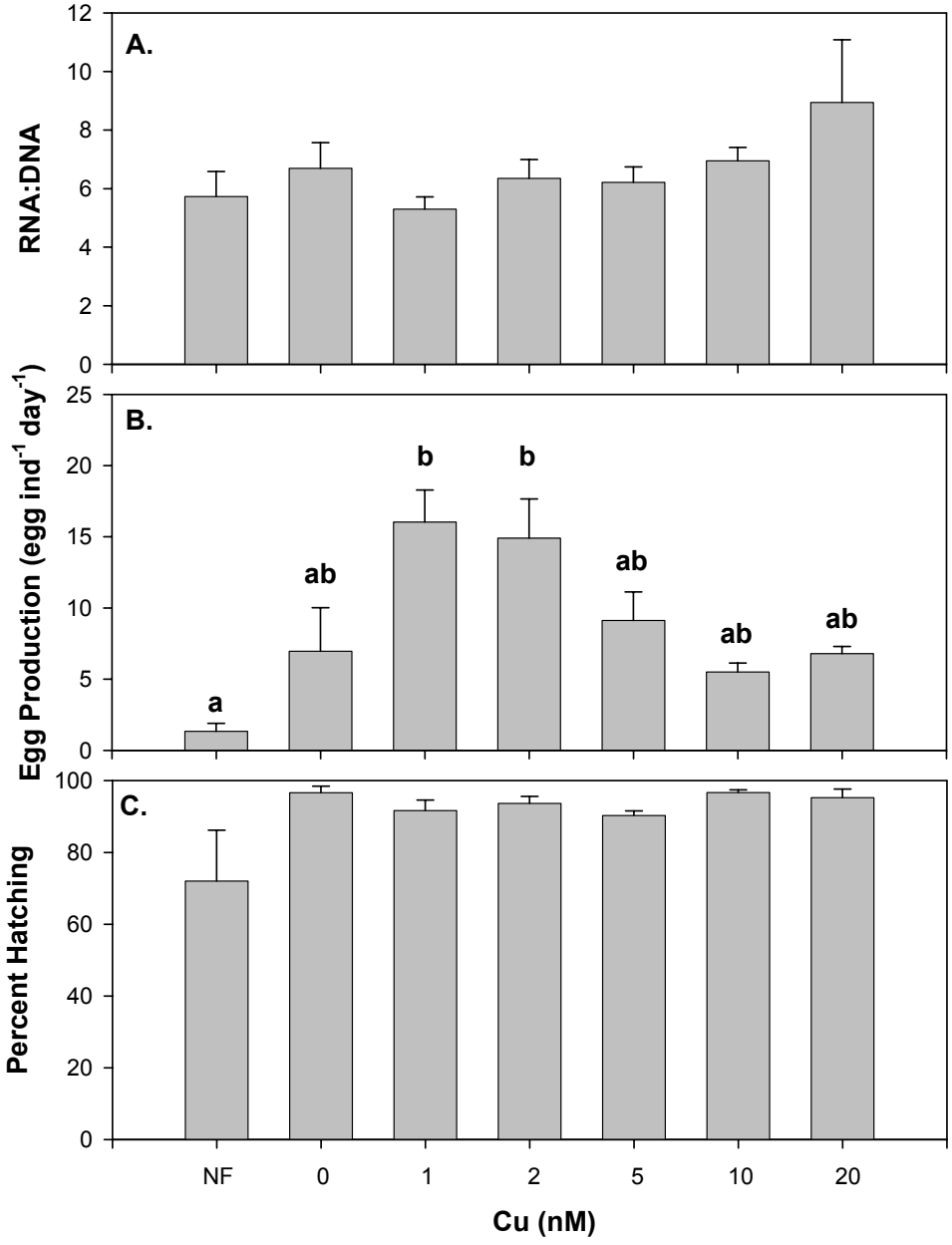


Figure 4.2. Changes in *Acartia tonsa* productivity under no food (NF) and 0 to 20 nM Cu contaminated algal diets; A. individual RNA:DNA ratios B. group estimates of egg production; C. group estimates of percent hatching; Tukey multiple comparisons $\alpha < 0.05$ and a is significantly different form b; error bars represent standard error.

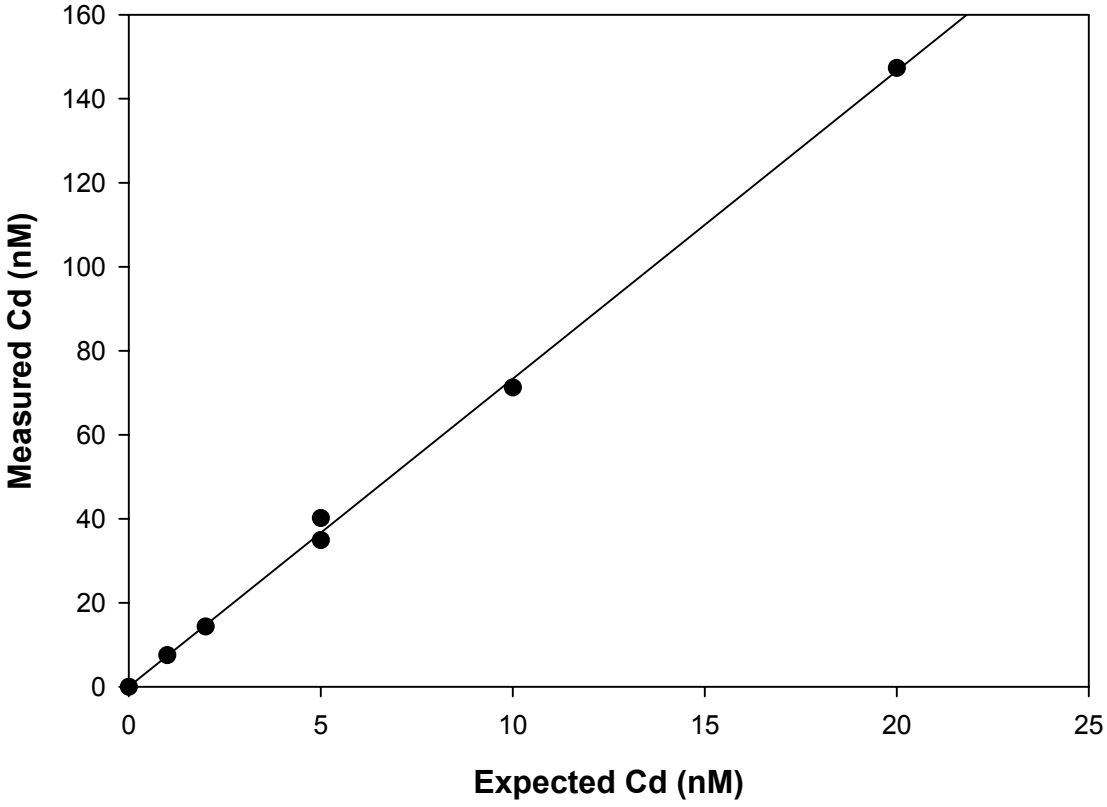


Figure 4.3. Concentration (nM) of Cd in f/2 media measured using inductively-coupled plasma mass spectrometry (ICP-MS); $y = 7.349x + 0.642$, $r^2 = 0.998$, $p < 0.001$.

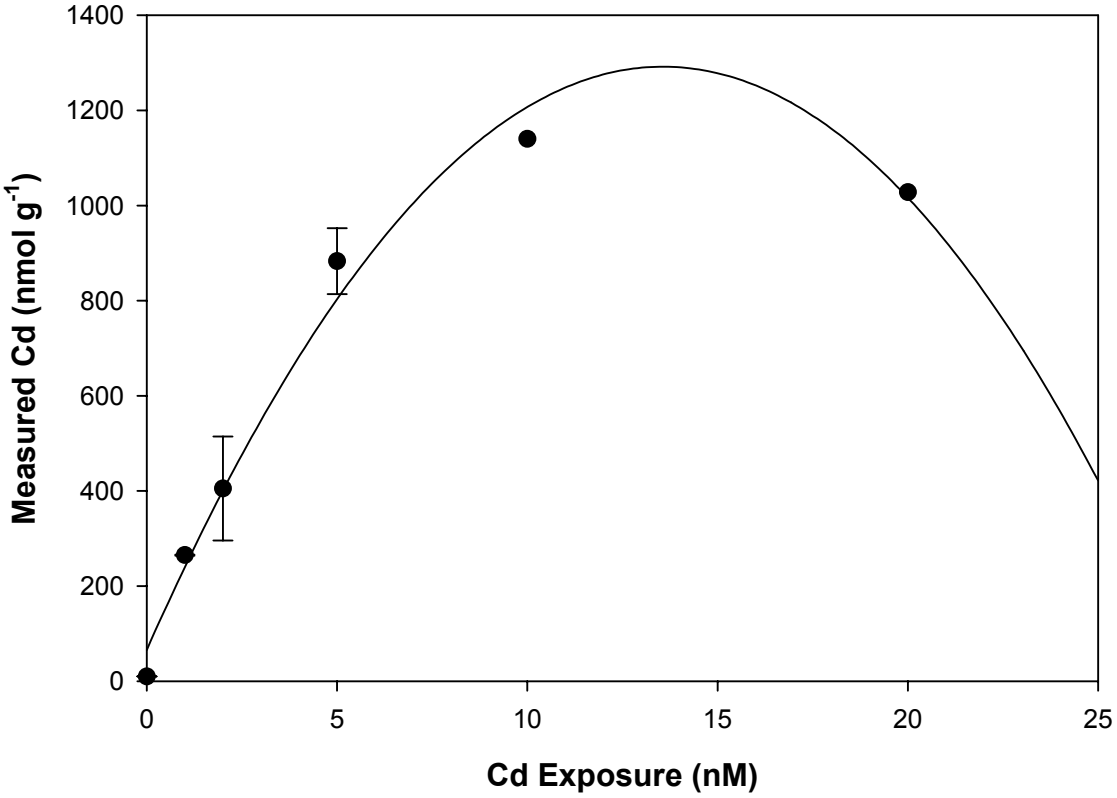


Figure 4.4. Concentration (nmol g^{-1} dry wt) of Cd measured using inductively-coupled plasma mass spectrometry (ICP-MS) in the alga *Thalassiosira* spp.; vertical lines represent standard error; $y = 65.396 + 180.740 + -6.660x^2$, $r^2 = 0.986$, $p < 0.001$.

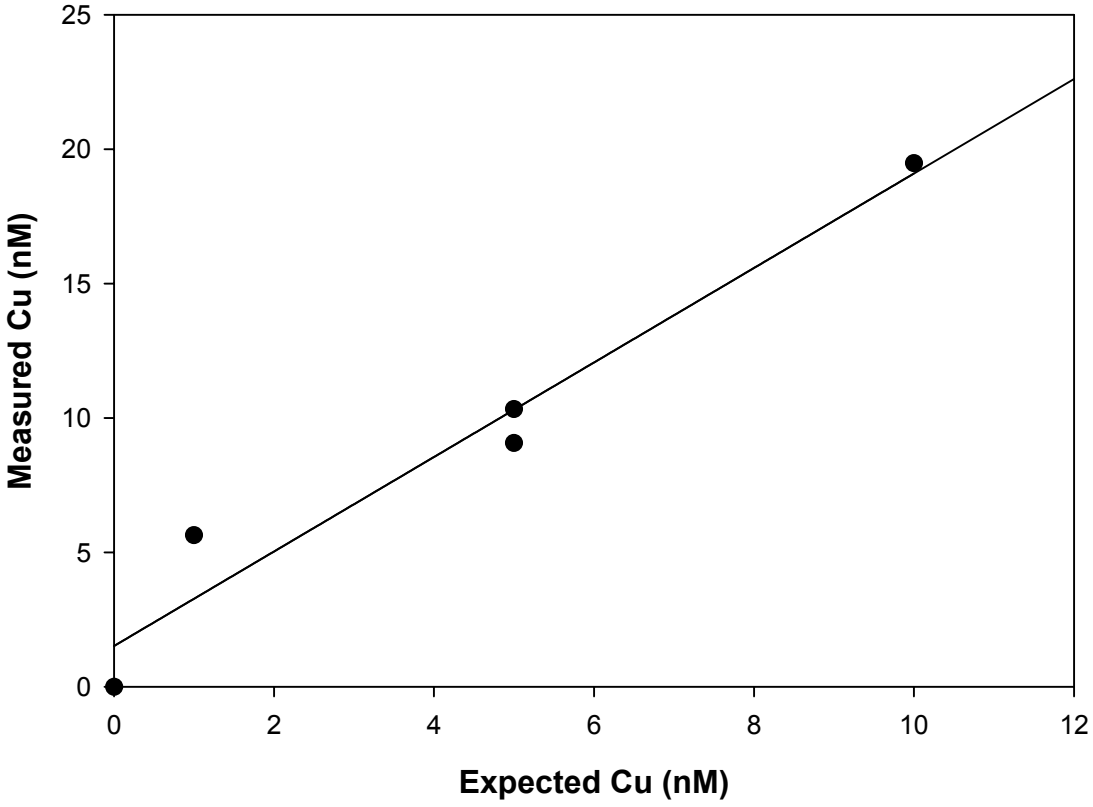


Figure 4.5. Concentration (nM) of Cu in f/2 media measured using inductively-coupled plasma mass spectrometry (ICP-MS); $y = 1.758x + 1.518$, $r^2 = 0.953$, $p = 0.004$.

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Vita

Christa Liane Speekmann, daughter of Judi and Walter Speekmann, was born on October 9, 1974, in Glendale, California. She graduated from Crescenta Valley High school in 1993. She received her Bachelor's degree at Occidental College in Biology with an emphasis in marine science and graduated *cum laude* with departmental honors in 1997. At Occidental College she studied the effects of ultraviolet radiation (UVR) on zooplankton. She continued her work on the harmful effects of UVR on zooplankton at San Francisco State University working with Dr. S. M. Bollens, where in 2000 she earned a Master of Art in Marine Biology. Her undergraduate and Master's work on UVR have been published in peer-reviewed journals. Christa furthered her graduate career by perusing a doctorate degree in Marine Science at The University of Texas Marine Science Institute (UTMSI). At UTMSI she worked with Dr. E. J. Buskey developing an indicator of zooplankton health by examining RNA:DNA ratios of copepods. Throughout her graduate career she has been a teaching assistant for several different biology/ecological classes. In addition, Christa was an active member of the graduate student associations and outreach activities.

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