

2011

# Vectoring algal toxin in marine planktonic food webs: sorting out nutritional deficiency from toxicity effects

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**VECTERING ALGAL TOXIN IN  
MARINE PLANKTONIC FOOD WEBS: SORTING OUT  
NUTRITIONAL DEFICIENCY FROM TOXICITY EFFECTS**

By

Amy Keita Burgess

Accepted in Partial Completion  
Of the Requirements for the Degree  
Masters of Science

Moheb A. Ghali, Dean of the Graduate School

ADVISORY COMMITTEE

Chair, Dr. Stephen D. Sulkin

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Dr. Ruth M. Sofield

## **MASTER'S THESIS**

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Amy Keita Burgess

November 4, 2011

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**ABSTRACT**

The present study determined whether increased mortality and delayed development of larval crabs fed heterotrophic prey that themselves have been fed toxin-containing algae is due to toxicity effects or nutritional deficiency. The effects on larval crabs of previous exposure to heterotrophic prey fed toxin-containing algae were examined. Effects of varying length of exposure of larvae to toxin-containing prey were also examined.

The rotifer *Brachionus plicatilis* was used as a heterotrophic prey source for three larval crab species (*Lophopanopeus bellus*, *Metacarcinus magister*, and *Glebocarcinus oregonensis*). Two rotifer treatments were created, one of rotifers fed a toxin-containing alga (*Alexandrium andersoni* or *A. fundyense*); the other of rotifers fed a non-toxic, nutritionally sufficient alga (*Isochrysis galbana*). To distinguish between toxic and nutritional effects, groups of larvae were fed various combinations of the two rotifer types. Diet treatments included the following ratios of toxin-containing algal fed and non-toxic algal fed rotifers: 100%/0%, 75%/25%, 50%/50%, 25%/75%, and 0%/100%. Larval crabs showed no differences in feeding rates or feeding preferences for the two rotifer diets. Crab survival was lower on the 100% toxin-containing algal fed rotifer diet when compared to the 100% non-toxin-containing algae fed rotifer diet for all three crab species. In all three crab species,

stage duration was also extended in larvae fed the 100% toxin-containing algal fed rotifers compared to the 100% non-toxin-containing algal fed rotifers. Increased survival and accelerated development when toxin-containing rotifers were replaced in treatments with non-toxin-containing rotifers implicates nutritional deficiency in the former diet rather than its potential toxic effects.

Reduction in time of exposure to a prey source reduced survival and extended development to a greater degree in toxin-containing rotifer treatments than in non-toxin-containing diets. There was no apparent effect of prior exposure to toxin-containing prey on survival or stage duration of later larval stage exposed to the same diet.

Larval crabs face an unpredictable and complex prey environment once they enter the plankton. Encounters with Harmful Algal Blooms (HAB) or heterotrophic prey that have ingested HAB species may injure larvae that have no other food source. While my research suggests that nutritional deficiency of the rotifers fed toxin-containing algae causes higher mortality rates and delayed development in the crab larvae, toxin transfer cannot be totally eliminated and a combination of the two factors is most likely causing the negative effects. If the prey environment for these larval crabs includes a nutritionally sufficient animal prey source, negative impacts (i.e. delay in stage duration and decreased survival) caused by exposure to HABs and prey that have ingested HABs can be supplemented.

## **ACKNOWLEDGEMENTS**

First, I would like to thank my advisor Dr. Stephen Sulkin, for his continual mentorship in all facets of my research and graduate education. His support, guidance, and knowledge have been invaluable to my growth as a scientist. He was the most important contributor to my graduate education.

I would also like to acknowledge my committee members, Dr. Brian Bingham for his instruction and assistance with statistics and Dr. Ruth Sofield for being available even when she was thousands of miles away.

I greatly appreciate the faculty and staff at the Shannon Point Marine Center faculty, all of whom have helped in my journey through graduate school, both in my research and education. I would like to thank Rachel Garcia for her wisdom and help with culturing organisms. I would like to acknowledge Nathan Schwarck for training and assistance in SCUBA for organism collection, as well as the undergraduate internship participants who assisted with collection of organisms and experiments.

Lastly I have a great appreciation for the Environmental Science Department and the Shannon Point Marine Center for providing financial support and training through teaching and graduate assistantships and finally my family for their never-ending support.

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## INTRODUCTION

Harmful algal blooms are an increasingly common occurrence throughout the world's oceans (Hallegraeff 1993, Landsberg 2002). Harmful algal blooms are detrimental to finfish in fish pens and can close down shellfish beds due to the potential for humans to contract paralytic shellfish poisoning (Landsberg 2002). *Alexandrium* is a genus of dinoflagelletes that has both toxin-containing and nontoxic strains known to form blooms (Anderson 1990). Toxicity varies among the toxin-containing *Alexandrium* species with some sufficiently toxic to harm other species that encounter the blooms (Anderson 1990). *Alexandrium* spp. and other HAB species could indirectly affect coastal system productivity by injecting algal toxin into the food web. However, a better understanding of the dynamics of algal toxin transfer among components of the food web must be developed before an accurate assessment of their broader impact will be possible.

Larval crabs are important members of the planktonic food web, linking the microbial loop to metazoans (Lehto et al. 1998, Sulkin et al. 1998b). Encounters with toxic algal blooms can have both population and community level. Crab recruitment as controlled by larval survival, development time and behaviors affect adult distribution and abundance. Community level effects relate to the role larval crabs play as dominant members of the meso-zooplankton at certain times and places, producing top-down control of other plankters via predation while serving as prey for larger plankton, jellyfish and finfish.

Crabs produce an independent, free-swimming, planktotrophic larva called the zoea. After hatching, zoeae enter the water column and begin feeding. The planktonic phase lasts for several weeks to months depending on the species and includes several molts before the zoeae reach a post-larval megalopa stage. This stage settles to the benthic habitat.

Zoeae must start feeding in the plankton soon after they hatch to sustain normal development and achieve metamorphosis (Staton and Sulkin 1991). Any delay in feeding results in reduced survival, even if feeding subsequently occurs (Staton and Sulkin 1991, Hartman and Sulkin 1999). Diets must provide both energy to sustain metabolism and specific nutrients necessary to support development to metamorphosis (Sulkin 1975, McConaugha 1985). For example, zoeae require long-chain polyunsaturated omega-3 fatty acids (PUFAs) in their diets to develop normally (Levine and Sulkin 1984a, 1984b). Zoeae are omnivores that will ingest almost anything of the proper size including phytoplankton, micro-zooplankton, meso-zooplankton (both holoplankton and meroplankton), and detritus (Levine and Sulkin 1984a, Lehto et al. 1998, Hinz et al. 2001).

Facing an unpredictable and complex prey environment, larval crabs are adapted to ingest a wide variety of prey, including prey that provide little or no nutritional value to them. Studies have shown that many types of phytoplankton are readily ingested, but will not sustain development. Larval diets must include long-chain, omega-3 polyunsaturated fatty acids to develop and metamorphose (Levine and Sulkin 1984). These polyunsaturated fatty acids can come from small animal prey (micro or small meso-plankton) and algal species.

Small animal prey may include lecithotrophic larvae of macroinvertebrates, heterotrophic larvae or holoplanktonic species. Larval crabs feeding on yolky larvae appear to be taking advantage of nutrients stored in the prey. If the crab feeds on heterotrophic prey, however its nutritional value may be dependent upon its own diet. Sulkin and McKeen (1999) reported that the ability of the rotifer *Brachionus plicatilis* to support larval



development depends upon its own algal prey, with rotifers fed the algae *Isochrysis galbana* being more nutritious than those fed *Dunaliella tertiolecta*. Clearly, larval crabs do better when fed a prey that itself has been eating a high quality diet. However, what happens if the prey has been feeding on a diet filled with toxins?

Garcia et al. (2011) raised rotifers on two types of toxin-containing algae. Previous research had shown that one algal species was ingested directly by larval crabs (*Alexandrium andersoni*) and accelerated mortality and the other toxin-containing alga was not ingested or was ingested for only a brief time (*A. fundyense*) (Hinz et al. 2001, Perez and Sulkin 2005). Garcia et al. (2011) found that the rotifer *Brachionus plicatilis* will itself ingest both algal species. Larval crabs fed these rotifers survived into the third zoeal stage at which point the experiments were terminated. Survival however, was lower than when larvae were fed rotifers that had been cultured on a non-toxic algal species (*Isochrysis galbana*). It is not clear, however, whether this reduced survival was due to the effects of algal toxin that has been transferred via the rotifer prey or to the possible nutritional deficiency of the rotifers themselves, resulting from their toxin-containing diet. The rotifer therefore provides an excellent opportunity to distinguish between nutritional deficiency and toxic effects on larval crabs.

Use of rotifers is of particular value experimentally, since the rotifer diet can potentially vector toxin that produce almost immediate mortality when ingested by crabs directly (Perez and Sulkin 2005). When the rotifer is ingested by the larval crab it can also introduce the crabs to a toxin containing species they will not feed on directly (Hinz et al.

2001). Thus, the heterotrophic rotifer prey can both ameliorate potential toxic effects and introduce toxins that would not be otherwise ingested.

The experimental approach of Dam and Colin (2005) was applied to sort out potential toxic effects from those caused by nutritional deficiency. Accordingly, mixtures of toxin-containing algal fed rotifers and non-toxic algal fed rotifers were given to larvae of three local crab species. This experimental approach requires that larvae ingest both rotifer prey types equally. The research therefore included experiments to determine if larval crabs showed different feeding rates on toxin-containing algal fed rotifers compared to non-toxin containing algal fed rotifers.

The results of Garcia et al. (2011) showing increased larval mortality on a diet of toxin-containing rotifers begs the question of whether there might be selection occurring as a result. Are surviving larvae less susceptible to exposure to algal toxin in later larval stages? If so, this should be revealed as reduced mortality when subsequent stages are exposed to toxin-containing algal fed rotifers, compared to larvae not exposed in early stages.

The purpose of my research was to study the dynamics of algal toxin transfer among components of the planktonic food web by applying a model for larval crab feeding. My research had three objectives. The main objective was to determine whether the reduction in crab larval survival and delay in development reported on the toxic algal-fed rotifer diet is a function of transfer of algal toxins or a consequence of reduced nutritional value of the rotifer fed on the toxin-containing algae. The second objective, to support the main objective, was to determine if continual feeding by larval crabs is occurring on a heterotrophic prey that has

consumed toxin-containing algae. The third objective was to determine if larvae that have been previously exposed to the nutritional or toxic stress associated with ingesting toxin-containing algal fed rotifers are more resistant to exposure to such prey in subsequent larval stages.

## METHODS

### Experimental Approach

The experimental approach is based on the technique reported by Dam and Colin (2005) to separate possible toxic versus nutritional deficiency effects on a developmental parameter as compared between a “good” diet and a “poor” diet. In the present study, *Isochrysis galbana* fed rotifers were defined as the “good” diet and *Alexandrium* spp. fed rotifers as the “poor” diet based on the results of Garcia et al. (2011).

The technique is based on the assumption that a toxic effect caused by ingesting a toxin-containing food cannot be masked by positive effects of adding a nontoxic prey to a mixture of the two. Adding a nontoxic prey to such a mixture, however, could supplement its nutritional value if the results of the “poor” diet were caused by nutritional deficiency and not by toxicity. Applying the Dam and Colin (2005) approach to the present study, acceleration of time to molt or percent survival was studied as a function of the proportion of the “good” food in the diet mixture. The technique involved plotting the percent molt acceleration or percent survival value of the 100% poor diet and the value of the 100% “good” diet. A straight line connects the two values. If, as the proportion of “good” food increases in a series of diet mixtures of varying proportions, the acceleration or percent values fall below the straight line, toxicity is inferred (e.g., the addition of the good diet is not sustaining or improving theoretical rates of acceleration or percent survival). If, however, the values fall above the straight line, it is inferred that acceleration or percent survival is being

increased by the improvement of the nutritional value of the prey mixture due to the increasing proportion of the “good” diet.

In the toxicity versus nutritional deficiency experiments, rotifers cultured on different algal species were combined in different ratios and fed to zoeae of three crab species, *Lophopanopeus bellus* Stimpson, *Metacarcinus magister* Dana, and *Glebocarcinus oregonensis* Dana. Daily survival through zoeal development and percent mortality and duration for individual stages I, II, III, and IV (depending on the species), were determined and compared among diet treatments.

To determine if zoeae fed on toxin-containing algal fed rotifers are ingesting prey on a continual basis, larvae were fed either continuously or four hours per day to determine if there were any differences in survival and development. These experiments were conducted using zoeae from two crab species, *Lophopanopeus bellus* and *Glebocarcinus oregonensis*. Daily survival, percent mortality for each stage, and stage duration for stages I and II were compared between diet exposure treatments.

The increased mortality of larvae fed rotifers that had been cultured on toxin-containing algae compared to larvae fed nontoxic rotifers through early development may be producing surviving larvae that are more resistant to the effects of subsequent exposure to toxin in the diets (i.e. the weak larvae die leaving only more toxin-resistant survivors). To determine if this is happening, stage III, day one larvae that had been previously fed either toxin-containing algal fed rotifers or non-toxin containing algal fed rotifers were given either

the same diet as previously fed or were switched to the other diet. Results from these experiments were recorded starting with stage III, day one larvae.

## **Experimental Organisms**

### **Algal Cultures**

The non-toxic (not containing saxitoxin) alga *Isochrysis galbana* (strain 1323) Parke was cultured in 20 L glass jugs with pasteurized 0.2  $\mu\text{m}$  filtered seawater (FSW). Cultures were kept at room temperature, aerated and kept in 24 hour light using a light box. The culture medium was F/2 medium.

Two species containing saxitoxin, *Alexandrium andersoni* (strain 1718) Balech and *A. fundyense* (strain 1719) Balech, were cultured in 0.5-1 L plastic Nalgene bottles and glass flasks in autoclaved 0.2  $\mu\text{m}$  FSW. Cultures were kept in an incubator at 20<sup>0</sup> C with a 12 hour light, 12 hour dark cycle. The culture medium was F/2 medium. Starter cultures were obtained from Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP, West Boothbay Harbor, ME, USA). To achieve desired algal densities 0.2  $\mu\text{m}$  FSW was used to dilute cultures.

### **Rotifers Cultures**

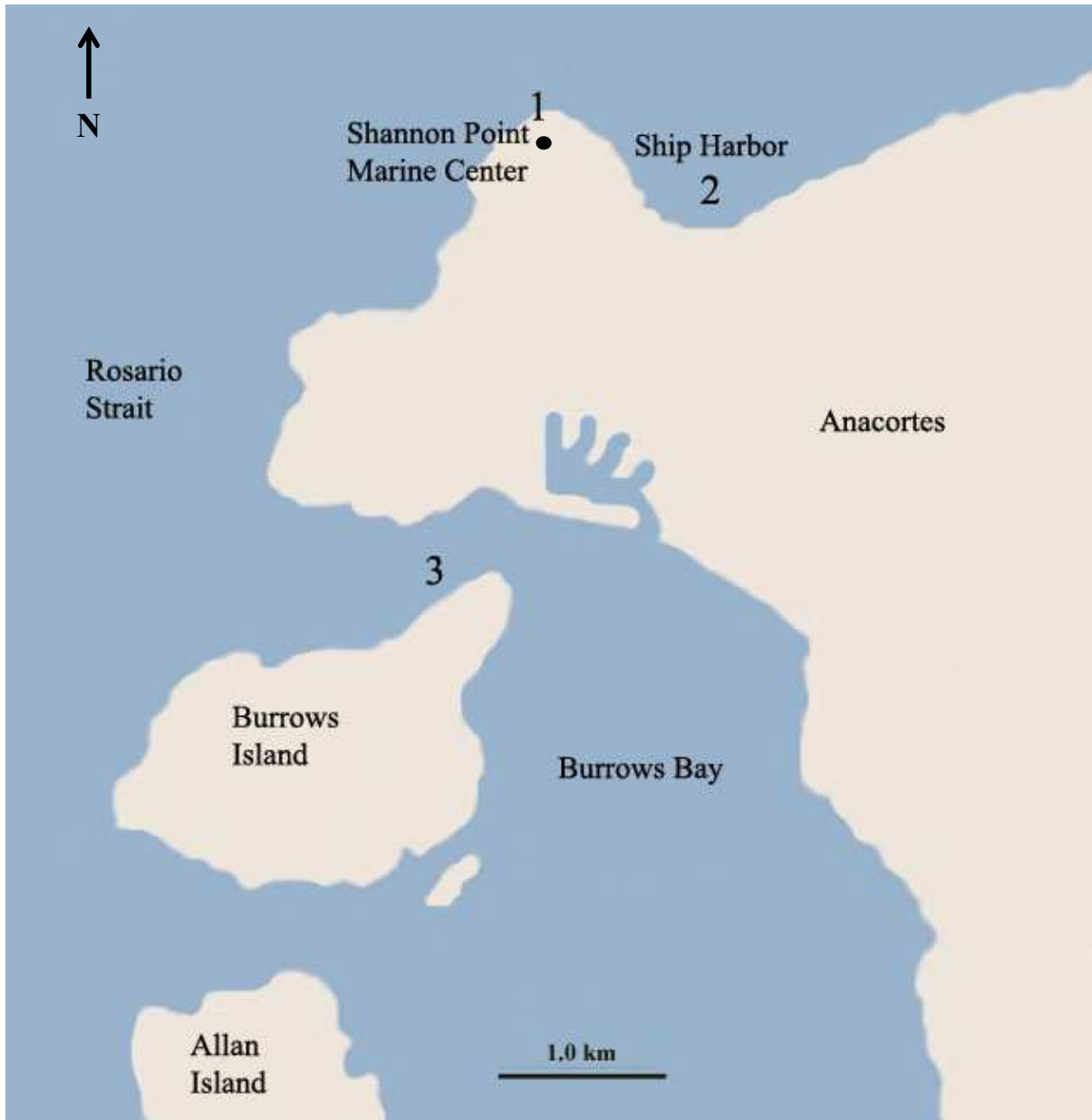
A culture of the rotifer *Brachionus plicatilis* was obtained from Reed Mariculture, Inc. (Campbell, CA, USA). Upon arrival, rotifers were put in an incubator at 20<sup>0</sup> C to adjust to the temperature. Rotifers were then filtered out of the algal food in which they were

originally suspended using an 80  $\mu\text{m}$  screen and rinsed with 0.2  $\mu\text{m}$  FSW. Rotifers were then divided into 600 mL beakers that contained 500 mL of high density (1.5 – 2 million cells  $\text{mL}^{-1}$ ) *Isochrysis galbana*. These are referred to hereafter as “stock cultures”. Rotifers were maintained on *I. galbana* and were periodically transferred to new beakers filled with algae to keep them in mictic phase. If necessary, excess rotifers were discarded so as to keep the cultures actively growing. Rotifers cultured on toxic algal species were maintained in 600 mL beakers in 500 mL of 800-1000 cells  $\text{mL}^{-1}$  concentration.

### **Ovigerous Females and Crab Larvae**

*Lophopanopeus bellus* ovigerous females were collected from Shannon Point beach, Anacortes, WA, USA from May 2010 through July 2010 (Figure 1). Females were held in flow-through seawater tables at ambient conditions (10-14<sup>0</sup> C; 28-30 psu) until eggs were near hatching. When heartbeats were observed in the eggs, females were transferred to 20 cm diameter glass bowls and kept in an incubator at 15<sup>0</sup> C with a 12 hour light, 12 hour dark cycle. The water in the bowls (5  $\mu\text{m}$  FSW; 30 psu) was changed every other day until hatching occurred. On days that two or more females hatched, their zoeae were collected using a pipette and combined for experiments. Pooled zoeae were used immediately to ensure that day one zoeae were used at the start of experiments.

*Metacarcinus magister* ovigerous females were collected by SCUBA at Ship Harbor, Anacortes, WA, USA on March 1, 2011 (Figure 1) and were kept in 10 L tanks with flowing seawater under ambient conditions (7-8<sup>0</sup> C; 30-32 psu). When two or more females began



**Figure 1.** Map of collection sites for crab ovigers. 1: *Lophopanopeus bellus* ovigers. 2: *Metacarcinus magister* ovigers. 3: *Glebocarcinus oregonensis* ovigers.



hatching on the same day, their tanks were drained and rinsed to remove all zoeae at the end of the day. Tanks were then refilled and left overnight. Larvae were collected the next morning using a 253  $\mu\text{m}$  screen, pooled and used for experiments. This ensured that all larvae were less than 24 hours old when experiments were initiated.

*Glebocarcinus oregonensis* ovigerous females were collected by SCUBA at Burrow's Island Pass, Anacortes, WA, USA on March 2, 2011 (Figure 1). Females were kept in containers with flowing seawater at ambient conditions (7-8<sup>0</sup> C; 30-32 psu) at Shannon Point Marine Center. When heartbeats were observed in the eggs, females were transferred to 20 cm diameter glass bowls and kept in an incubator at 15<sup>0</sup> C with a 12 hour light, 12 hour dark cycle. The water in the bowls (5  $\mu\text{m}$  FSW; 30 psu) was changed every other day until hatching occurred. On days that two or more females hatched, their zoeae were collected using a pipette and combined for experiments. Pooled zoeae were used immediately in experiments to ensure day one (less than 24 hour old) zoeae were used at the start of experiments.

### **Algal Ingestion by Rotifers**

The *Isochrysis galbana* fed rotifers used in experiments were taken from stock cultures. Rotifers that were maintained on toxin-containing algae were used in experiments after they had been fed the algae for a minimum of two days to ensure full gut saturation of the specified diet treatment.

The toxin-containing algal-fed rotifers used in experiments were taken from *Isochrysis galbana* stock cultures, filtered using an 80 µm screen, rinsed with 0.2 µm FSW to remove excess algal cells, and placed in 600 mL beakers filled with 500 mL of 800-1000 cells mL<sup>-1</sup> of the toxin-containing algae. These rotifers were cultured in these beakers for at least two days before being used in experiments.

Preliminary experiments were done to determine how long algae that had been ingested by rotifers would be maintained in the gut if the rotifers were subsequently starved. These experiments were conducted over the course of seven days and used epifluorescence microscopy to document the presence of chlorophyll, which indicates the presence of algae, in rotifer guts. These experiments determined that within 48 hours, rotifer guts had been cleared of chlorophyll. Since rotifers being fed toxin-containing algal species were initially from the *Isochrysis galbana* stock culture they were used in experiments after being fed on the *Alexandrium andersoni* or *A. fundyense* for 2-4 days to ensure that *I. galbana* was no longer in the rotifer guts. Rotifers fed the toxin-containing algae were not used after being fed the toxin-containing algae for more than four days because rotifer cultures began to decline as egg production ceased and mortality took its toll.

### **Larval Ingestion Rates on Rotifers**

To determine the larval ingestion rates of the different rotifer diets, the Frost equation (Frost 1972) was used (Figure 2). This approach accounts for growth (positive or negative) of the rotifers when no predators are present. Each experiment thus includes two treatments

### Frost Equation (Frost 1972)

$$\mu = 1/t \ln(N_{t\text{control}}/N_0) \quad \text{eq. 1}$$

$$g = \mu - [1/t \ln(N_{t\text{zoeae}}/N_0)] \quad \text{eq. 2}$$

$$F = Vg/\#\text{predators} \quad \text{eq. 3}$$

$$\langle C \rangle = N_0 [e^{(\mu-g)t} - 1]/t(\mu-g) \quad \text{eq. 4}$$

$$I = \langle C \rangle F \quad \text{eq. 5}$$

$\mu$  = growth rate (rotifers day<sup>-1</sup>)

$g$  = grazing rate (rotifers day<sup>-1</sup>)

$F$  = clearance or filtration rate (ml zoeae<sup>-1</sup> day<sup>-1</sup>)

$\langle C \rangle$  = time averaged cell concentration (rotifers ml<sup>-1</sup>)

$I$  = ingestion rate (# of rotifers ingested zoea<sup>-1</sup> day<sup>-1</sup>)

$t$  = time in days

$N_{t(\text{control or zoeae})}$  = final rotifer  
Concentration

$N_0$  = initial rotifer concentration

$V$  = experimental volume

$N$  = zoeae per experimental unit

**Figure 2.** Frost Equation for ingestion rates

carried out simultaneously; one in which rotifers are maintained without predators and an identical one in which a specified number of crab larvae predators are included.

Rotifer cultures were filtered (80  $\mu\text{m}$  screen) and rinsed to separate rotifers from the algae and rotifers were suspended in 0.2  $\mu\text{m}$  FSW. Rotifer densities were determined using a Sedgwick rafter counting cell. Three 2-mL aliquots were taken from each rotifer diet and fixed using 0.5 mL 90% ethanol in 20 mL scintillation vials. Counts from the aliquots were averaged and cultures were adjusted (by adding 0.2  $\mu\text{m}$  FSW) to standardize the densities of rotifers to 50-150 rotifers  $\text{mL}^{-1}$ . Densities of both the toxin-containing algal fed rotifers and non-toxic algal fed rotifers, hereafter referred to “toxic rotifers” and “non-toxic rotifers” respectively, were the same during any given set of experiments.

Two experimental approaches were used to determine feeding rates of larvae on rotifers. In the first, 100 mL of suspended rotifers (prepared as described above) were transferred (via pouring) into 250 mL Erlenmeyer flasks. Flasks were haphazardly assigned to one of two treatments (per rotifer diet); a control (without crab larvae) or an experimental set with crab larvae. For each treatment, 5-8 replicates were completed. If a flask was assigned to have larvae, 25, day one zoeae were placed in the flask. After 24 hours, three 2-mL aliquots containing rotifers were taken from each flask, put into 20 mL scintillation vials and fixed using 0.5 mL of 90% ethanol. One mL of each aliquot was counted using the Sedgwick rafter counting cell to determine final rotifer concentrations. The aliquot counts were averaged to determine rotifers  $\text{mL}^{-1}$  in each flask. Averaged counts from initial and final vials were analyzed using the Frost equation (Figure 2) to determine feeding rates.

Frequent experimental error occurred when using flasks to determine ingestion rates. Errors were occurring in the transferring of rotifers from beakers to flasks resulting in negative ingestion rates. To reduce these errors ingestion rates for the winter hatching crab species were carried out using a 325 mm x 220 mm x 50 mm plastic container that was divided into 55 mm x 55 mm x 50 mm cells. Each cell was filled with 100 mL of either toxic rotifers or non-toxic rotifers (rotifers prepared as described above). Rotifers were placed into the cells and densities were checked as described above. Cells were gently stirred to evenly distribute rotifers, when rotifers were no longer moving (due to stirring) a plastic divider was inserted diagonally into the cell to create two chambers of approximately equal rotifer density. Twenty-five day one zoeae were then added to one side of the divided cell while the other side was kept free of larvae. After 24 hours, three, 2-mL aliquots were removed from each side of the divider and preserved in 0.5 mL of 95% ethanol in 20 mL scintillation vials. Counting and determination of feeding rates were conducted as described above. Feeding rates, for both methods, were compared using two-sample independent t-tests to determine if there was a difference between treatments.

### **Rotifer Preference by Larval Crabs**

To determine if zoeae were preferentially feeding on toxic versus non-toxic rotifers, selection experiments were conducted. To distinguish between the two types of rotifers a cell tracker probe was used to label the toxin-containing algal cells ingested by the toxin-containing algal fed rotifers. CellTracker Blue CMAC (7-amino-4-chloromethylcoumarin) was obtained from Molecular Probes, Inc. Eugene, OR, USA. The probe contains a thiol

reactive chloromethyl group that interacts with cellular thiols within living cells to form an intracellular fluorescent dye. Using an epifluorescence microscope, the blue fluorescence is visible when the excitation filter transmits 354 nm wavelength light to the sample, which then emits blue fluorescent (466 nm) light. This probe “stains” the target cells, which are then fed to rotifers. In this way rotifers that were fed toxin-containing algal cells could be differentiated from rotifers that had been fed non-toxic algal cells, using epifluorescence microscopy. An equal mixture of the two rotifer types were fed to larvae and counts of each type of rotifer were determined after 24 hours.

A sample of 300 mL ( $1000 \text{ cells mL}^{-1}$ ) of the *Alexandrim* spp. was inoculated with 150  $\mu\text{l}$  of CellTracker Blue (cells with CellTracker Blue are hereafter referred to as “stained”). Beakers with toxin-containing cells and CellTracker Blue were held in a  $15^{\circ} \text{C}$  incubator for four hours. After cells had taken up the label they were checked under epifluorescence microscopy to verify staining. Labeled cells were filtered and rinsed of excess label using  $0.2 \mu\text{m}$  FSW and a  $10 \mu\text{m}$  mesh filter. Labeled cells were then suspended in  $0.2 \mu\text{m}$  FSW and held in a  $15^{\circ} \text{C}$  incubator, with a 12 hour light, 12 hour dark cycle, for 24 hours. Labeled cells were then fed to rotifers for two days. After two days, rotifers were separated from the algae using an  $80 \mu\text{m}$  screen, rinsed with  $0.2 \mu\text{m}$  FSW and suspended in  $0.2 \mu\text{m}$  FSW at ambient conditions ( $15^{\circ} \text{C}$ , 28-30 psu). Rotifer densities was determined by taking three, 2-mL aliquots from the rotifers and fixing them with 0.5 mL 90% ethanol and using a Sedgwick rafter counting cell to determine rotifer densities. Counts were averaged and densities of rotifers were diluted to equalize the densities of non-toxic rotifers and toxic rotifers. Rotifer densities ranged from 50-150 rotifers  $\text{mL}^{-1}$  throughout these experiments.

Densities of both the toxic rotifers and non-toxic rotifers were approximately the same during any given sets of experiments.

After dilutions were completed, 50 mL of toxic and 50 mL of non-toxic rotifers were added to each of ten 250 mL Erlenmeyer flasks. Five replicates each of the control (no larvae) and the selection (*Lophopanopeus bellus* larvae) treatments were used to determine if larvae were feeding preferentially between the two types of rotifers by determining if the 50:50 ratio of the two rotifer types changed over time. Larvae used in preferential feeding experiments were day one zoeae from mixed broods of crabs. After feeding was allowed to occur for 24 hours, three 2-mL aliquots were taken from each flask and fixed with 0.5 mL 90% ethanol in 20 mL scintillation vials. Densities of each rotifer type were determined using Sedgwick rafter counting cells under epifluorescence and the mean ratio determined. Preferential feeding counts were determined by looking at the aliquots using epifluorescence microscopy. When a rotifer came into view on the scope, a 354 nm filter was used to see whether it had fed on the toxin-containing *Alexandrium* spp. (i.e. it fluoresced blue). If no blue fluorescence was observed, the rotifer was counted as non-toxic. Densities of each type of rotifer from 1-ml of each aliquot were recorded and the mean ratio was calculated to determine if preferential feeding between the rotifer types was occurring. Control flasks, with no larvae, were counted to determine if there were natural density changes due to differential death or reproduction rates.

The second round of selection experiments, using *Glebocarcinus oregonensis* larvae, conducted during winter 2011, used light microscopy and visual differences to distinguish

non-toxic and toxic rotifers. The cell tracker probe was not used in these experiments due to time constraints and lack of confirmation of cell tracker uptake by the *Alexandrium* cells.

These experiments were conducted using a 325 mm x 220 mm x 50 mm plastic container that was divided into 55 mm x 55 mm x 50 mm cells. Each cell was filled with 50 mL of toxic rotifers and 50 mL of non-toxic rotifers. Rotifers were mixed together using pipettes, when rotifers were no longer moving (due to stirring) a plastic divider was inserted diagonally into the cell to create two chambers. Twenty-five day one zoeae were added to one side of the divided cell while the other side was kept larva free. After feeding was allowed to occur for 24 hours, three 2-mL aliquots were removed from each side of the divider and preserved in 0.5 mL of 95% ethanol in 20 mL scintillation vials. Rotifer ratios (toxic: non-toxic) were determined.

Ratios were compared using a Chi Square test to determine any changes in ratios of rotifers without larvae. Then ratios were compared in the same manner but looking at the rotifers with larvae present to determine if there was a preference.

### **Toxicity Effects versus Nutritional Deficiency experiments**

To determine whether the decrease in survival and increase in stage duration seen previously in larval crabs when fed rotifers cultured on toxin-containing algae were due to toxicity or to nutritional deficiency, experiments were conducted following the methods of Dam and Colin (2005). Larval crabs were given a series of diet treatments that differed in the ratio of non-toxic to toxic rotifers. There were six diet treatments: Unfed (control), 100%



toxic rotifers (control), 75% toxic rotifers/25% nontoxic rotifers, 50% toxic rotifers/50% nontoxic rotifers, 25% toxic rotifers/75% nontoxic rotifers, and 100% nontoxic rotifers.

### ***Lophopenopeus bellus* experiments**

Experiments were started when two or more broods hatched on the same day. Larvae from broods were mixed together. Zoeae were haphazardly assigned to wells containing 1 mL of 0.2  $\mu\text{m}$  FSW. Trays contained 12 wells with one zoea per well. After trays were filled with zoeae, diet treatments were haphazardly assigned to trays and diets were distributed to each larva in the tray accordingly. For each diet treatment, three trays were set up for each brood set. The whole experiment was replicated with three different brood sets. The three brood sets were started on July 27, 2010 (two broods), July 28, 2010 (two broods), and August 1, 2010 (four broods).

Rotifer cultures that had been fed *Isochrysis galbana* or *Alexandrium andersoni* for at least two days were separated from the algae using an 80- $\mu\text{m}$  screen and any excess algal cells were removed by rinsing with 0.2  $\mu\text{m}$  FSW. Rotifers were suspended in 0.2  $\mu\text{m}$  FSW and counts were done using the same methods as described in the feeding rate experiments. After rotifers from both diets were diluted to equal densities, the specified ratios were set up and fed to the zoeae (Table 1). A Finnpiquette repeater pipette was used to distribute diets to larval crabs.

On a daily basis each zoea was transferred with a pipette to a clean tray, into the corresponding well, with fresh 0.2  $\mu\text{m}$  FSW and the appropriate diet. Mortality and molting were recorded daily. This was done until zoeae molted to the megalopa stage or for 40-43

**Table 1.** Milliliter amounts of 0.2- $\mu\text{m}$  FSW, non-toxic rotifers, toxic rotifers, and *A. fundyense* cells given to each well in specified diet treatments for toxicity vs. nutritional experiments.

Diet	FSW (ml)	Non-toxic rotifers (ml)	Toxic rotifers (ml)	<i>Alexandrium fundyense</i> cells <sup>†</sup> (ml)
Unfed	3.0	0.0	0.0	0.0
100% non-toxic rotifers	1.0	2.0	0	0.0
75% non-toxic rotifers; 25% toxic rotifers	1.0	1.5	0.5	0.0
50% non-toxic rotifers; 50% toxic rotifers	1.0	1.0	1.0	0.0
25% non-toxic rotifers; 75% toxic rotifers	1.0	0.5	1.5	0.0
100% toxic rotifers	1.0	0.0	2.0	0.0
<i>Alexandrium fundyense</i> cells	1.0	0.0	0.0	2.0

<sup>†</sup> Cell concentration was 800 cells ml<sup>-1</sup>

days (experiments one and two were carried out until day 43; experiment three was carried out for 40 days), whichever came first.

### ***Metacarcinus magister* experiments**

Experiments were started when larvae from four broods that hatched on the same day were mixed together (collection of larvae were conducted using the same methods described in the *Metacarcinus magister* ingestion rate experiments). Zoeae were haphazardly assigned to wells with 1 mL of 0.2  $\mu\text{m}$  FSW. Trays contained 12 wells with one zoea per well. After trays were filled with zoeae, diet treatments were haphazardly assigned to trays and diets were distributed to each larva. Five trays of each diet treatment were started on March 5, 2011. Rotifers for the diet treatments were filtered, rinsed, counted, diluted, and distributed as described above for *Lophopanopeus bellus*. For *M. magister* experiments the toxin-containing *Alexandrium fundyense* was used for feeding rotifers. A diet treatment of *A. fundyense* cells at 800 cells  $\text{mL}^{-1}$  was included as an additional treatment to look at the impacts of direct algal cell ingestion by larval crabs.

Zoeae were transferred and fed as described for *Lophopanopeus bellus*. Mortality and molting were recorded daily. This was repeated daily through stage I.

### ***Glebocarcinus oregonensis* experiments:**

Larvae from three females hatching on the same day were mixed together (larvae collected as described in *Glebocarcinus oregonensis* feeding rate experiments). Zoeae were haphazardly assigned to wells containing 1 mL 0.2  $\mu\text{m}$  FSW. Trays contained 12 wells with

one zoea per well. After trays were filled with zoeae, diet treatments were haphazardly assigned to trays and diets were distributed to each larva. Five trays for each treatment were started on March 25, 2011. Rotifers for the diet treatments were filtered, rinsed, counted, diluted, and distributed as described above for *Lophopanopeus bellus*. For *G. oregonensis* experiments, the toxin-containing algal species *Alexandrium fundyense* was used for feeding rotifers. In addition to the rotifer diet treatments, a diet treatment of *A. fundyense* cells at 800 cells mL<sup>-1</sup> was included as an additional diet treatment.

Zoeae were transferred and fed as described for *Lophopanopeus bellus*. Mortality and molting was recorded daily. This was repeated daily for 41 days.

#### ***Data Analysis:***

Statistical analyses were conducted using SPSS v. 18. Daily survival percentages were calculated for each tray. Daily percent survival from each tray were averaged among all trays to produce daily survival curves. Stage mortality (e.g., mortality rate that occurred during each stage) was calculated for each stage and averaged among trays for each diet treatment. Stage duration was calculated as the number of days it took a zoea to molt to the next stage. A nested one-way ANOVA was used to compare stage duration among diet treatments. In this analysis, if there was no tray effect, it was pooled with error. The treatment was then treated against the pooled error. The ANOVA equal variance assumption was tested. If it was not met, data were log transformed. If variances were still unequal, a non-parametric Kruskal-Wallis test was used. If significance was found using the ANOVA, a Tukey's Honestly Significant Difference (HSD) post-hoc test was conducted to compare

each treatment against the 100% *Isochrysis galbana* fed rotifer treatment as the control. If the variance assumption was not met in the ANOVA test, a Dunnett's T3 post-hoc test was conducted to compare the treatments against the 100% *I. galbana* fed rotifer treatment.

To determine a toxicity versus nutritional deficiency effect, the average percent stage acceleration or percent survival for each diet treatment was plotted on a scatterplot. A reference line (dilution line) connected the 100% toxic and non-toxic rotifer treatments. Intermediate points from the other diet treatments were then plotted on the same scatterplot. Based on where the intermediate points fall (above, below, or on the reference line) toxic effects or nutritional deficiency effects of the suspected prey (the toxic rotifers) on the larval crabs were determined (Dam and Colin 2005).

### **Continuous versus 4 hour per day feeding experiments**

#### ***Lophopanopeus bellus* experiments**

Experiments were started when two or more broods hatched on the same day. Larvae from broods were mixed together. Zoeae were haphazardly assigned to wells with 1 mL of 0.2  $\mu\text{m}$  FSW. Trays contained 12 wells with one zoea per well. After trays were filled with zoeae, diet treatments were haphazardly assigned to trays and diets (2 mL well<sup>-1</sup>) were distributed to each larva.

Rotifer cultures that had been fed *Isochrysis galbana* or *Alexandrium andersoni* for at least two days were filtered using an 80  $\mu\text{m}$  screen and excess algae were rinsed using 0.2  $\mu\text{m}$  FSW. Rotifers were suspended in 0.2  $\mu\text{m}$  FSW and counts were done using the

methods described in the feeding rate experiments. After counts were made, rotifers from both diets were diluted to equal densities and diet treatments were prepared.

The following five diet treatments were administered: Unfed, Non-toxic rotifers 24 hours, Non-toxic rotifers 4 hours, Toxic rotifers 24 hours, Toxic rotifers 4 hours. Three trays for each diet treatment were used for each of the brood sets. The brood sets were started on August 7, 2010 (two broods), August 8, 2010 (three broods), and August 11, 2010 (one brood). Rotifers for the diet treatments were filtered, rinsed, counted, and diluted as described above.

On a daily basis each zoea was transferred with a pipette to a clean tray, into the same corresponding well, with fresh 0.2  $\mu\text{m}$  FSW and the appropriate diet (Table 2). Zoeae in the 24 hour treatments were continuously exposed to rotifers. The zoeae in the 4 hour treatments were exposed to rotifers for 4 hours per day and then transferred to new trays that contained only 3 mL of 0.2  $\mu\text{m}$  FSW. Mortality and molting was recorded daily. This was repeated daily for 30 days.

### ***Glebocarcinus oregonensis* experiments**

Larvae from three broods that hatched on the same day were mixed together. Zoeae were haphazardly assigned to wells with 1 mL of 0.2  $\mu\text{m}$  FSW. Trays contained 12 wells with one zoea well<sup>-1</sup>. After trays were filled with zoeae, diet treatments were haphazardly assigned to trays and diets were distributed to each larva. Five trays were set up for each diet treatment and were started on March 25, 2011. Rotifers for the diet treatments were filtered,

**Table 2.** Milliliter amount of 0.2- $\mu$ m FSW, non-toxic rotifers, and toxic rotifers given to each well in specified diet treatments for continuous versus 4 hour per day feeding experiments. Last column indicates if larval crabs were transferred to 0.2- $\mu$ m FSW after feeding for 4 hours.

<b>Diet</b>	<b>FSW (ml)</b>	<b>Non-toxic rotifers (ml)</b>	<b>Toxic rotifers (ml)</b>	<b>Switch to 3 ml FSW after 4 hours</b>
Unfed	3.0	0.0	0.0	NO
Non-toxic rotifers 24 hours	1.0	2.0	0.0	NO
Non-toxic rotifers 4 hours	1.0	2.0	0.0	YES
Toxic rotifers 24 hours	1.0	0.0	2.0	NO
Toxic rotifers 4 hours	1.0	0.0	2.0	YES

rinsed, counted, diluted, and distributed as described above in the *Lophopanopeus bellus* experiment. Daily larval transfer and rotifer diet treatments distribution was conducted as described above. The toxin-containing algal species used in this experiment was *Alexandrium fundyense*. This was repeated daily until zoeae died or molted to stage II (approximately 15 days). Data were analyzed as described above for toxicity versus nutritional deficiency experiments.

### **Diet Change experiments**

Experiments were started when two *Lophopanopeus bellus* broods hatched on the same day. Larvae were mixed together, divided into two groups, and kept in 0.2  $\mu\text{m}$  FSW in 20 cm diameter glass bowls at 15<sup>0</sup> C with a 12 hour light, 12 hour dark cycle. Bowls were haphazardly assigned to one of two diet treatments. The diets administered to larvae were *Isochrysis galbana* fed rotifers and *Alexandrium andersoni* fed rotifers. Rotifer cultures that had been fed *I. galbana* or *A. andersoni* for at least two days were separated from the algae using an 80- $\mu\text{m}$  screen and any excess algal cells were removed by rinsing with 0.2  $\mu\text{m}$  FSW. Every other day larvae were transferred to clean bowls with 0.2  $\mu\text{m}$  FSW and given rotifers in excess. Every day bowls were checked for molts from the larvae.

When larvae were nearing the second molt they were transferred into wells containing 1 mL of 0.2  $\mu\text{m}$  FSW. Trays contained 12 wells with one zoea well<sup>-1</sup>. Zoeae continued to be fed on the initial rotifer diet. After zoeae had been transferred to trays, on a daily basis each zoea was transferred with a pipette to a clean tray, with fresh 0.2  $\mu\text{m}$  FSW and the



appropriate diet. This permitted me to follow individual larvae and determine when molting to stage III had occurred.

On days that molting occurred, stage III, day one zoeae were transferred to new trays with fresh 0.2  $\mu\text{m}$ . Once all stage III, day one zoeae were separated into new trays, equal numbers of larvae, from each original diet treatment, were haphazardly assigned to a new diet treatment. The diets administered to larvae were *I. galbana* fed rotifers and *A. andersoni* fed rotifers.

The following four diet treatments were administered. The first rotifer type in the treatment name was originally fed to larvae through the first two stages, the second rotifer type in the treatment name was fed to larvae beginning on day one of stage III: *Isochrysis galbana* fed rotifers – *I. galbana* fed rotifers, *I. galbana* fed rotifers – *Alexandrium andersoni* fed rotifers, *A. andersoni* fed rotifers – *A. andersoni* fed rotifers, *A. andersoni* fed rotifers - *I. galbana* fed rotifers. Rotifers for the diet treatments were filtered and rinsed as described above. Rotifer treatments were administered in excess to each well using a pipette.

Four trays for each diet treatment were used. Because larvae do not molt synchronously, trays were filled as zoeae molted from stage II to stage III. On a daily basis zoea were transferred with a pipette to a clean tray, into the corresponding well, with fresh 0.2  $\mu\text{m}$  FSW and the appropriate diet. Mortality and molting were recorded daily starting on day one of stage III. This was done until zoeae molted to stage IV.

Statistical analyses were conducted using SPSS v. 18. Because molting in larvae is not synchronous, data were collected for each individual larva and normalized to stage III,

day one. Normalized data were analyzed as described above for the toxicity versus nutritional deficiency experiments.

A flow chart showing which of the experiments were conducted on the three crab species can be found on the following page (Figure 3).

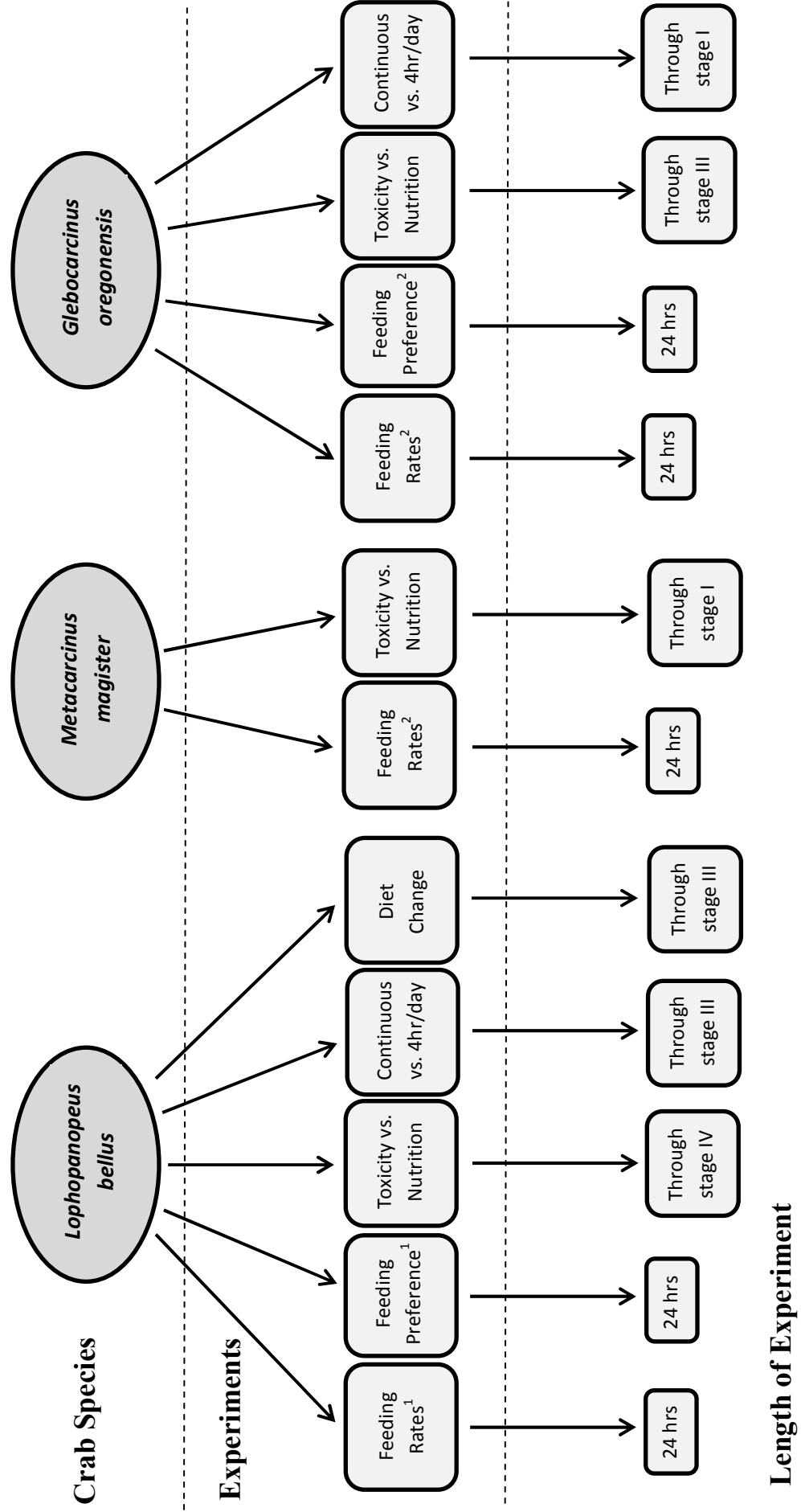


Figure 3. Flow chart of crab species used in experiments. 1: original method, 2: second method (winter method)

## RESULTS

### Larval Ingestion Rates on Rotifers

The experimental approach used to distinguish between toxic effect and nutritional deficiency (Dam and Colin 2005) requires that the crab larvae ingest *Isochrysis galbana* (nontoxic) fed rotifers and *Alexandrium andersoni* or *A. fundyense* (toxin containing) fed rotifers at the same rate. Ingestion rates for *Lophopanopeus bellus*, *Metacarcinus magister*, and *Glebocarcinus oregonensis* on non-toxic and one of the toxin-containing rotifer diets are shown in Table 3. In all three crab species, no significant differences were found in the ingestion rates between the two diets (Table 3, two-sample t-test, p values > 0.05).

### Rotifer Preference by Larval Crabs

The experimental approach of Dam and Colin (2005) also assumes that there is no preferential ingestion of one prey type over the other in mixed prey treatments. Because rotifers reproduce asexually, it is possible that there might be differential growth of the two prey types over time that could influence the final ratios even if preferential ingestion did not occur. To test whether that was a complicating factor, changes in comparative prey densities of mixed prey in an initial 1:1 ratio were tested after 24 hours with no larval predators present. The results indicated that no differential growth was occurring (*Lophopanopeus bellus*:  $\chi^2=2.09$ , df=3, p=0.15; *Glebocarcinus oregonensis*:  $\chi^2=1.09$ , df=4, p=0.30). Therefore any changes in the 1:1 ratio that occurred over time when larval predators were present can be attributed to preferential ingestion.

**Table 3.** Ingestion rates for the three crab species used in experiments. Ingestion rate values are mean (rotifers zoea<sup>-1</sup> day<sup>-1</sup>) ± one standard error. Resulting p-values are from independent t-tests (SPSS v.19). When variances assumptions were not met, 'equal variances not assumed' p-values were used. Values shown under 'equal variances not assumed' are noted with an asterisk. Non-toxic rotifer refers to *Isochrysis galbana* fed rotifers. Toxic rotifer refers to *Alexandrium fundyense* fed rotifers, except where noted.

Species	Nontoxic rotifer ingestion	n	Toxic rotifer ingestion	n	p value
<i>Lophopanopeus bellus</i> <sup>^</sup>	83.88 ± 25.70	4	50.82 ± 14.86	4	0.296
<i>Metacarcinus magister</i>	132.0 ± 8.070	5	129.6 ± 0.374	5	0.791*
<i>Glebocarcinus oregonensis</i>	22.05 ± 14.39	5	29.88 ± 7.430	5	0.614

<sup>^</sup>Toxic rotifer refers to *A. andersoni* fed rotifers.

When predators were present in the case of *Glebocarcinus oregonensis*, the ratio of prey types did not change over the course of 24 hours, indicating no preferential ingestion ( $\chi^2=0.06$ ,  $df=4$ ,  $p=0.81$ ). The results for larvae of *Lophopanopeus bellus* were more equivocal. The ratio did change in the tests, with more *Alexandrium andersoni* fed rotifers apparently ingested ( $\chi^2=5.00$ ,  $df=3$ ,  $p=0.03$ ). These tests were run, however, using a method for estimating prey density that was questionable (see Methods) and thus are not as reliable as those with *G. oregonensis*. Based on these results, I determined that preferential ingestion was not a complicating factor in the nutrition versus toxicity tests, although the *L. bellus* results could have been compromised.

### **Toxicity Effects versus Nutritional Deficiency experiments**

#### ***Lophopanopeus bellus***

When larvae were fed either of the toxin-containing algae directly, all larvae died by day 10 for *Alexandrium andersoni* and by day 12 for *A. fundyense*. No larvae molted to stage II in either treatment. A starved control also showed 100% mortality by day 10, with no molting to stage II. To examine these data further, mean days of death were compared between unfed larvae and those fed one of the toxin-containing cells directly (Table 4). Larvae fed *A. andersoni* cells showed significantly faster mortality than did the unfed controls, while *A. fundyense* fed larvae showed a significant delay in mortality (Table 4; Kruskal-Wallis,  $\chi^2=65.94$ ,  $df=2$ ,  $p<0.001$ ).

**Table 4.** Mean days of death for stage I larvae of the indicated crab species when starved and fed *Alexandrium fundyense* cells (*L. bellus* larvae show both *A. andersoni* cells (Aa) and *A. fundyense* cells (Af)). Day of death values are means  $\pm$  one standard error. One-way ANOVA tests were used, if ANOVA assumptions were not met, a non-parametric Kruskal-Wallis test was used (SPSS v.19). Resulting p-values are presented; p-values from Kruskal-Wallis tests are noted with an asterisk.

Species	Mean Day of death $\pm$ standard error				p value
	Unfed	n	Toxic cells	n	
<i>Lophopanopeus bellus</i> - Aa	7.39 $\pm$ 0.17	59	6.47 $\pm$ 0.23	60	<0.001*
<i>Lophopanopeus bellus</i> - Af	7.39 $\pm$ 0.17	59	8.95 $\pm$ 0.30	60	<0.001*
<i>Metacarcinus magister</i>	4.82 $\pm$ 0.15	60	4.78 $\pm$ 0.19	58	0.868
<i>Glebocarcinus oregonensis</i>	7.05 $\pm$ 0.26	60	9.79 $\pm$ 0.36	60	<0.001

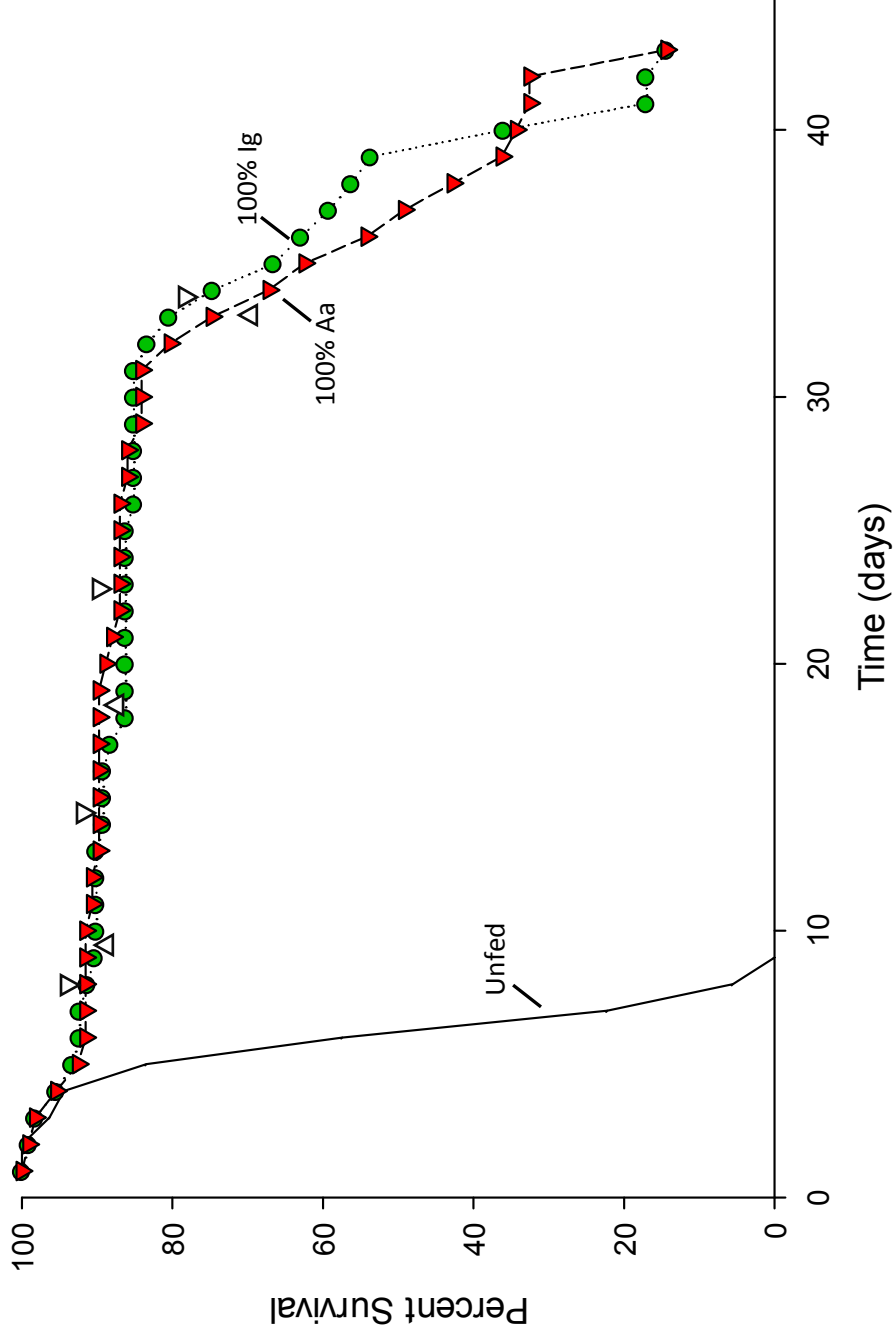
There appeared to be little difference in daily survival between larvae fed diets of 100% *Isochrysis galbana*-fed rotifers (Ig-rot) and 100% *Alexandrium andersoni*-fed rotifers (Aa-rot) (Figure 4), with high survival on both diets through stage III and rapid decline in survival after day 30. When diets including mixtures of these two diets are added to the analysis, little difference in daily survival is seen among treatments (Figure 5).

To examine this further, percent mortality that occurred during each zoeal stage was calculated and compared among all diet treatments (Table 5). The results of a series of one-way ANOVA's for each zoeal stage show significant difference among treatments only for stage III where mortality on the 100% Aa-rot diet is significantly higher than on any of the other rotifer diet treatments (Kruskal-Wallis  $p < 0.001$ , Tukey's HSD test  $\alpha = 0.05$ ).

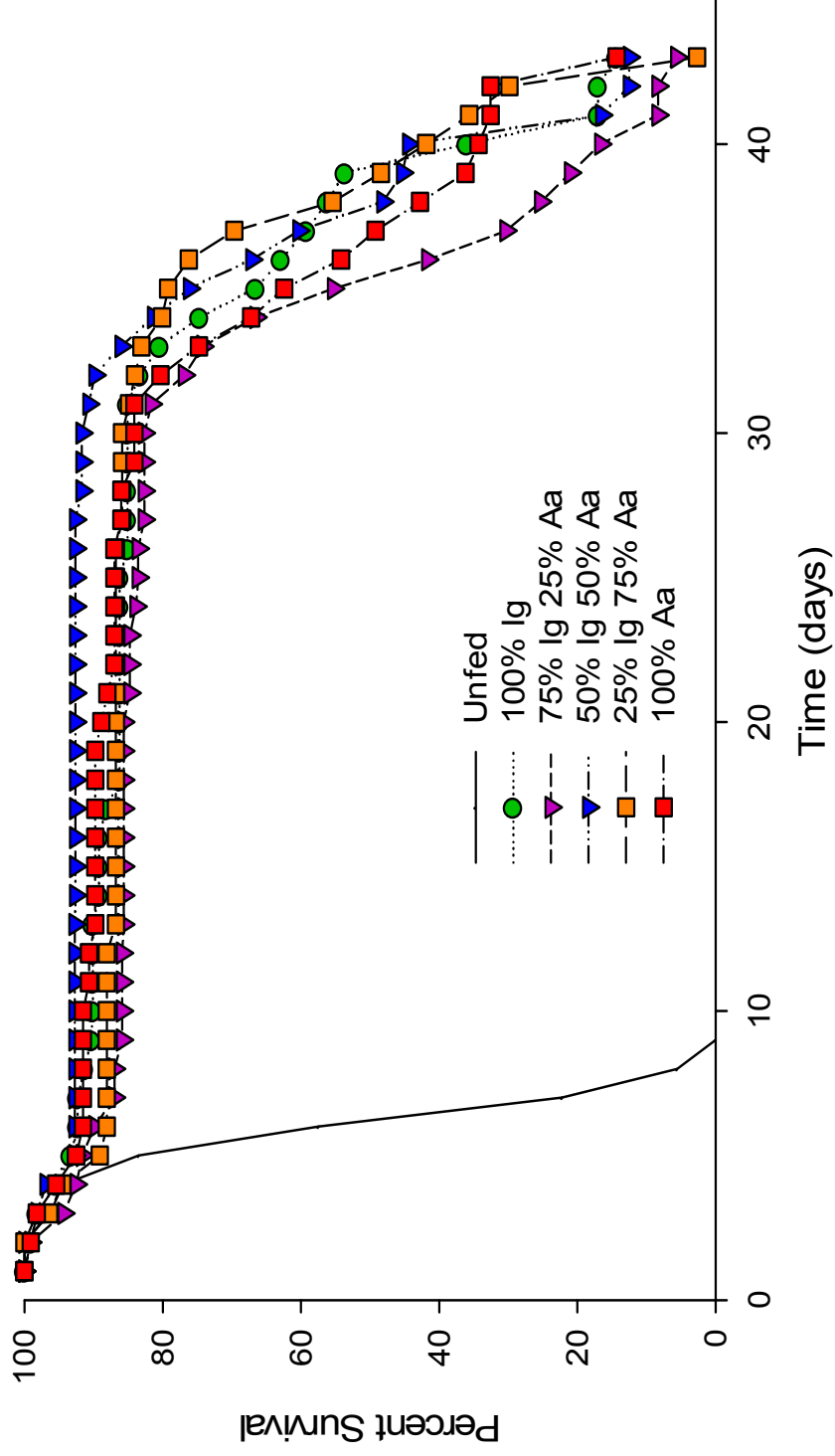
Duration in days for each zoeal stage on all diet treatments is shown in Table 6, along with the results of a one-way ANOVA for each stage. In each case, there was a significant difference among treatments. Figure 6 shows the results of pairwise contrasts ( $\alpha = 0.05$ ) among treatments for each zoeal stage. In stages I-III, the 100% Aa-rot diet was significantly longer in duration than for any other treatment, while the relationships among the other treatments varied, but in general, as Ig-rot proportions declined, duration was extended. The pattern was the same for stage IV larvae.

Based on the fact that there were no significant differences in mortality among treatments, I determined that for *Lophopanopeus bellus*, only stage duration could be used to assess the toxicity versus nutritional deficiency distinction using the method of Dam and Colin (2005). Figures 7 - 9 show the results of that analysis for zoeal stages I-III respectively.





**Figure 4.** Daily percent survival of *Lophopanopeus bellus* zoeae fed the indicated diet treatment. “Ig” indicates *Isochrysis galbana* fed rotifers and “Aa” indicates *Alexandrium andersoni* fed rotifers. Open triangles indicate the mean day of molt for stages I-III.



**Figure 5.** Daily percent survival of *Lophopanopeus bellus* zoeae fed the indicated diet treatment. “Ig” indicates *Isochrysis galbana* fed rotifers and “Aa” indicates *Alexandrium andersoni* fed rotifers.

**Table 5.** *Lophopanopeus bellus* mean stage mortality (%) during each stage for stages I-IV larvae fed indicated diet treatments. Ig indicates rotifers that were fed *Isochrysis galbana* and Aa indicates rotifers that were fed *Alexandrium andersoni*. Mortality values are means  $\pm$  standard error. Resulting p-values are from one-way ANOVA test; except when ANOVA assumptions were not met, in which case a non-parametric Kruskal-Wallis test was used (SPSS v.19). Resulting p-values from Kruskal-Wallis tests are noted with an asterisk. Pairwise contrast (Tukey's HSD; SPSS v.19) results for stage III are indicated by letters, shared letters indicates no significant treatment difference ( $\alpha = 0.05$ ). NA indicates that no larvae molted from stage IV to the megalopa stage before experiments were terminated.

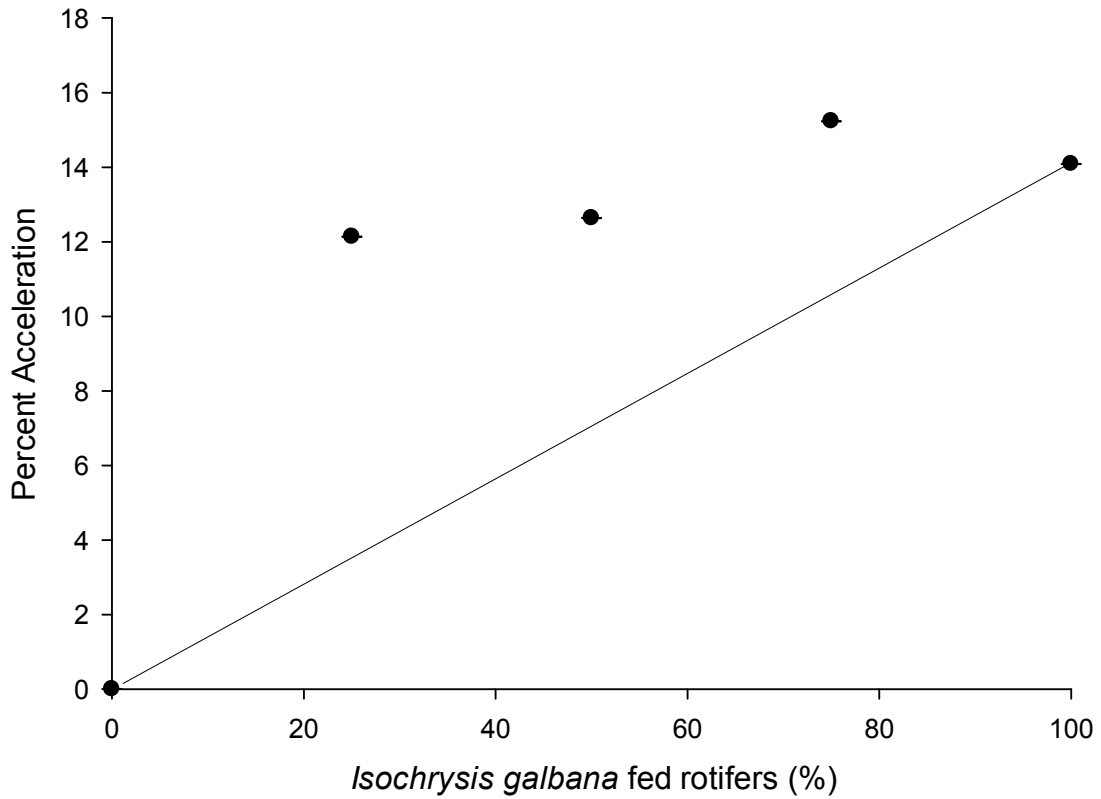
Stage	Mean Stage Mortality (%) $\pm$ standard error						p value
	100% Ig	75% Ig 25% Aa	50% Ig 50% Aa	25% Ig 75% Aa	100% Aa		
I	8.67 $\pm$ 4.95	13.9 $\pm$ 3.68	6.13 $\pm$ 3.04	11.9 $\pm$ 3.70	8.44 $\pm$ 3.13		0.636
II	2.22 $\pm$ 1.47	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	1.56 $\pm$ 1.56	5.11 $\pm$ 2.47		0.082*
III	4.78 $\pm$ 2.07 (a)	1.11 $\pm$ 1.11 (a)	0.00 $\pm$ 0.00 (a)	4.00 $\pm$ 2.13 (a)	66.8 $\pm$ 8.91 (b)		<0.001*
IV	56.1 $\pm$ 12.4	59.6 $\pm$ 10.5	55.4 $\pm$ 13.6	68.2 $\pm$ 7.84	NA		0.839

**Table 6.** *Lophopanopeus bellus* mean stage duration (days) for stages I-IV larvae fed indicated diet treatments. Ig indicates rotifers that were fed *Isochrysis galbana* and Aa indicates rotifers that were fed *Alexandrium andersoni*. Duration values are mean  $\pm$  standard error. Resulting p-values are from one-way ANOVA tests; except when ANOVA assumptions were not met in which case a non-parametric Kruskal-Wallis test was used (SPSS v.19). Resulting P-values from Kruskal-Wallis tests are noted with an asterisk. NA indicates that no larvae molted to the megalopa stage before experiments were terminated.

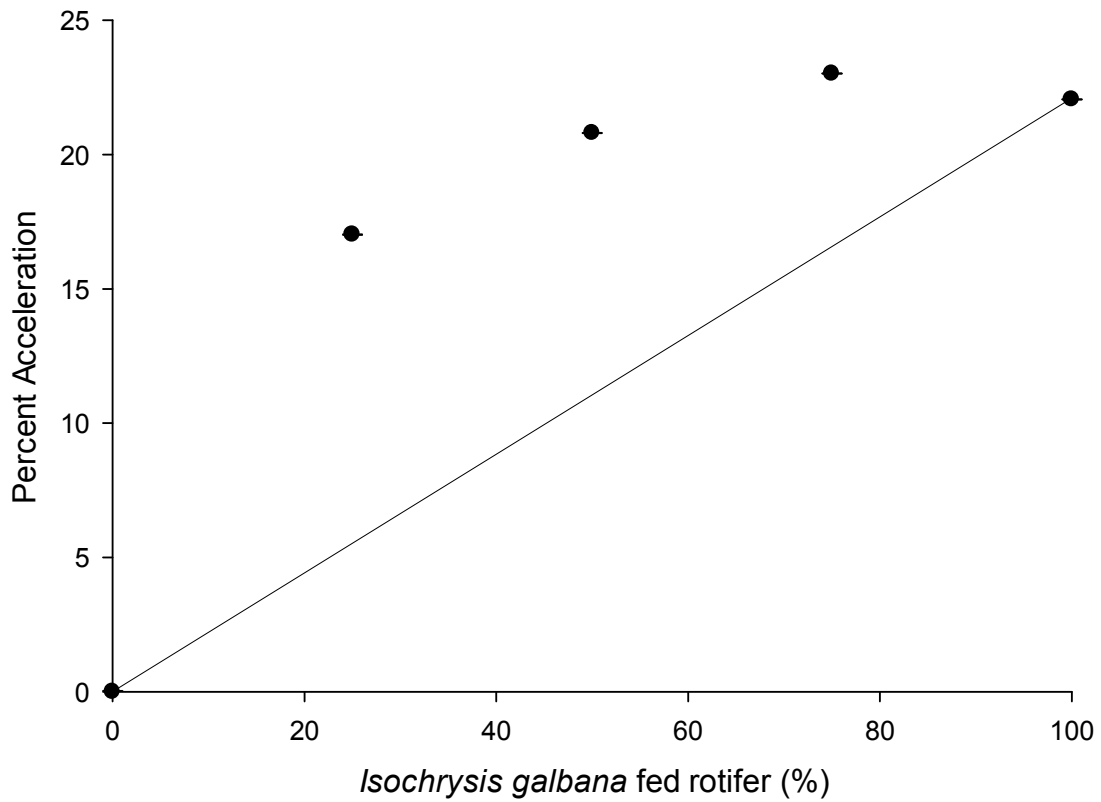
Stage	Mean Stage Duration (days) $\pm$ standard error												p value	
	100% Ig	n	75% Ig	25% Aa	n	50% Ig	50% Aa	n	25% Ig	75% Aa	n	100% Aa		n
I	8.34 $\pm$ 0.05	98	8.23 $\pm$ 0.04		93	8.48 $\pm$ 0.06		90	8.53 $\pm$ 0.06		95	9.70 $\pm$ 0.08	98	<0.001*
II	6.43 $\pm$ 0.06	93	6.35 $\pm$ 0.07		93	6.53 $\pm$ 0.07		90	6.85 $\pm$ 0.10		94	8.25 $\pm$ 0.27	84	<0.001
III	8.09 $\pm$ 0.08	90	7.77 $\pm$ 0.08		88	8.25 $\pm$ 0.09		89	8.85 $\pm$ 0.11		88	15.3 $\pm$ 0.54	33	<0.001*
IV	10.8 $\pm$ 0.15	43	10.8 $\pm$ 0.12		38	11.4 $\pm$ 0.14		40	13.3 $\pm$ 0.17		29	NA	NA	<0.001

Stage	Diets fed to larvae				
I	<u>75% Ig/25% Aa</u>	<u>100% Ig</u>	50% Ig/50% Aa	25% Ig/75% Aa	<u>100% Aa</u>
II	<u>100% Ig</u>	75% Ig/25% Aa	<u>50% Ig/50% Aa</u>	25% Ig/75% Aa	<u>100% Aa</u>
III	<u>100% Ig</u>	<u>50% Ig/50% Aa</u>	<u>75% Ig/25% Aa</u>	<u>25% Ig/75% Aa</u>	<u>100% Aa</u>
IV	<u>100% Ig</u>	<u>75% Ig/25% Aa</u>	<u>50% Ig/50% Aa</u>	<u>25% Ig/75% Aa</u>	

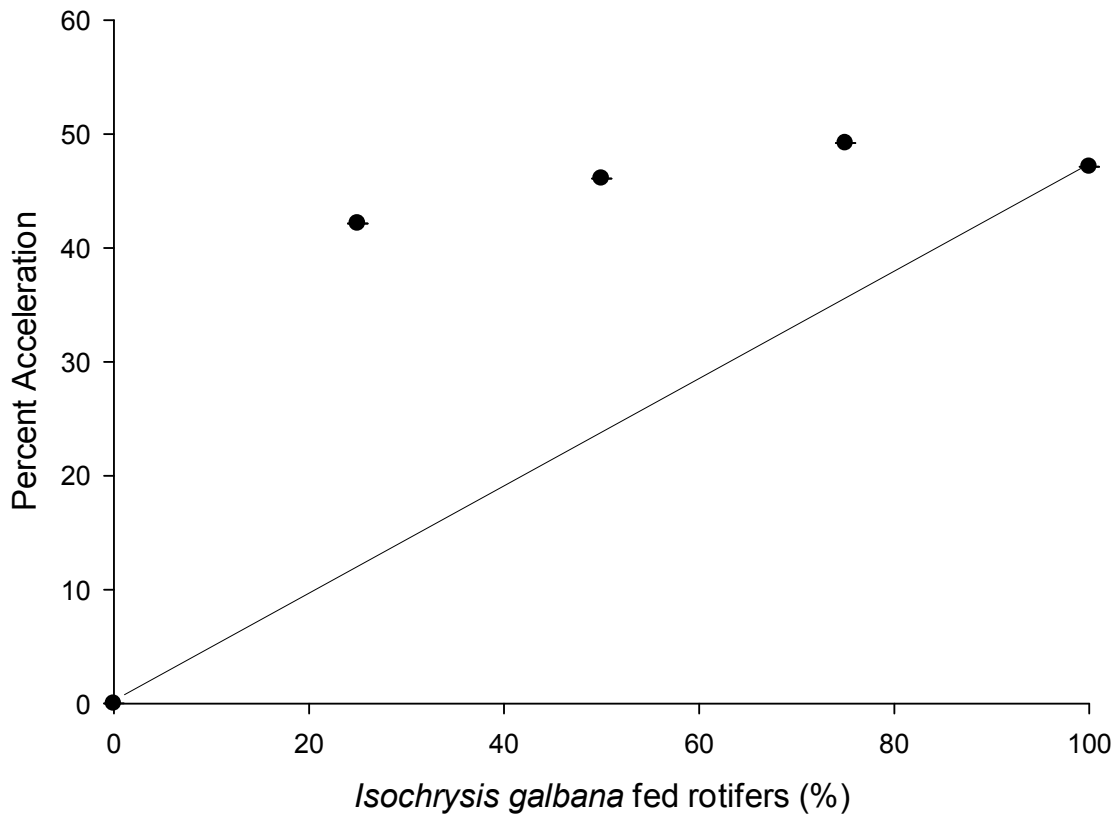
**Figure 6.** Pairwise contrast results for stage duration for stages I-IV *Lophopanopeus bellus* larvae fed indicated diet treatments. Ig indicates rotifers that were fed *Isochrysis galbana* and Aa indicates rotifers that were fed *Alexandrium andersoni*. For stages I and III stage duration for the diets were contrasted using a Dunnett T3 test and stages II and IV were contrasted using a Tukey's HSD test. Results are contrasts of stage duration by diet during each stage. Shared lines represent no significant treatment difference ( $\alpha=0.05$ ).



**Figure 7.** *Lophopanopeus bellus* stage I duration in the mixed diet (*Alexandrium andersoni* fed rotifers and *Isochrysis galbana* fed rotifers) experiment. Points represent the mean and bars represent  $\pm$  one standard error. Reference line connects the 100% rotifer diets.



**Figure 8.** *Lophopanopeus bellus* stage II duration in the mixed diet (*Alexandrium andersoni* fed rotifers and *Isochrysis galbana* fed rotifers) experiment. Points represent the mean and bars represent  $\pm$  one standard error. Reference line connects the 100% rotifer diets.



**Figure 9.** *Lophopanopeus bellus* stage III duration in the mixed diet (*Alexandrium andersoni* fed rotifers and *Isochrysis galbana* fed rotifers) experiment. Points represent the mean and bars represent  $\pm$  one standard error. Reference line connects the 100% rotifer diets.



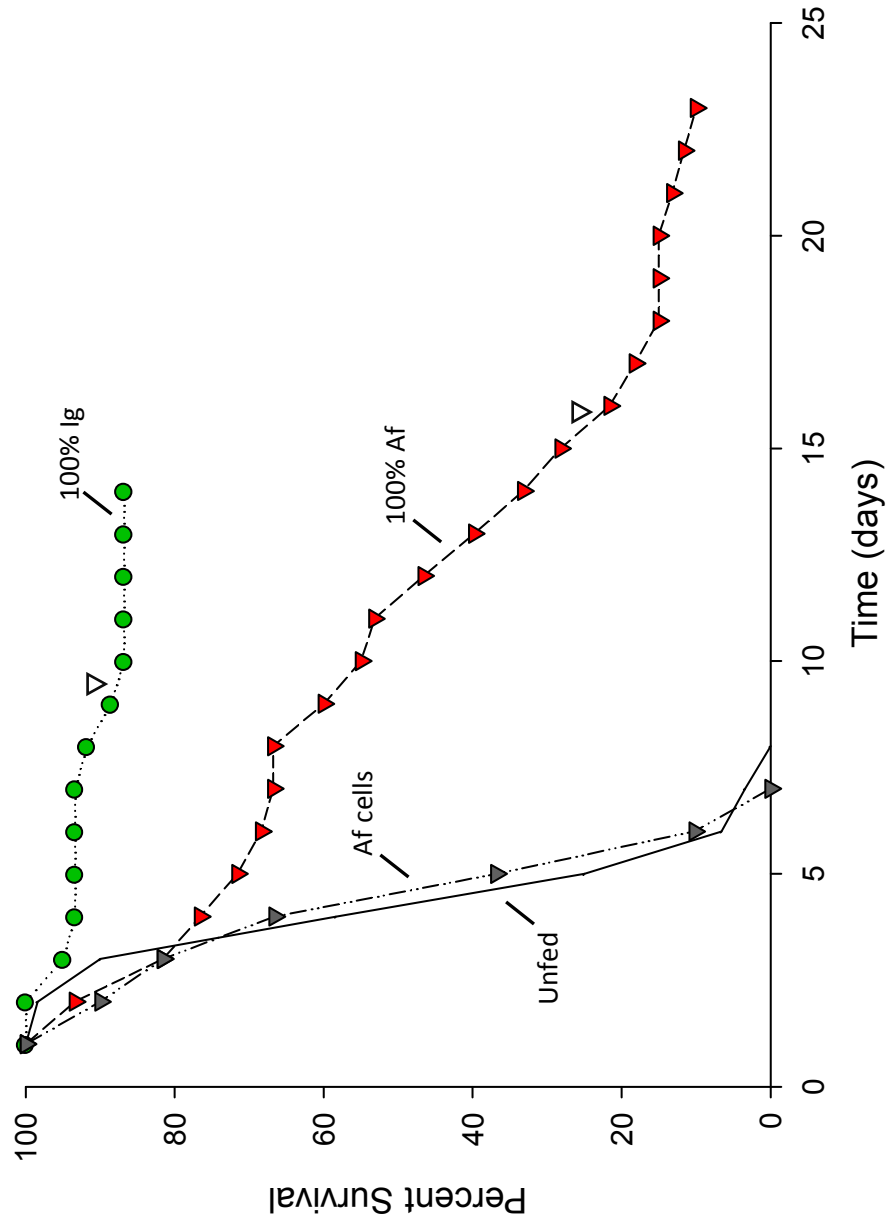
The Y-axis uses percent acceleration as compared to the 100% Aa-rot treatment to permit the analysis. The durations of all three mixtures containing some *Isochrysis*-fed rotifers fall above the reference line, indicating that adding Ig-rot to the mix, even as little as 25%, accelerates development, implicating nutritional deficiency of the Aa-rot diet.

### ***Metacarcinus magister***

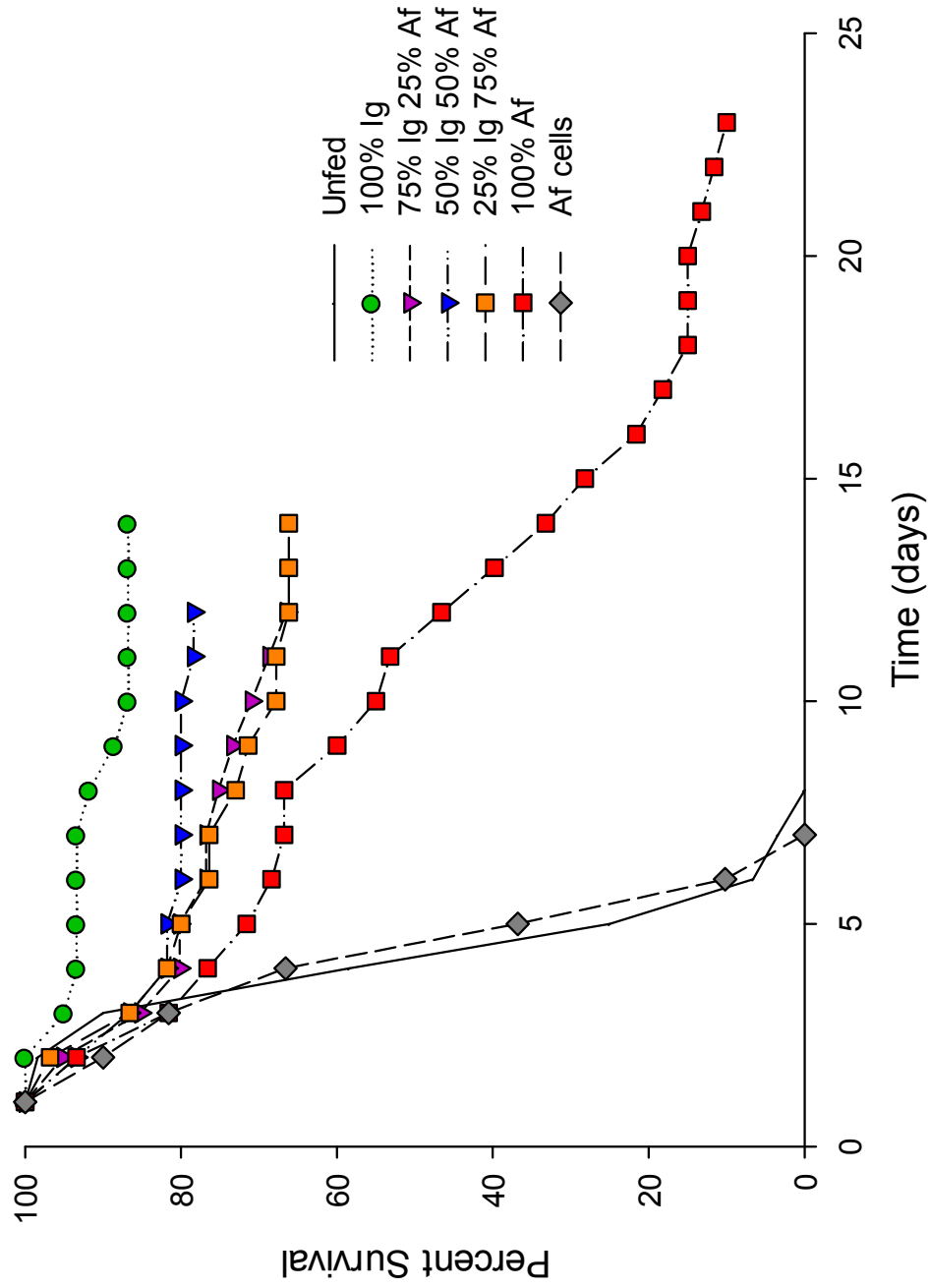
When larvae were fed the toxin-containing *Alexandrium fundyense* cells directly, all larvae had died by day seven and no larvae had molted to stage II (Figure 10). A starved control also showed 100% mortality by day eight, with no molting to stage II (Figure 10). A comparison of mean days of death between starved and *A. fundyense* cell treatments (Table 4) showed no significant difference between the treatments (Table 4; ANOVA,  $F=0.028$ ,  $p=0.868$ ).

Larval survival was highest on the 100% *Isochrysis galbana* fed rotifer (Ig-rot) diet, while survival on the 100% *Alexandrium fundyense* fed rotifer (Af-rot) diet steadily declined over the course of the experiment (Figure 10). Survival on the mixed diet treatments fell between the 100% Ig-rot and 100% Af-rot diets (Figure 11). All rotifer fed diet treatments experienced molting to stage II, at which point the experiments were terminated.

Percent mortality that occurred during stage I was calculated and compared among all diet treatments (Table 7). The results from a one-way ANOVA analysis showed significant differences among treatments (Table 7; ANOVA,  $F=23.20$ ,  $p<0.001$ ). The *Alexandrium fundyense* fed rotifer diet treatment had significantly higher mortality during stage I, while all other rotifer diet treatments were not significantly different from one another.



**Figure 10.** Daily percent survival of *Metacarcinus magister* zoeae fed the indicated diet treatment. “Ig” indicates *Isochrysis galbana* fed rotifers, “Af” indicates *Alexandrium fundyense* fed rotifers and “Af cell” indicates *A. fundyense* cells. Open triangles indicate the mean day of molt for stage I.



**Figure 11.** Daily percent survival of *Metacarcinus magister* zoeae fed the indicated diet treatment. “Ig” indicates *Isochrysis galbana* fed rotifers, “Af” indicates *Alexandrium fundyense* fed rotifers and “Af cell” indicates *A. fundyense* cells.

**Table 7.** *Metacarcinus magister* mean stage mortality (%) for stage I larvae fed indicated diet treatments. Ig indicates rotifers that were fed *Isochrysis galbana* and Af indicates rotifers that were fed *Alexandrium fundyense*. Mortality values are means  $\pm$  standard error. The resulting p-value is from a one-way ANOVA test (SPSS v.19). Pairwise contrast (Tukey's HSD) results are indicated by letters; shared letters indicate no significant treatment differences ( $\alpha=0.05$ ).

Stage I	Mean Stage Mortality $\pm$ standard error						p value
	100% Ig	75% Ig 25% Af	50% Ig 50% Af	25% Ig 75% Af	100% Af		
	13.2 $\pm$ 3.43	33.5 $\pm$ 4.91	21.6 $\pm$ 9.70	33.8 $\pm$ 5.25	90.0 $\pm$ 6.12		<0.001
Tukey's HSD	a	a	a	a	b		

Duration in days for stage I zoeae on all diet treatments is shown in Table 8, along with the results of a one-way ANOVA. There was a significant difference among treatment (Table 8; ANOVA,  $F=60.84$ ,  $p<0.001$ ). Larvae fed the 25% Ig-rot /75% Af-rot diet and 100% Af-rot diet experienced significant delay in development compared to the other diet treatments (Table 8). The 100% Af-rot diet had significantly longer duration compared to the 25% Ig-rot/75% Af-rot diet.

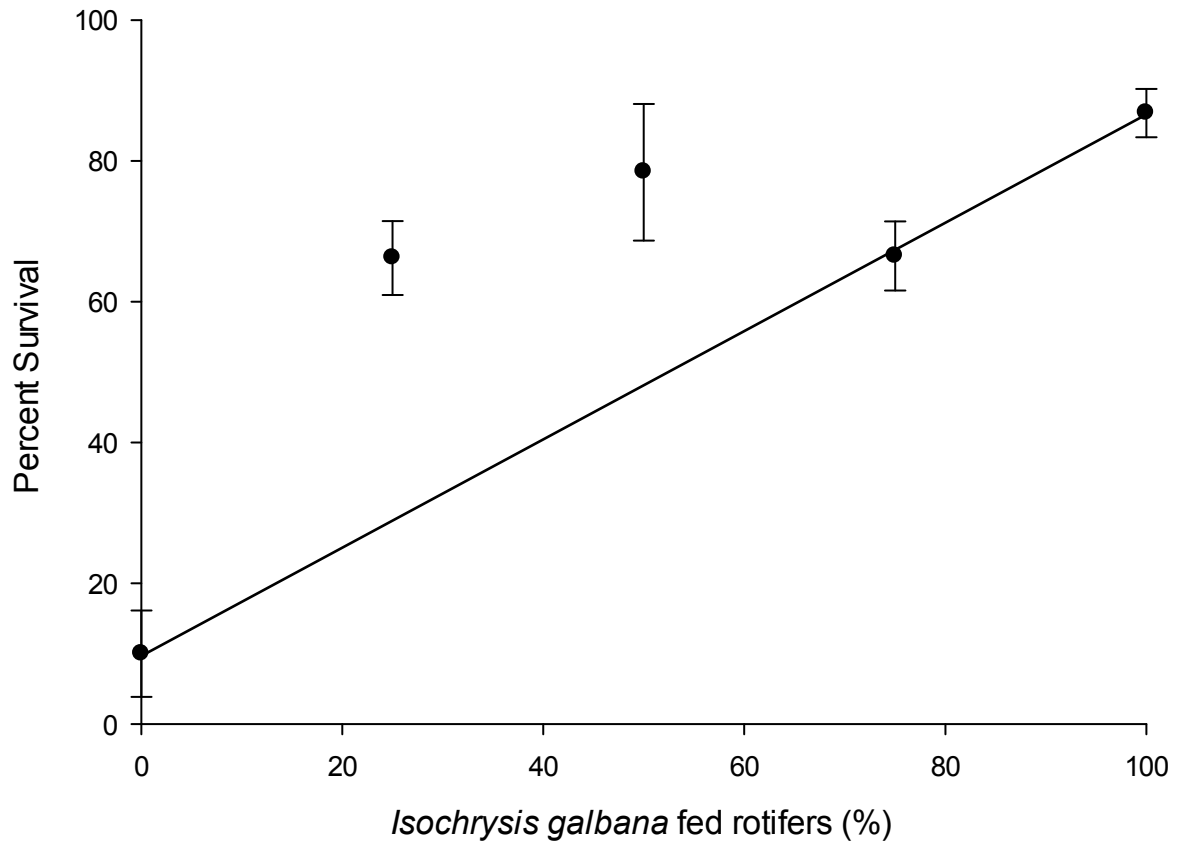
Based on these results, I determined that for *Metacarcinus magister*, both stage survival and duration during the stage could be used to assess the toxicity versus nutritional deficiency distinction using the method of Dam and Colin (2005). Figure 12 shows the results of the percent survival analysis for stage I. The percent survival of the 50% Ig-rot/50% Af-rot diet and the 25% Ig-rot/75% Af-rot diet fall above the reference line, indicating that adding Ig-rot to the mix, even as little as 25%, increases survival during the stage, implicating nutritional deficiency of the Af-rot diet. The percent survival for the 75% Ig-rot/25% Af-rot diet falls at the reference line, indicating no additional nutritional value. Figure 13 shows the results of the stage duration analysis for stage I. The Y-axis uses percent acceleration of each treatment as compared to the 100% Af-rot treatment to permit the analysis. The durations of all three mixtures containing some *Isochrysis galbana* fed rotifers fall above the reference line, indicating that adding Ig-rot to the mix, even as little as 25%, accelerates development, implicating nutritional deficiency of the Af-rot diet.

### ***Glebocarcinus oregonensis***

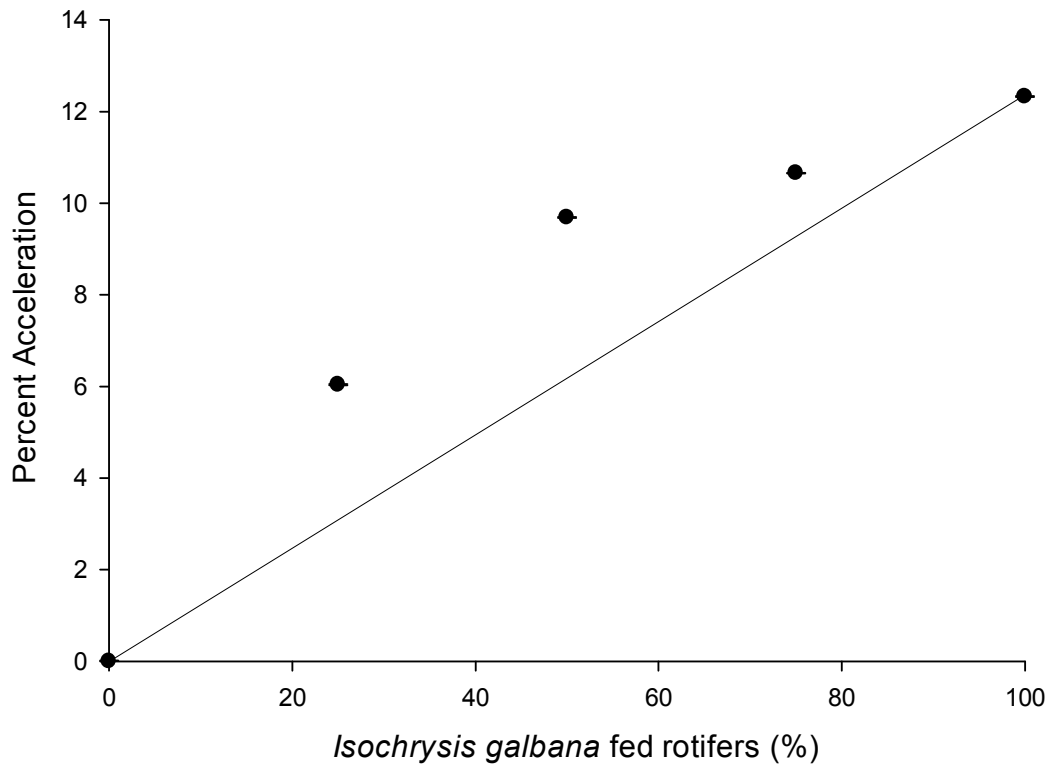
When larvae were fed toxin-containing *Alexandrium fundyense* cells directly, all larvae had died by day 15 (Figure 14), with no larvae molting to stage II. A starved control

**Table 8.** *Metacarcinus magister* mean stage duration (days) for stage I larvae fed indicated diet treatments. Ig indicates rotifers that were fed *Isochrysis galbana* and Af indicates rotifers that were fed *Alexandrium fundyense*. Duration values are means  $\pm$  standard error. The resulting p-value is from a one-way ANOVA test (SPSS v.19). Pairwise contrast (Tukey's HSD) results are indicated by letters, shared letters indicate no significant treatment differences ( $\alpha=0.05$ ).

Stage I Tukey's HSD	Mean Stage Duration (days) $\pm$ standard error													
	100% Ig	n	75% Ig	25% Af	n	50% Ig	50% Af	n	25% Ig	75% Af	n	100% Af	n	p value
a	10.27 $\pm$ 0.12	52	10.31 $\pm$ 0.13	a	32	10.55 $\pm$ 0.10	a	47	11.67 $\pm$ 0.17	b	39	15.33 $\pm$ 0.42	6	<0.001

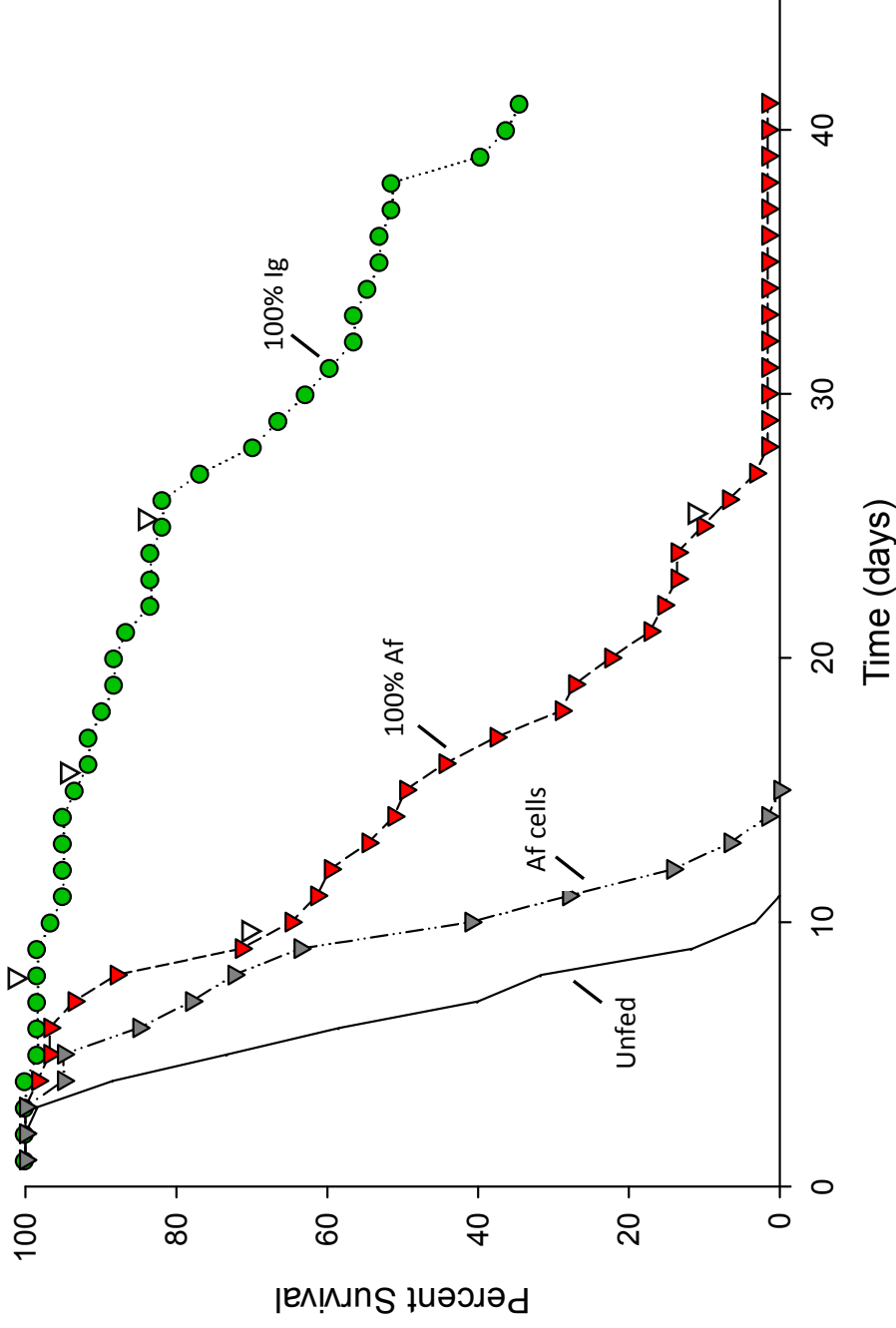


**Figure 12.** *Metacarcinus magister* survival during stage I in the mixed diet (*Alexandrium fundyense* fed rotifers and *Isochrysis galbana* fed rotifers) experiment. Points represent the mean and bars represent  $\pm$  one standard error. Reference line connects the 100% rotifer diets.



**Figure 13.** *Metacarcinus magister* stage I duration in the mixed diet (*Alexandrium fundyense* fed rotifers and *Isochrysis galbana* fed rotifers) experiment. Points represent the mean and bars represent  $\pm$  one standard error. Reference line connects the 100% rotifer diets.



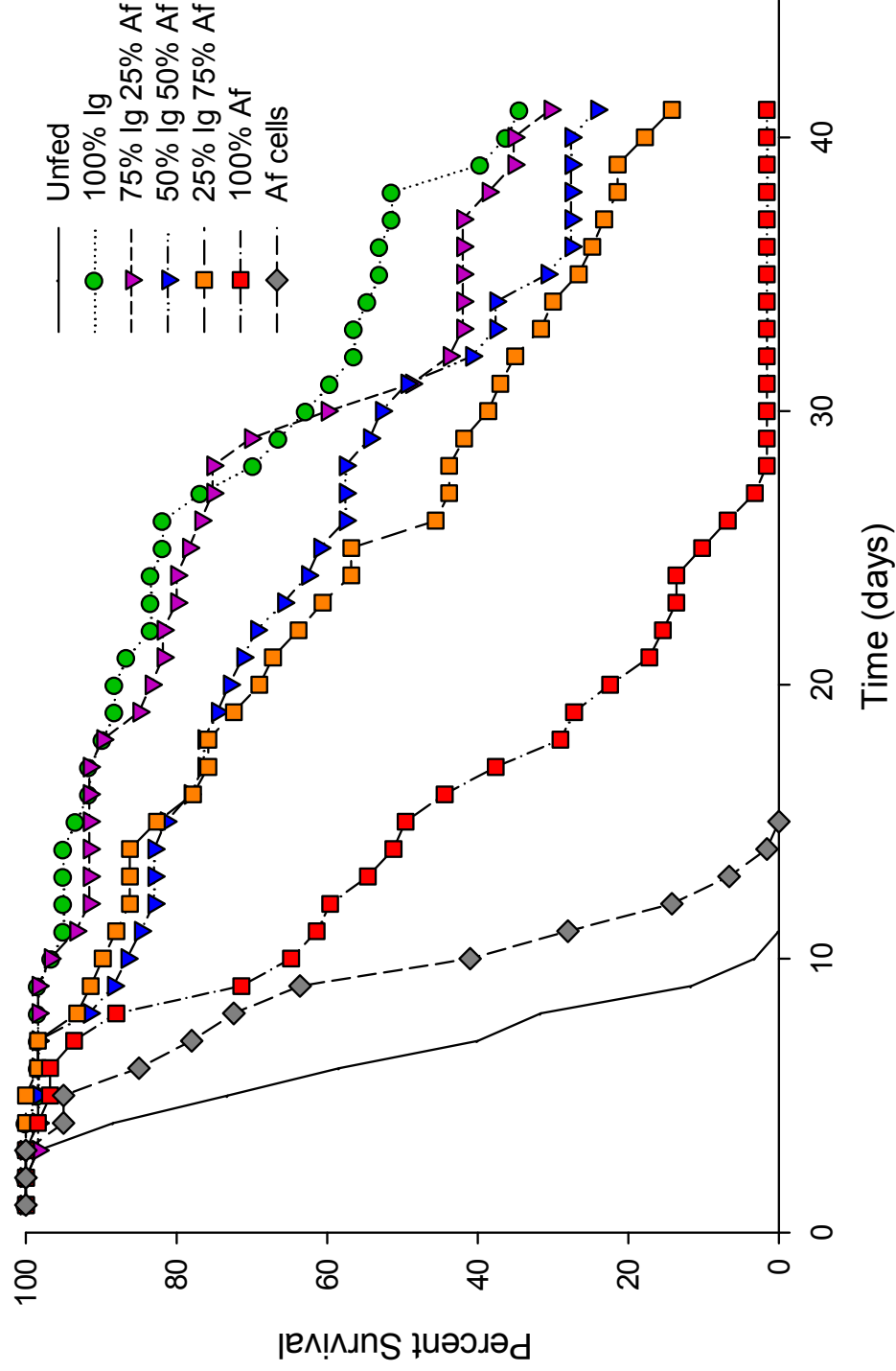


**Figure 14.** Daily percent survival of *Glebocarcinus oregonensis* zoeae fed the indicated diet treatment. “Ig” indicates *Isochrysis galbana* fed rotifers, “Af” indicates *Alexandrium fundyense* fed rotifers and “Af cell” indicates *A. fundyense* cells. Open triangles indicate the mean day of molt for stage I-III.

showed 100% mortality by day 11 (Figure 14), also with no molting to stage II. The mean days of death were calculated for each treatment (Table 4). Larvae fed *A. fundyense* cells showed a significant delay in mortality when compared to the starved control (Table 4; ANOVA,  $p < 0.001$ ).

Larval survival was high for the 100% Ig-rot treatment, while the 100% Af-rot treatment survival experienced a decline throughout the experiment (Figure 14). When the mixed diet results are added to the analysis, survival falls between the two 100% treatments (Figure 15). Larval survival was high for all rotifer diet treatments until approximately day seven; at this point, survival steadily declined for all diet treatments (Figure 15). The 100% Ig-rot diet and the 75% Ig-rot/25% Af-rot diet maintained the highest survival rate through the first 30 days of the experiment. Of the rotifer diet treatments, the 100% Af-rot diet had the lowest survival after day five. All rotifer diet treatments except 100% Af-rot were able to support some survival through the third stage (experiments were terminated at day 41).

Percent mortality that occurred during each zoeal stage was calculated and compared among all diet treatments (Table 9). The results of a series of one-way ANOVA's for each zoeal stage show significant differences during stages I and II (Table 9). Figure 16 shows the results of pairwise contrasts ( $\alpha = 0.05$ ) among treatments for each zoeal stage. In stages I and II, the 100% Af-rot diet had significantly higher mortality than any other treatment. During stage I there was no difference between the 100% Ig-rot and mixed diet rotifer treatments, while the relationships among these treatments varied in stage II (Figure 16). Experiments were terminated on day 41, before any zoeae from the 100% Af-rot treatment molted to stage IV.



**Figure 15.** Daily percent survival of *Glebocarcinus oregonensis* zoeae fed the indicated diet treatment. “Ig” indicates *Isochrysis galbana* fed rotifers, “Af” indicates *Alexandrium fundyense* fed rotifers and “Af cell” indicates *A. fundyense* cells.

**Table 9.** *Glebocarcinus oregonensis* mean stage mortality (%) during each stage for stages I-III larvae fed indicated diet treatments. Ig indicates rotifers that were fed *Isochrysis galbana* and Af indicates rotifers that were fed *Alexandrium fundyense*. Survival values are means  $\pm$  standard error. Resulting p-values are from one-way ANOVA tests. NA indicates that no larvae molted to stage IV before experiments were terminated.

Stage	Mean Percent Stage Mortality $\pm$ standard error						p value		
	100% Ig	75% Ig	25% Af	50% Ig	50% Af	25% Ig		75% Af	100% Af
I	1.60 $\pm$ 1.60	1.60 $\pm$ 1.60	1.60 $\pm$ 1.60	10.0 $\pm$ 3.21	10.0 $\pm$ 3.21	6.60 $\pm$ 3.16	6.60 $\pm$ 3.16	33.6 $\pm$ 4.39	<0.001
II	10.2 $\pm$ 3.18	18.8 $\pm$ 5.61	18.8 $\pm$ 5.61	35.8 $\pm$ 5.35	35.8 $\pm$ 5.35	43.6 $\pm$ 7.90	43.6 $\pm$ 7.90	86.0 $\pm$ 2.80	<0.001
III	29.2 $\pm$ 6.51	43.8 $\pm$ 5.20	43.8 $\pm$ 5.20	51.4 $\pm$ 14.9	51.4 $\pm$ 14.9	59.0 $\pm$ 6.60	59.0 $\pm$ 6.60	NA	0.165

Stage	Diets fed to larvae				
I	100% Ig	75% Ig/25% Af	50% Ig/50% Af	25% Ig/75% Af	100% Af
II	100% Ig	75% Ig/25% Af	50% Ig/50% Af	25% Ig/75% Af	100% Af

**Figure 16.** Pairwise contrast results for percent mortality through the stage for stages I and II *Glebocarcinus oregonensis* larvae fed indicated diet treatments. Ig indicates rotifers that were fed *Isochrysis galbana* and Af indicates rotifers that were fed *Alexandrium fundyense*. Tukey’s HSD tests were used for the contrasts. Results are contrasts of percent mortality by diet during each stage. Shared lines represent no significant treatment difference ( $\alpha=0.05$ ).

Duration in days for each zoeal stage on all diet treatments is shown in Table 10, along with the results of a one-way ANOVA for each stage. In each case, there was a significant difference among treatments. Figure 17 shows the results of pairwise contrasts ( $\alpha=0.05$ ) among treatments for each zoeal stage. In stages I and II, the 100% Af-rot diet was significantly longer than for diet treatments that contained 50% or less of Af-rots, while the relationships among the other treatments varied (Figure 17).

Based on these results, I determined that for *Glebocarcinus oregonensis* both stage survival and duration during the stage could be used to assess the toxicity versus nutritional deficiency distinction using the method of Dam and Colin (2005). Figures 18 and 19 show the results of the percent survival during the stage analysis for zoeal stages I and II respectively. The percent survival of all three mixtures containing some *Isochrysis*-fed rotifers falls above the reference line, indicating that when Ig-rots are added to the diet, even as little as 25%, survival during the stage increases, implicating nutritional deficiency of the Af-rot diet. Figures 20 and 21 show the results of the stage duration analysis for zoeal stages I and II respectively. The Y-axis uses percent acceleration of each treatment as compared to the 100% Af-rot treatment to permit the analysis. The durations of all three mixtures containing some *Isochrysis*-fed rotifers fall above the reference line, indicating that when Ig-rots are added to the diet, even as little as 25%, acceleration of development occurs, implicating nutritional deficiency of the Af-rot diet.

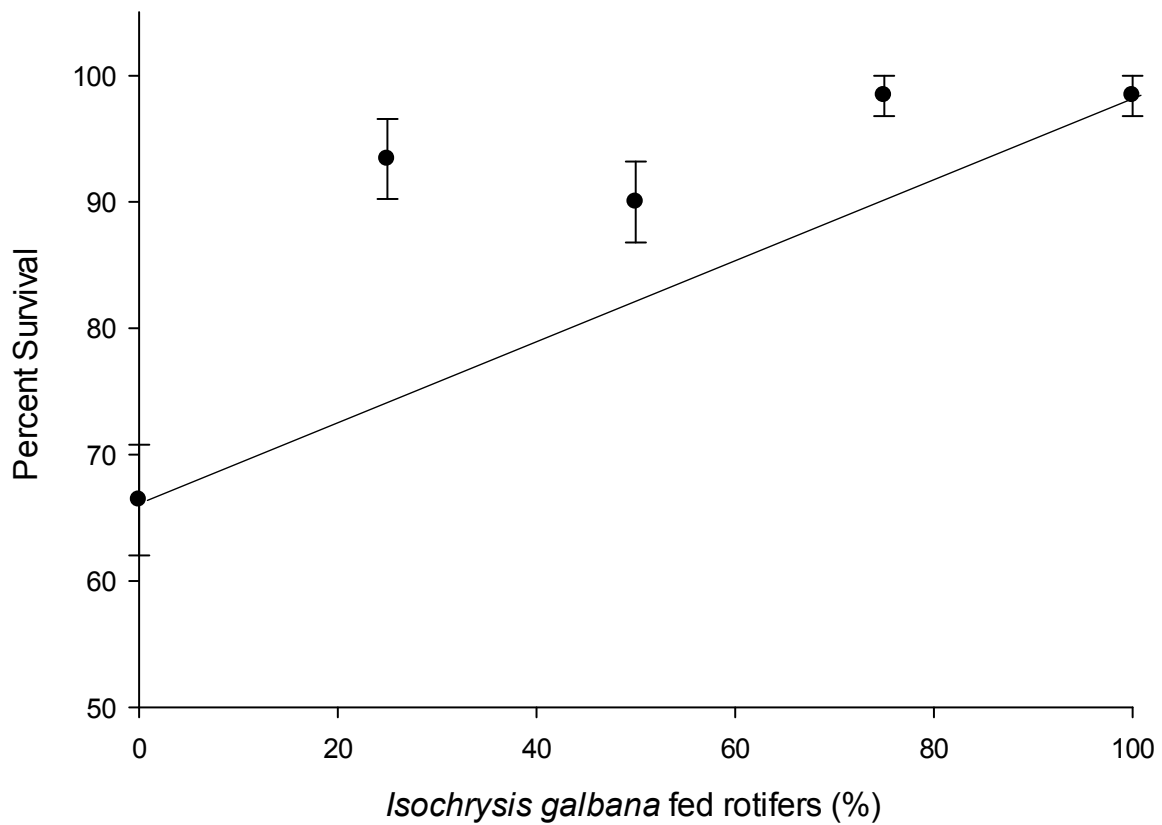
**Table 10.** *Glebocarcinus oregonensis* mean stage duration (days) for stages I-III larvae fed indicated diet treatments. Ig indicates rotifers that were fed *Isochrysis galbana* and Af indicates rotifers that were fed *Alexandrium fundyense*. Duration values are means  $\pm$  standard error. Resulting p-values are from one-way ANOVA tests; except when ANOVA assumptions were not met in which case a non-parametric Kruskal-Wallis test was used (SPSS v.19). Resulting p-values from Kruskal-Wallis tests are noted with an asterisk. NA indicates that no larvae molted to stage IV before experiments were terminated. No standard error is included for stage II 100% Af due to only one larvae molting to stage III.

Stage	Mean Stage Duration (days) $\pm$ standard error												n	p value
	100% Ig	n	75% Ig	25% Af	n	50% Ig	50% Af	n	25% Ig	75% Af	n	100% Af		
I	8.00 $\pm$ 0.07	59	8.15 $\pm$ 0.08	8.24 $\pm$ 0.08	59	8.24 $\pm$ 0.08	54	8.57 $\pm$ 0.11	54	9.13 $\pm$ 0.19	40	<0.001*		
II	7.91 $\pm$ 0.18	53	9.31 $\pm$ 0.18	10.5 $\pm$ 0.39	48	10.5 $\pm$ 0.39	35	11.5 $\pm$ 0.47	30	16.0	1	<0.001*		
III	9.57 $\pm$ 0.19	37	8.81 $\pm$ 0.27	9.35 $\pm$ 0.15	27	9.35 $\pm$ 0.15	17	10.4 $\pm$ 0.24	11	NA	NA	<0.002		

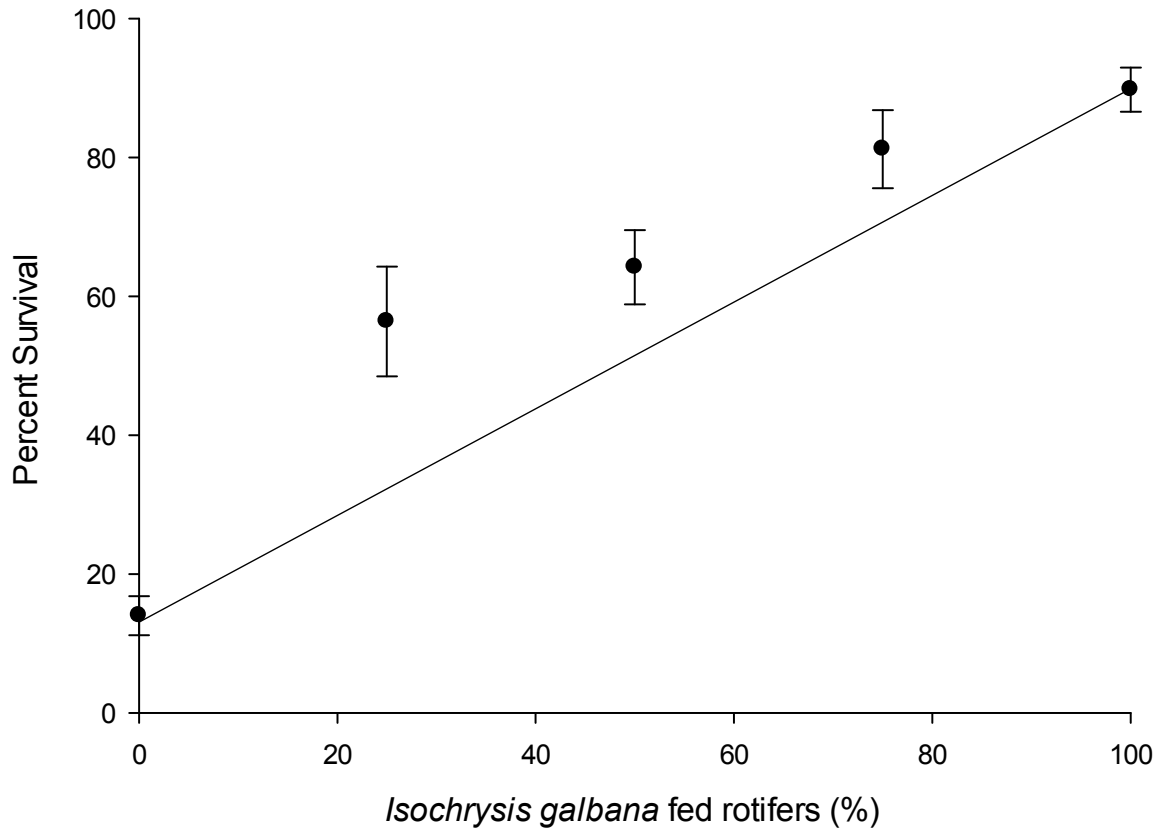
Stage	Diets fed to larvae				
I	100% Ig	75% Ig/25% Af	50% Ig/50% Af	25% Ig/75% Af	100% Af
II	100% Ig	75% Ig/25% Af	50% Ig/50% Af	25% Ig/75% Af	
III	75% Ig/25% Af	50% Ig/50% Af	25% Ig/75% Af	100% Ig	

**Figure 17.** Pairwise contrast results for stage duration for stages I-III *Glebocarcinus oregonensis* larvae fed indicated diet treatments. Ig indicates rotifers that were fed *Isochrysis galbana* and Af indicates rotifers that were fed *Alexandrium fundyense*. For stages I and II stage duration for the diets were contrasted using a Dunnett T3 test and stage III was contrasted using a Tukey's HSD test. Results are contrasts of stage duration by diet during each stage. Shared lines represent no significant treatment difference ( $\alpha=0.05$ ). During stage II only one larva from the 100% *Alexandrium fundyense* fed rotifer treatment molted to stage III, that number was not used in pairwise comparisons.

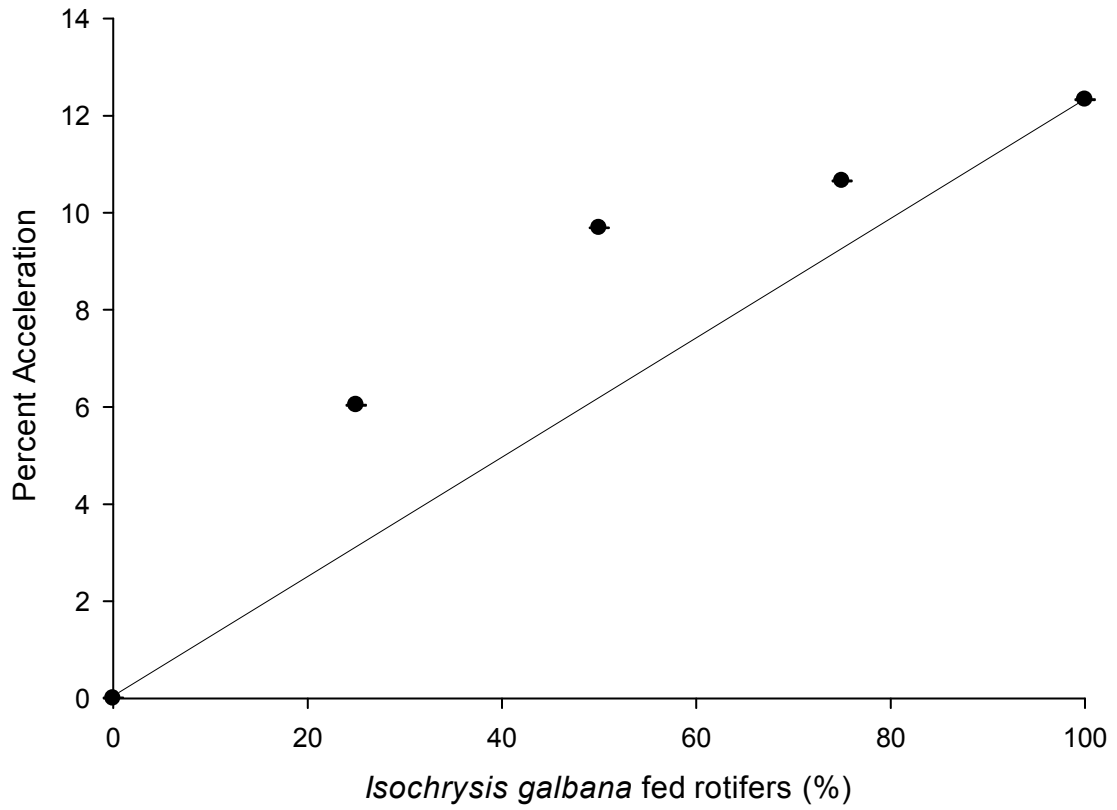




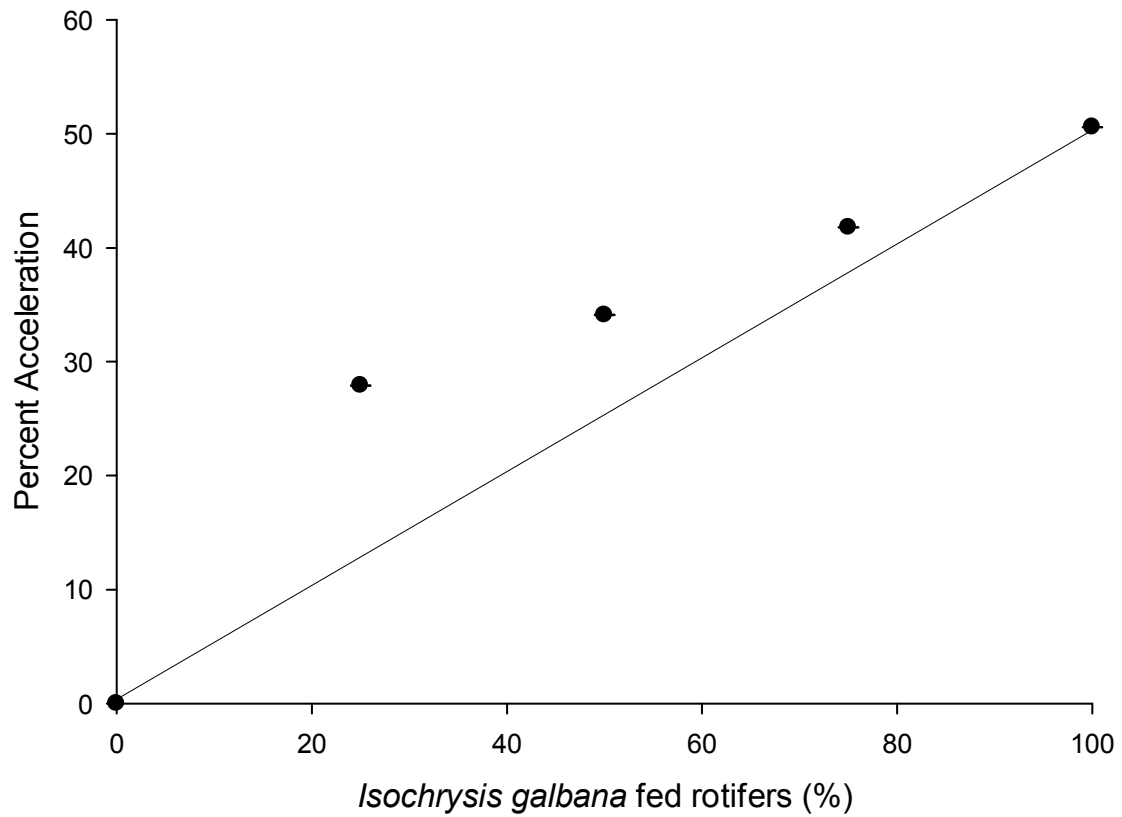
**Figure 18.** *Glebocarcinus oregonensis* survival during stage I in the mixed diet (*Alexandrium fundyense* fed rotifers and *Isochrysis galbana* fed rotifers) experiment. Points represent the mean and bars represent  $\pm$  one standard error. Reference line connects the 100% rotifer diets.



**Figure 19.** *Glebocarcinus oregonensis* survival during stage II in the mixed diet (*Alexandrium fundyense* fed rotifers and *Isochrysis galbana* fed rotifers) experiment. Points represent the mean and bars represent  $\pm$  one standard error. Reference line connects the 100% rotifer diets.



**Figure 20.** *Glebocarcinus oregonensis* stage I duration in the mixed diet (*Alexandrium fundyense* fed rotifers and *Isochrysis galbana* fed rotifers) experiment. Points represent the mean and bars represent  $\pm$  one standard error. Reference line connects the 100% rotifer diets.



**Figure 21.** *Glebocarcinus oregonensis* stage II duration in the mixed diet (*Alexandrium fundyense* fed rotifers and *Isochrysis galbana* fed rotifers) experiment. Points represent the mean and bars represent  $\pm$  one standard error. Reference line connects the 100% rotifer diets.

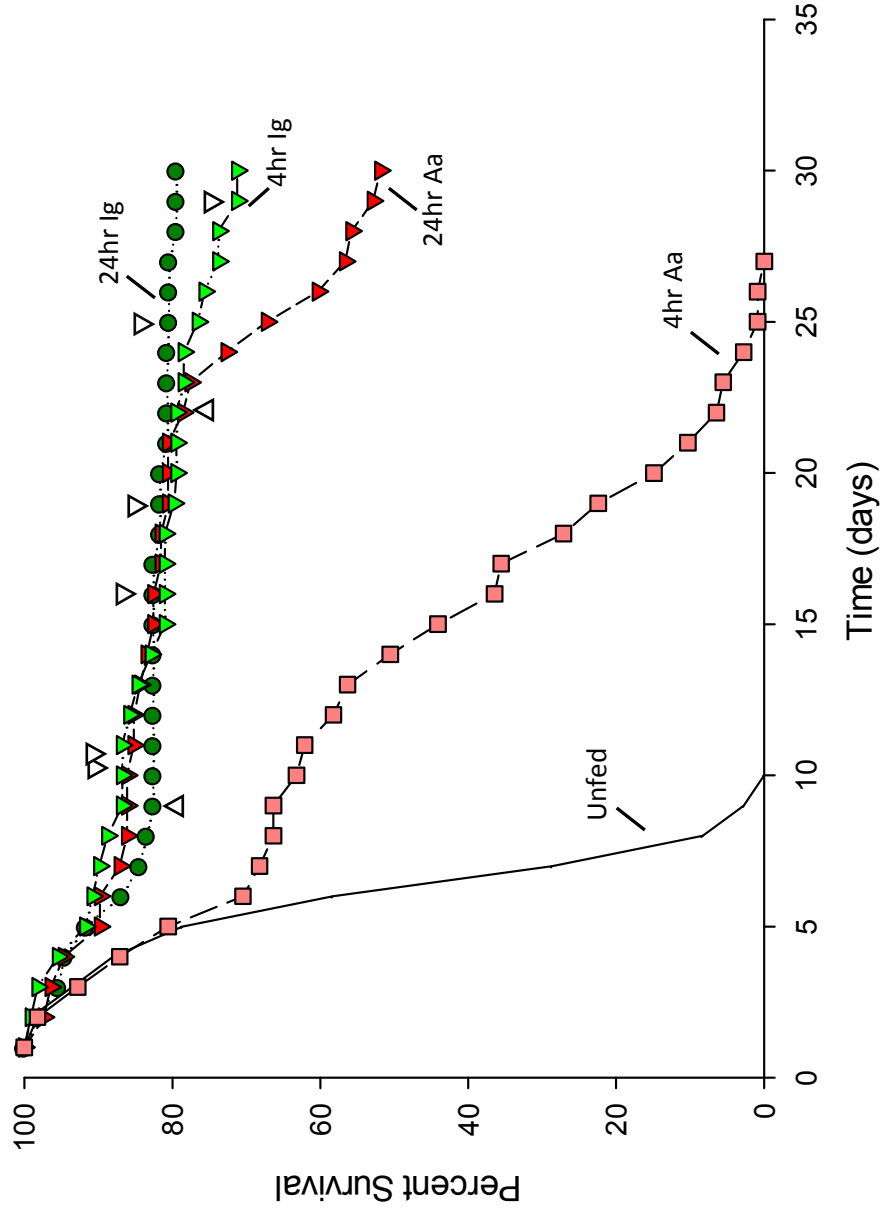
## Continuous versus 4 hour per day feeding experiments

### *Lophopanopeus bellus*

Larval survival was high until day 24, for all rotifer diets except for the four hour *Alexandrium andersoni* fed rotifer treatment, which experienced steady mortality throughout the experiment (Figure 22). Sibling larvae that were starved reached 100% mortality by day 10 with no larvae molting to stage II. Larvae on the four hour *A. andersoni* fed rotifer treatment reached 100% mortality by day 27, with no larvae molting to stage II. Larvae in the 24 hour *Isochrysis galbana* fed rotifer and four hour *I. galbana* fed rotifer treatments molted to stage III. The 24 hour *A. andersoni* fed rotifer treatment larvae molted to stage II, but no larvae from this treatment molted to stage III before experiments were terminated (Figure 22).

Mortality during stage I was compared among rotifer treatments (excluding the four hour *Alexandrium andersoni* treatment) and no significant differences were found (Table 11). Mortality during stage II was compared and found that the 24 hour *A. andersoni* fed rotifer treatment had significantly higher mortality during the stage (Table 11). For stage III the *Isochrysis galbana* fed rotifer treatments were compared since no larvae from the 24 hour *A. andersoni* fed rotifer treatment molted to stage IV during the experiment. The 24 hour *I. galbana* fed rotifer treatment had significantly lower mortality during stage III compared to the four hour *I. galbana* fed rotifer treatment (Table 11).

Stage duration was also affected by diet treatment (Table 12). When compared to the 24 hour *Isochrysis galbana* fed rotifer treatment, the 24 hour *Alexandrium andersoni* fed rotifer and four hour *I. galbana* fed rotifer treatments experienced a significant delay in



**Figure 22.** Daily percent survival of *Lophopanopeus bellus* zoeae given indicated diet treatment. The “24 hour” treatments were fed continuously and the “4 hour” treatments were fed for four hours and changed to filtered seawater. “Ig” indicates *Isochrysis galbana* fed rotifers and “Aa” indicates *Alexandrium andersoni* fed rotifers. Open triangles indicate the mean day of molt for stages I-III.

**Table 11.** *Lophopanopeus bellus* mean stage mortality (%) through each stage for stages I-III larvae fed indicated diet treatments. Ig indicates rotifers that were fed *Isochrysis galbana* and Aa indicates rotifers that were fed *Alexandrium andersoni*. The 24 hour treatments were fed continuously and 4 hour treatments were fed rotifers for four hours and then changed to filtered sea water. Survival values are means  $\pm$  standard error. Resulting p-values are from one-way ANOVA tests; except when ANOVA assumptions were not met in which case a non-parametric Kruskal-Wallis test was used (SPSS v.19). Resulting p-values from Kruskal-Wallis tests are noted with an asterisk. NA indicates no larvae molted to stage IV before experiment termination. Pairwise contrast (Dunnnett T3) results are indicated by letters, shared letters indicates no significant treatment difference ( $\alpha=0.05$ ). The stage mortality in stage III of the 4 hour Ig treatment shows the percent mortality at the end of the experiment, not all larvae in this treatment molted to stage IV before the termination of the experiment.

Stage	Mean Percent Stage Mortality $\pm$ standard error			p value
	24 hour Ig	4 hour Ig	24 hour Aa	
I	16.9 $\pm$ 5.88	19.0 $\pm$ 3.12	17.6 $\pm$ 6.00	0.369
II	1.78 $\pm$ 1.18 (a)	5.11 $\pm$ 2.02 (a)	37.8 $\pm$ 10.8 (b)	<0.001*
III	2.22 $\pm$ 1.48	8.09 $\pm$ 3.08	NA	<0.001*

**Table 12.** *Lophopanopeus bellus* mean stage duration (days) for stages I-III larvae fed indicated diet treatments. Ig indicates rotifers that were fed *Isochrysis galbana* and Aa indicates rotifers that were fed *Alexandrium andersoni*. The 24 hour treatments were fed continuously and 4 hour treatments were fed rotifers for four hours and then changed to filtered sea water. Duration values are means  $\pm$  standard error. Resulting p-values are from one-way ANOVA tests; except when ANOVA assumptions were not met in which case a non-parametric Kruskal-Wallis test was used (SPSS v.19). Resulting p-values from Kruskal-Wallis tests are noted with an asterisk. NA indicates no larvae molted to stage IV before experiments were terminated. Pairwise contrast (stage I: Dunnett T3; stage II: Tukey's HSD) results are indicated by letters, shared letters indicates no significant treatment difference ( $\alpha=0.05$ )

Stage	Mean Stage Duration (days) $\pm$ standard error						
	24 hour Ig	n	4 hour Ig	n	24 hour Aa	n	p value
I	9.15 $\pm$ 0.11 (a)	88	10.2 $\pm$ 0.24 (b)	85	10.2 $\pm$ 0.12 (b)	89	<0.001*
II	6.92 $\pm$ 0.08 (c)	86	8.73 $\pm$ 0.17 (d)	80	12.2 $\pm$ 0.19 (e)	54	<0.001
III	7.88 $\pm$ 0.07	84	10.1 $\pm$ 0.16	35	NA	NA	<0.001

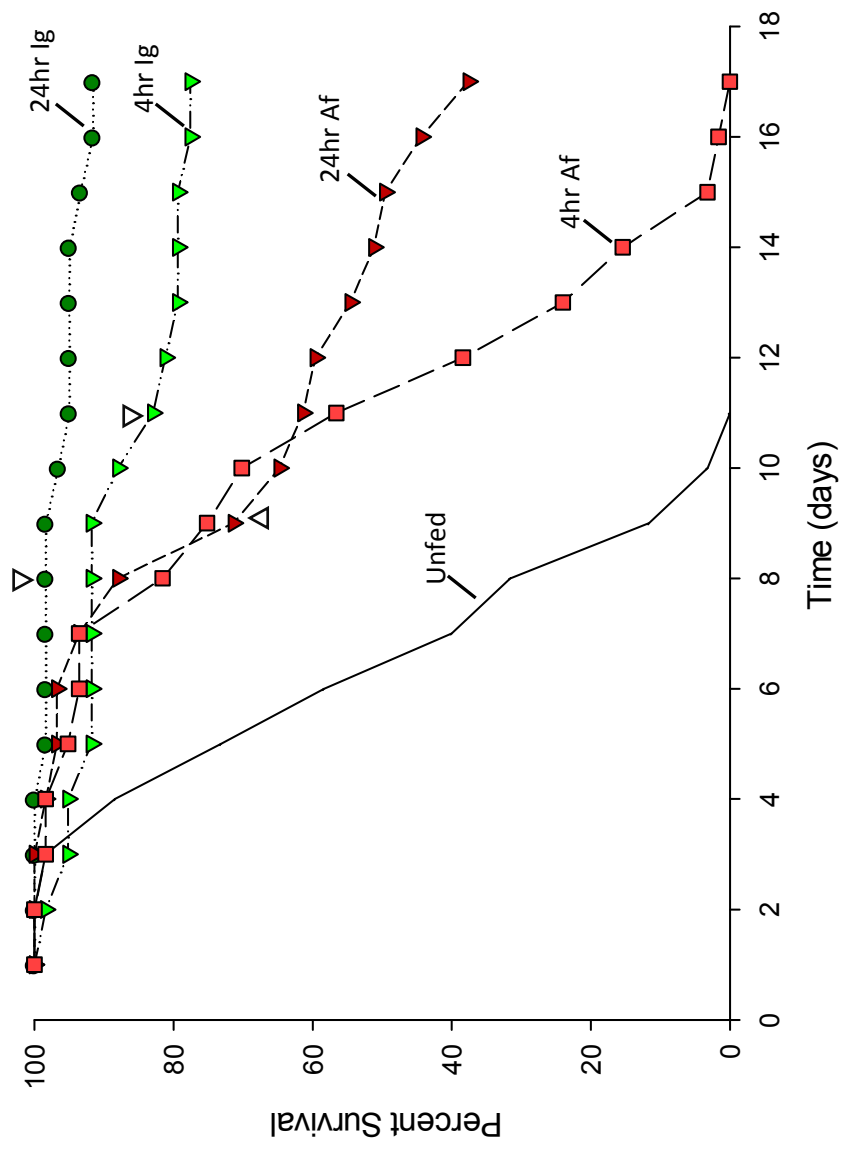


development during stages I-III. There was no difference in stage duration between the 24 hour *Alexandrium andersoni* fed rotifer and the four hour *I. galbana* fed rotifer treatment during stage I. For stage II duration the 24 hour *A. andersoni* fed rotifer treatment continued to be delayed (Table 12) and was significantly longer than both *I. galbana* fed rotifer treatments.

### ***Glebocarcinus oregonensis***

Survival was high throughout the experiment for larvae in the *Isochrysis galbana* fed rotifer treatments, with the 24 hour *I. galbana* fed rotifer treatment experiencing the highest overall survival (Figure 23). Survival for the *Alexandrium fundyense* fed rotifer treatments were high until day seven at which point both treatments started to steadily decline (Figure 23). The 24 hour *A. fundyense* fed rotifer diet treatment was able to support development into stage II while the four hour *A. fundyense* fed rotifer treatment did not. The larvae in the four hour *A. fundyense* fed rotifer treatment reached 100% mortality by day 17, no larvae molted to stage II. Sibling larvae that were starved reached 100% mortality by day 11 with no larvae molting to stage II (Figure 23).

Mortality during stage I was also affected by diet treatment (Table 13). The 24 hour *Isochrysis galbana* fed rotifer treatment had the lowest mortality during stage I, while the 24 hour *Alexandrium fundyense* fed rotifer treatment had the highest mortality. The four hour *I. galbana* fed rotifer treatment mortality during stage I fell between the two 24 hour diet treatments and was not significantly different from either treatment (Table 13). Stage duration was also affected by diet treatments (Table 13). Stage duration was delayed in the 24 hour *A. fundyense* fed rotifer treatment as well as the four hour *I. galbana* fed rotifer



**Figure 23.** Daily percent survival of *Gleboecarcinus oregonensis* zoeae given indicated diet treatment. The “24 hour” treatments were fed continuously and the “4 hour” treatments were fed for four hours and changed to filtered seawater. “Ig” indicates *Isochrysis galbana* fed rotifers and “Af” indicates *Alexandrium fundyense* fed rotifers. Triangles indicate the mean day of molt for stage I.

**Table 13.** *Glebocarcinus oregonensis* mean stage mortality (%) and mean stage duration (days) for stage I larvae fed indicated diet treatments. Ig indicates rotifers that were fed *Isochrysis galbana* and Aa indicates rotifers that were fed *Alexandrium andersoni*. The 24 hour treatments were fed continuously and 4 hour treatments were fed rotifers for four hours and then changed to filtered sea water. Survival and duration values are means  $\pm$  standard error. Resulting p-values are from one-way ANOVA tests (SPSS v.19). Pairwise contrast (Tukey's HSD) results are indicated by letters, shared letters indicates no significant treatment difference ( $\alpha=0.05$ ).

	Stage I Stage Mortality (%) and Duration (days)			p value
	24 hour Ig	4 hour Ig	24 hour Af	
<b>Mortality</b>	1.60 $\pm$ 1.60 (a)	20.4 $\pm$ 7.67 (a, b)	33.6 $\pm$ 4.39 (b)	0.003
<b>Duration</b>	8.00 $\pm$ 0.07 (c)	11.0 $\pm$ 0.14 (d)	9.12 $\pm$ 0.19 (e)	<0.001

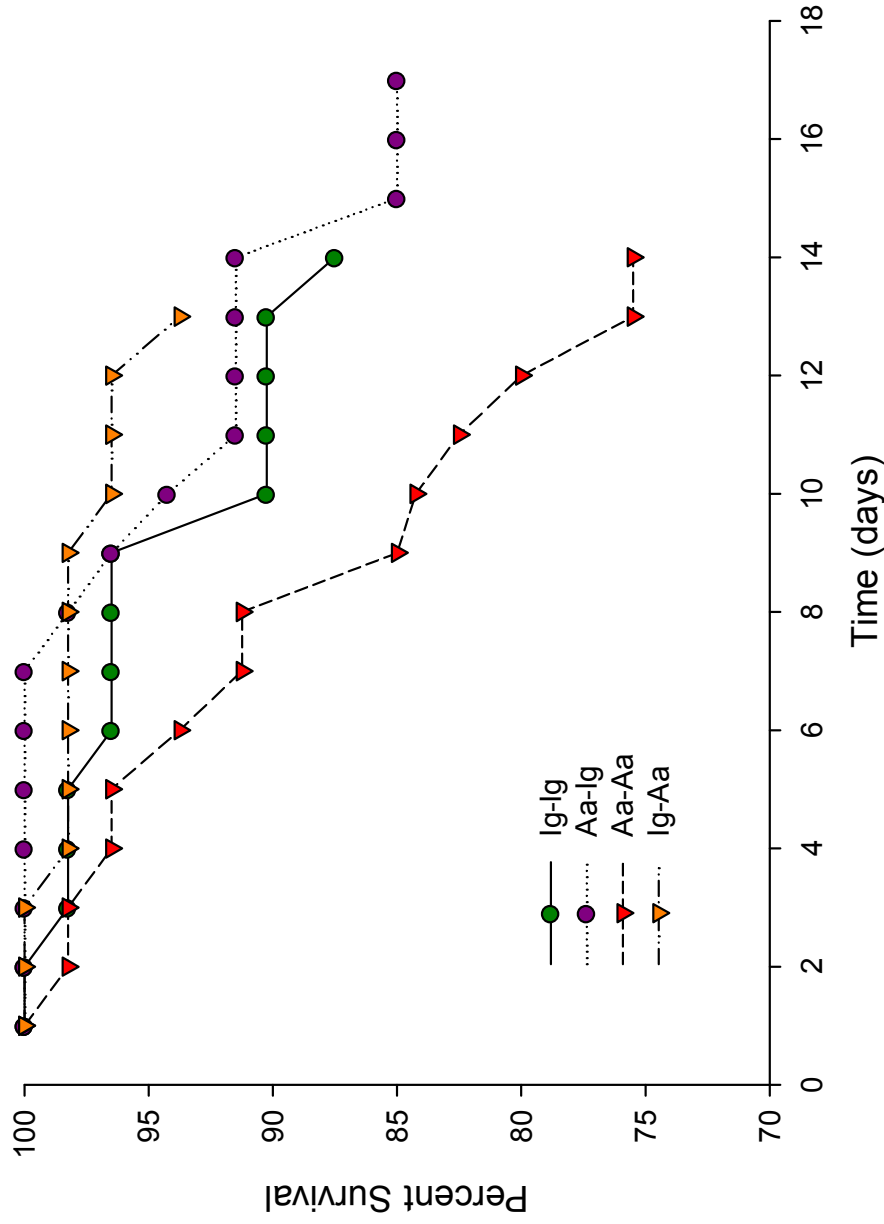
treatment. All three diet treatments that supported development were significantly different from one another in terms of stage duration, the four hour *I. galbana* fed rotifer treatment stage duration was delayed the longest of the three treatments (Table 13).

## **Diet Change Experiment**

### ***Lophopanopeus bellus***

The increased mortality of larvae fed toxic rotifers compared to larvae fed non-toxic rotifers through early development may produce surviving larvae that are more resistant to the effects of subsequent exposure to toxin. To look at the effects of exposure to algal toxin and possible selection during early larval stages, larvae were exposed to a given treatment for a period before experimental treatments were applied. For this experimental approach, stage III day one larvae that had been previously fed either Aa-rot (toxic) or Ig-rot (nontoxic) rotifers were used. Results from these experiments were recorded starting with stage III day one larvae. Because molting in larvae is not synchronous, data were collected for each individual larva and normalized to stage III, day one to compare stage survival and duration during stage III.

Survival during stage III declined in all four diet treatments (Figure 24). Survival at the end of stage III appeared highest in larvae originally fed Ig-rot then switched to Aa-rots on day one of stage III. Survival steadily declined and was the lowest for the larvae that were continually fed an Aa-rot diet. All treatments experienced molting to stage IV, at which point the experiments were terminated.



**Figure 24.** Daily percent survival of *Lophopanopeus bellus* zoeae fed the indicated diet treatment starting at day one of stage III. “Ig” indicates *Isochrysis galbana* fed rotifers, “Aa” indicates *Alexandrium andersoni* fed rotifers. The first set of letters indicates the initial diet treatment, the second set of letters indicates the diet treatment administered from day one of stage III through the third zoeal stage. Time is from the start of the second diet treatment (stage III, day one).

Percent mortality that occurred during stage III was calculated and compared among diet treatments (Table 14). The results from a one-way ANOVA analysis found no significant difference among the treatments (Table 14; ANOVA,  $F=2.21$ ,  $p=0.14$ ).

Duration in days for stage III zoeae on all diet treatments are shown in Table 14, along with the results of a Kruskal-Wallis test. There was a significant difference among treatments (Table 14; Kruskal-Wallis,  $\chi^2=35.05$ ,  $df=3$ ,  $p<0.001$ ). The larvae that were fed Aa-rots during stage III experienced significant delay in development compared to the larvae continually fed Ig-rots (Table 14). The larvae that were originally fed Aa-rots and switched to Ig-rots on day one of stage III did not experience a delay in development when compared to the larvae continually fed Ig-rots, but these larvae were also not significantly different from the other diet treatments (Table 14).

**Table 14.** *Lophopanopeus bellus* mean stage mortality (%) and mean stage duration (days) for stage III larvae fed indicated diet treatments. Ig indicates rotifers that were fed *Isochrysis galbana* and Aa indicates rotifers that were fed *Alexandrium andersoni*. The first set of letters indicates the initial diet treatment, the second set of letters indicates the diet treatment administered through the third zoeal stage. Survival and duration values are means  $\pm$  standard error. Resulting P-values are from one-way ANOVA tests; except when ANOVA assumptions were not met in which case a non-parametric Kruskal-Wallis test was used (SPSS v.19). Resulting p-values from Kruskal-Wallis tests are noted with an asterisk. Pairwise contrast (Dunnnett T3) results are indicated by letters, shared letters indicates no significant treatment difference ( $\alpha=0.05$ ).

	Stage III Mean Percent Mortality & Mean Stage Duration (days)			p value	
	Ig-Ig	Aa-Ig	Aa-Aa		Ig-Aa
<b>Mortality</b>	12.5 $\pm$ 3.18	15.0 $\pm$ 7.04	24.5 $\pm$ 5.11	6.25 $\pm$ 4.25	0.140
<b>Duration</b>	8.49 $\pm$ 0.11 (a)	9.28 $\pm$ 0.36 (a, b)	9.70 $\pm$ 0.19 (b)	9.24 $\pm$ 0.10 (b)	<0.001*

## DISCUSSION

### Algal Cell Ingestion by Larval Crabs

During these experiments, *Lophopanopeus bellus* larvae were fed *Alexandrium andersoni* cells directly, resulting in accelerated mortality when compared to a starved control, supporting previous studies that implicated toxicity (Perez and Sulkin 2005, Garcia et al. 2011). The toxins from the algal cells have an immediate effect on the larvae, which is seen by their accelerated mortality over the unfed control. Larvae of *L. bellus* were also given *A. fundyense* cells directly mortality of these crabs was delayed beyond that of the starved control, suggesting that the toxin-containing cells are providing nutrition to the larvae that overcome any toxic effects that may be present. While *Metacarcinus magister* larvae fed *A. fundyense* cells directly were not significantly different from the starved control, *Glebocarcinus oregonensis* larvae that were fed *A. fundyense* cells also delayed mortality when compared to the starved control. This result supports previous research that shows delayed mortality for larvae fed *A. fundyense* cells (Perez and Sulkin 2005, Garcia et al 2011). Hinz et al. (2001) and Perez and Sulkin (2005) both reported that larval ingestion of *A. fundyense* was only brief when it occurred. This may suggest that toxin build up in the larvae and therefore toxic effects may be limited because ingestion rates are low (Garcia et al. 2011). It is the case though, that ingestion of this alga, however limited, is providing at least some nutritional benefit, resulting in delayed mortality. The present research also confirms previous research that showed that although some algal species may contribute nutrition and delay mortality, algae alone are not sufficient to support larval crab development (Sulkin 1975).



## Mixed Diet Experiments

When a heterotrophic prey source is used as a diet for larval crabs, the diet of the heterotrophic prey itself affects its nutritional value, therefore affecting the development and survival of the larval crabs. Sulkin and McKeen (1999) reported that larval crab development varied depending on the diet of the rotifer *Brachionus plicatilis* that was fed to the crabs. When rotifers were fed *Isochrysis galbana*, they supported higher larval crab survival than rotifers fed *Dunaliella tertiolecta*. Garcia et al. (2011) found that *B. plicatilis* will ingest both *Alexandrium andersoni* and *A. fundyense* and when these rotifers are fed to larval crabs they supported development through the third stage. Garcia et al. (2011) found, however, that these larvae had significantly lower survival than larvae fed *I. galbana* fed rotifers. The results from the present experiments confirm the results of Sulkin and McKeen (1999) and Garcia et al. (2011) that the ability of the rotifer *B. plicatilis* to support development of larval crabs is dependent on the food source of the rotifers. The lower survival seen in larvae fed toxin-containing algal fed rotifers (Garcia et al. 2011) leads to the question of whether the rotifers are transferring toxins to the larvae or if the rotifers are nutritionally insufficient.

In distinguishing a nutritional deficiency from a toxic effect, the term nutritional deficiency needs to be defined. With a rotifer diet, a nutritional deficiency to the larval crabs could be due either to a reduced ingestion rate of the toxic rotifers by the larval crabs or to a reduction in nutritional value of the rotifers that had ingested the toxic algae due to some element unrelated to the toxin. Prey experiments found no significant differences in ingestion rates between the two types of rotifers and no larval feeding preference (Table 3).

Accordingly, for my experiments, the nutritional deficiency was due to a reduction in value of the rotifer.

The approach used in these experiments to tease out nutritional deficiencies from toxicity effects is taken from methods used by Dam and Colin (2005). Their experiment looked at whether the dinoflagellate *Prorocentrum minimum* was nutritionally insufficient or toxic to the copepod *Acartia tonsa*. Their technique is based on the premise that growth (egg production in their case) is linearly dependent upon the percentage of good and poor foods in a mixed diet. If egg production is lower than predicted by dilution of the poor food, toxicity of the poorer food is suggested; whereas, if egg production is greater than predicted by dilution of the poor food, the good food complements the poor food's nutritional insufficiency.

Using mixed diets consisting of differing proportions of *P. minimum* (toxic) and *Thalassiosira weissflogii* (nontoxic), Dam and Colin (2005) found that the addition of *T. weissflogii* increased egg production, suggesting the *P. minimum* was not a sufficiently complete food source for egg production (Dam and Colin 2005). For my experiments, I used the two differing types of rotifers (one fed toxin-containing algae of unknown nutritional value and one fed non-toxin-containing, nutritionally sufficient algae) in mixed proportions to attempt to distinguish between toxic effects and nutritional effects on larval crabs. The experiments run by Dam and Colin (2005) require that there be no prey selection. This assumption was confirmed in the rotifer preference by larval crab experiments (Table 3).

### *Lophopanopeus bellus*

In the present experiments, exposure to rotifers fed on toxin-containing algae did not increase mortality through zoeal stage III in *Lophopanopeus bellus* directly, although it did delay development as early as stage I. Faster development for larval crabs leads to a higher probability of reaching settlement, which is beneficial to their overall survival. The longer larvae are present in the water column, the more likely they are to suffer mortality from predation, degradation of water quality, harmful algae blooms, competition or nutritional stress. Therefore, factors that extend stage duration have the potential to increase overall mortality prior to metamorphosis.

Although there did not seem to be significant differences among treatments in daily survival, when calculated on a stage basis, there was high mortality during stage III on toxin-containing rotifers (Table 5). The increased mortality is somewhat disguised in Figures 3 and 4 because development among treatments is not synchronous. The increase in mortality during stage III of the zoeae fed toxin-containing rotifers occurs simultaneously with the stage IV mortality seen in the treatments that have some proportion of non-toxic rotifers.

The nutritional deficiencies of the Aa-rots are seen when looking at the mixed diet analysis (Figures 6-8) for this species. The analysis concludes that since the percent acceleration of mixed diet values fall above the reference line, the delay in development of larvae fed 100% toxic rotifers was due to nutritional deficiency.

### ***Metacarcinus magister***

Hinz et al. (2001) showed that larval crabs will ingest *A. andersoni* cells continually, while *A. fundyense* cells are initially ingested but subsequently rejected by the larvae. With the introduction of the rotifer into these experiments, larvae can no longer avoid toxins potentially introduced by the *A. fundyense* since it has been ingested by the rotifer.

*Metacarcinus magister* larvae in these experiments showed reduced survival on the 100% Af-rot diet as compared to the 100% Ig-rot diet. This is consistent with Garcia et al. (2011) where this crab species was fed toxic and non-toxic rotifers. The survival results for *M. magister* show that as long as there are Ig-rots present in the diet, even as little as 25%, the survival of the larvae increases over that obtained when the diet consists solely of Af-rot prey.

The nutritional deficiencies of the Af-rots are illustrated by the mixed diet analysis (Figures 11-12). Since both the mixed diet percent acceleration and mixed diet percent survival fall above the reference line, delay in development and reduced survival on 100% *Alexandrium fundyense* fed rotifers is due to nutritional deficiency of the prey.

### ***Glebocarcinus oregonensis***

*Glebocarcinus oregonensis* larvae in these experiments had the highest survival on the 100% Ig-rot diet and the lowest survival on the 100% Af-rot diet. This is consistent with previous research where this species was fed toxic and non-toxic rotifers (Garcia et al. 2011). The survival results for *G. oregonensis* show that as long as there are Ig-rots present in the

diet, even as little as 25%, the survival of the larvae increases over that obtained when the diet consists solely of Af-rot prey.

During the *Glebocarcinus oregonensis* experiments, the 100% Ig-rot diet supported the highest survival, but survival for all diet treatments steadily declined over the course of the experiment (Figure 14). These results for the control treatment differ from previous results (Sulkin and McKeen 1999, Garcia et al. 2011), in which *G. oregonensis* larvae showed consistently high survival when fed Ig-rots. It is not surprising therefore that the results for survival for Af-rot fed larvae were also lower than reported by Garcia et al. (2011). However, the present experiment is internally consistent since Af-rot diet results were compared directly to the Ig-rot diet results.

The nutritional deficiencies of the Af-rots are observed in the mixed diet analysis (Figures 16-19). Since the mixed diet percent acceleration and mixed diet percent survival values fall above the reference line, development results seen when larvae are fed 100% *Alexandrium fundyense* rotifers are due to nutritional deficiency.

Overall, larvae that were fed 100% Af-rots from the *Metacarcinus magister* and *Glebocarcinus oregonensis* mixed diet experiments experienced significant decreases in survival compared to the other treatments. Unlike those species, *Lophopanopeus bellus* larvae fed solely on toxic rotifers did not experience a significant decrease in survival in the present experiments. Perhaps what we are seeing in the *G. oregonensis* and *M. magister* experiments are the effects of the *A. fundyense* toxins. *A. fundyense* has higher saxitoxin levels than *A. andersoni* and these toxins presumably are retained in the rotifer when algal

cells are ingested (Garcia et al. 2011). The steady decline in survival seen in the *G. oregonensis* and *M. magister* experiments may be due to higher toxin levels in the *A. fundyense* fed rotifers.

Reasons behind this difference may also be a function of the species of crab. The crab *Lophopanopeus bellus* belongs to the family Xanthidae, while species used in similar experiments in previous research were generally from the families Cancridae and Varunidae (Sulkin and McKeen 1999, Garcia et al. 2011). Adult xanthid crabs can accumulate toxins in their tissues without lethal consequences (Llewellyn 1997). The same thing may be occurring in the larvae of *L. bellus*.

The survival results for all three species used in these experiments confirm that as long as there are Ig-rots present in the diet, even as little as 25%, the survival of the larvae increases. These results support the conclusion that the Ig-rots are nutritionally supplementing the toxin containing algal fed rotifers.

### **Toxicity versus nutritional deficiency**

We know that rotifers fed the toxin containing algae were ingesting the algae, at least initially, and sequestering the toxins (Garcia et al. 2011). ELISA analyses of the sample rotifer cultures confirmed that they contained saxitoxin, with the *Alexandrium fundyense* fed rotifers having a higher saxitoxin level than the *A. andersoni* fed rotifers (Garcia et al. 2011). White (1981) found that barnacle nauplii and copepods could ingest toxic *A. tamarense* and accumulate toxins with no apparent adverse effect and that toxins were retained in the organisms for several days after gut evacuation. It has also been found that *Alexandrium* spp.

toxins accumulate in several species of copepods (Turrieff et al. 1995, Teegarden and Cembella 1996). In the present experiments, the rotifers may have ingested the *Alexandrium* cells for a period of time after which ingestion may have decreased. The rotifers could have sequestered the toxins from the algae, but if ingestion by the rotifer on the algae decreased or stopped, nutritional value of the rotifer would begin to drop because the rotifer is basically experiencing a starved condition.

From the results of the continuous versus 4-hour per day feeding experiments, we found that longer exposure to the Aa or Af-rots supported higher survival, which is an indication that the crab larvae were ingesting the Aa or Af-rots for more than a limited period. These results also support the argument that the lower survival and delay in development of larval crabs fed toxin containing algal fed rotifers can be contributed to a nutritional deficiency of the rotifers. Otherwise, one would expect longer exposure would have a more deleterious effect compared to shorter exposure.

Other heterotrophic prey sources recognize algal cells that contain PSP toxin, a toxin found in some *Alexandrium* species (Teegarden 1999). Teegarden (1999) found that cells containing PSP toxin can be discerned by grazers prior to ingestion. The grazers Teegarden (1999) used in experiments were copepods, but it may be possible that rotifers also recognize toxins and decrease or limit ingestion of toxic cells. If this is occurring, the nutritional value of the rotifer might decrease over time and therefore would not support normal development if ingested by larval crabs. Yan et al. (2009) determined that when the rotifer *Brachionus plicatilis* was fed differing species of algae, some toxic, some nontoxic, there were significantly lower ingestion rates on the toxic species. Preliminary experiments were

conducted in the present study to determine if the rotifers were ingesting the algal species. Using gut fluorescence under epifluorescence to indicate the presence of chlorophyll *a*, ingestion of all algal species used in these experiments was confirmed. These experiments however were not able to quantify the amount of algae ingested and rotifers may have reduced ingestion rates of the toxic algal cells.

In addition, as cell concentrations of toxin-containing *Alexandrium* spp. increases, algal ingestion deterrents may also increase (Colin and Dam 2003). If cell deterrents were present the rotifers may have been deterred from eating a sufficient number of toxic cells, therefore reducing the nutrition to the rotifer. If rotifers were not ingesting a sufficient amount of algal cells this would lead to a nutritionally deficient rotifer. If this was occurring (no experiments looking at the number of algal cells ingested by rotifers were conducted, but positive ingestion was confirmed using epifluorescent microscopy) rotifers would not be getting the needed nutrients to pass on to larval crabs. This would therefore show up as a nutritional deficiency on the part of the rotifer, rather than a toxic effect. It is hard to tease out toxicity completely from these results. It may be that due to the toxins and ingestion deterrents found within *Alexandrium* cells, rotifers became nutritionally deficient because they could not ingest enough cells to support normal development when ingested by larval crabs. Nevertheless, it would still be true that reduction in survival of the larvae was the result of nutritional factors rather than direct toxicity.

Although my results cannot completely tease out toxicity from nutritional deficiency, we can conclude that relatively high larval crab survival can be supported when larvae are presented with nutritionally sufficient rotifers (in this case the Ig-rots), even when toxin-



containing rotifers (Aa-rots and Af-rots) are present in the diet. This research is supported by previous research that the nutritional quality of the food given to a heterotrophic prey source will affect the survival and development of the larval crab that ingest that heterotrophic prey (Sulkin and McKeen 1999). We can speculate that in nature as long as there is a mixed diet present, with some source of animal prey, larval crab survival will be higher than if only toxic algal species were present.

### **Continuous versus 4 hour per day feeding experiments**

It is apparent that length of exposure to prey had an important impact on larvae of both *Lophopanopeus bellus* and *Glebocarcinus oregonensis*, but varied according to prey type. Larvae fed the toxin-containing rotifers were particularly affected by the reduced period of exposure. As expected from the previous results, larvae fed continuously on Ig-rots fared better than those fed continuously on the *Alexandrium* sp-fed rotifers. The critical issue, however, is that reduced exposure to the toxin-containing rotifer has a greater negative impact on the larvae than did reduced exposure to the Ig-rots. This is consistent with the argument offered previously that the constraint to development induced by the *Alexandrium andersoni* or *A. fundyense* rotifers is due to nutritional deficiency. If the reduction in crab survival and delayed development was due to introduction of toxins, one would expect continuous exposure to the toxin-containing rotifers to increase zoeae mortality and delay development more than limited exposure to these rotifers.

Garcia et al. (2011) suggested that one possible reason for the increased mortality and slower development of larvae fed toxic rotifers is that after an initial period of ingesting toxic

rotifers, larvae may temporarily stop feeding, thus resulting in a lack of nutrition from feeding. My results suggest, furthermore, that in the mixed prey experiments, the larvae were not ingesting the toxin-containing rotifers for a limited period of time since the latter results reflected the continuous treatment rather than the four hour per day treatment. Although the results are confounded by being carried out on different crab species, there is also the suggestion that Af-rots are nutritionally superior to Aa-rots, with the onset of significant mortality occurring sooner in the latter treatment (Figures 21-22).

These results differ from those of Sulkin et al. (1998a), who found that larvae did well after limited exposure to prey. However, Sulkin et al. (1998a) used freshly-hatched nauplii of the brine shrimp *Artemia* sp., a prey that is highly nutritious to larval crabs. It seems likely that the rotifer is a far more limited prey in terms of its nutritional contributions, particularly with respect to its lipid content (Levine and Sulkin 1984a, Sulkin and McKeen 1999) and that it is necessary for larvae to have longer exposure to the prey as a result.

The results suggest that laboratory conditions that typically expose larvae to continuous access to prey may be producing different results from those seen in nature. Larval crabs in nature will only occasionally be exposed to high densities of favorable prey. The present results, and those of Sulkin et al. (1998), suggest that the differences between laboratory and field results may be substantial, and will be related to prey type. Since prey types will likely be mixed in nature, the relationship between laboratory and field results are unlikely to be resolved unless subjected to modeling of the various possible prey exposure scenarios.

### **Early Exposure to Toxin Impact on Later Stages**

Exposure of larvae to prey containing algal toxins in the early zoeal stages had little impact on the outcome of either mortality or stage duration on a later zoeal stage. If there had been such impact, one would expect those larvae surviving exposure to the Aa-Aa treatment to fare better in stage III than those exposed to the Ig-Aa treatment, but that was not the case. Larvae robust enough to survive the first two stages on the Aa-Aa treatment were just as susceptible to the Aa-rot treatment as were those that survived the less rigorous Ig-Aa treatment. Any treatment exposed to *Isochrysis galbana* rotifers, early or late, experienced higher survival than the *Alexandrium andersoni* fed rotifer treatment, and any treatment exposed to *A. andersoni* fed rotifers experienced a delay in development. This may again be consistent with the assertion that the impact of toxin-containing algal fed rotifers is due more to nutritional considerations than to toxicity, although again, the latter cannot be entirely ruled out. The bottom line is that if late stage larvae encounter toxic algal conditions in which they either ingest the algae directly or via an intermediate heterotrophic prey, their fate is unlikely to be affected by a previous history of such encounters.

Larval crabs encountering a bloom of toxin-containing algae will be exposed to a complex prey environment. The zoeae would likely be faced with consuming the algae directly or ingesting heterotrophic prey that itself had ingested the toxin-containing algae. If the larvae were exposed only to the toxin-containing algae and heterotrophic prey that had

ingested this algae, these prey sources might not be nutritionally sufficient to support normal development. Depending on hatching of larval crabs and timing of harmful algal blooms, many zoeae from winter hatching crab species may not encounter toxin-containing algae until the later zoeal stages, if at all. Again due to the nutritional deficiencies of potential heterotrophic prey of zoeae that have ingested toxin-containing algae, exposure to these prey sources later in development would not have an increased negative effect compared to their effects on freshly hatched crab larvae that are exposed to toxin-containing prey sources. Natural prey exposure times are difficult to determine and lab experiments with larval crab feeding are generally exposing larvae to optimal feeding (i.e. continuous exposure to prey). In nature crab larvae will be exposed to areas, or patches, of higher food source densities that will have differing concentrations of prey, combinations of prey, and varying sizes of prey patches. Determining the differences between laboratory and natural prey exposure proves to be difficult and discrepancies between the two situations are unlikely to be resolved without modeling of various prey exposure scenarios.

It is more likely that as crab larvae enter the water column, exposure to numerous prey sources, such as algal species (both toxic and nontoxic), detritus, holoplankters, and other invertebrate larvae, will occur in varying patches. If among these prey sources, the larval crabs ingest a mixed diet, with some source of nutritionally sufficient animal prey, normal survival and development can be supported through the zoeal stages. If crab larvae ingest these nutritionally insufficient prey, which cause delayed development, this can be overcome if a nutritionally sufficient prey source is subsequently ingested.

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