ROTIFER GROWTH UNDER ASTAXANTHIN ENRICHMENT

A Thesis
Presented to
The Academic Faculty

by

Emma Jewell Siegfried

In Partial Fulfillment
of the Requirements for the Degree
Biology in the
School of Sciences

Georgia Institute of Technology
December 2017

ROTIFER GROWTH UNDER ASTAXANTHIN ENRICHMENT

Approved by:

Dr. Terry W. Snell, Advisor
School of Biology
Georgia Institute of Technology

Dr. Frank Stewart
School of Biology
Georgia Institute of Technology

Date Approved: May 2, 2017

ACKNOWLEDGEMENTS

I would like to thank Sustainable Aquatics, for their support during this experiment, providing all of the astaxanthin products used, and providing the space and the resources for the mass culture and population density experiments.

TABLE OF CONTENTS

LIST OF FIGURES

Page

SUMMARY

Rotifers and astaxanthin both play an important part in the aquaculture industry. Rotifers are used as a substitute for copepods, the main source of food for larval fish in natural systems, due to the ease with which they can be cultured. Astaxanthin is a carotenoid and antioxidant which brightens the coloring of fish and improves fish health. Rotifers are believed to be a method through which astaxanthin can be bioencapsulated and vectored to larval fish. As a result, it is important to understand the effect of astaxanthin on rotifers themselves. This experiment uses a multitude of different protocols to determine how different astaxanthin compounds effects rotifers on both the individual and population levels. Reproductive tables and fluorescent imaging were used to assess the health of individual rotifers; population density measurements in mass cultures were used to assess rotifer population health. The reproductive ability of rotifers was significantly different from control under multiple astaxanthin treatments. Astaxanthin enrichment also created a higher stable population density in the mass cultures. The fluorescent imaging showed that the rotifers reached peak astaxanthin concentration within the rotifer gut after 3 hours, and but concentration returned to control levels within 24 hours of removal from astaxanthin. These results all point to the fact that astaxanthin helps to increase rotifer health and fitness, and that these rotifers could be used as a vector for astaxanthin to larval fish.

CHAPTER I

INTRODUCTION

Astaxanthin has come to play a large role in the aquaculture industry. Astaxanthin is an antioxidant and carotenoid used in salmonids, crustaceans and other common fishery species to provide their natural-looking reddish coloration, a quality which increases appeal to buyers (Higuera-Ciapara et al. 2006). More significantly, astaxanthin has been shown to increase both the growth and survival of fish fry of the species *Salmo salar* (Christiansen et al. 1995). These results also suggest that astaxanthin is a precursor to vitamin A (Christiansen et al. 1995). Astaxanthin also decreases the amount of liver damage done over time and an improvement in liver structure (Nakano et al. 1995). Nakano et al. also suggest that treatment with astaxanthin has positive metabolic effects on fish populations and overall fish health (1995).

In natural systems, astaxanthin builds up in algae where it then is taken into the rest of the food chain (Lorenz and Cysewski 2000). Importantly for production, astaxanthin has been shown to build up in *Haematococcus pluvialis* (Lorenz and Cysewski 2000). On top of this, astaxanthin naturally reaches fish larvae through feeding on copepods, their most common food source. These copepods produce astaxanthin depending on the concentration and type of feed they are receiving (Andersson et al. 2003). While copepods are what larval fish consume most abundantly in natural settings, the culture of copepods for aquaculture has proven to be difficult.

As a result, rotifers play a large role in the aquaculture industry. For the first part of life, larval fish only consume live feeds before being moved onto a dry feed diet. Rotifers have become a more common live feed as they can be easily kept in mass

culture, unlike copepods. Copepods have been shown to produce carotenoids throughout their development (Lotocka and Styczynska-Jurewicz 2001), a quality which rotifers do not possess, and result in rotifers having a lower nutritional value in comparison to copepods. Multiple studies have been done to see how carotenoids affect rotifer life, and to determine if enrichment is a viable option for the transfer of carotenoids to larval fish. One study found that in mass cultures enriched with lutein, a vitamin A and astaxanthin predecessor, a shorter incubation time resulted in a higher concentration of lutein in the rotifer gut, and over time the amount of carotenoids in the rotifer body decreases (Bergeijk et al. 2013). This result also suggests that lutein passes through the gut, and is not incorporated into rotifer tissues (Bergeijk et al. 2013). Dominguez et al. found similar results when astaxanthin was used instead of lutein. In this experiment, the astaxanthin in the rotifer body was higher after 2 hours of enrichment than after 24 hours (2005). They also found that only one-third of the astaxanthin in the rotifer remained in the body when the rotifer underwent 24 hours of starvation post astaxanthin enrichment – again suggesting that astaxanthin is not accumulated in the rotifer tissues (Dominguez et al. 2005). Larval fish of the species Red Sea Bream (*Pagrus major*)
and Tiger Puffer (*Takifugu rubripes*) fed with enriched rotifers also showed higher survival than a control group, and it is possible that with astaxanthin additives, healthier larvae are produced (Yagi et al. 2001).

Fish in closed aquaculture systems do not have access to astaxanthin naturally (Higuera-Ciapara et al. 2006), and as a result, it is necessary to add astaxanthin to their diets through their feeds. Without enrichment themselves, rotifers provide are inferior in providing nutrients to fish in comparison to copepods (Hamre 2016). As a result,

enrichment with astaxanthin could be important for rotifers to reach peak nutritional value.

This experiment follows multiple paths of questioning involving how astaxanthin affects rotifer life. The effect of astaxanthin on rotifer population growth, reproduction, and stress resistance would be very important, as rotifers present a possible pathway to provide fish species with astaxanthin. The effects of astaxanthin on rotifer populations and individuals was assessed using (1) reproductive tables to determine differential reproductive rates under varying astaxanthin enrichment concentrations, (2) fluorescent imaging of the rotifer gut to determine if astaxanthin is incorporated into rotifer tissues after ingestion, and (3) population density measurements in mass cultures to assess possible maximum stable population densities and reproductive rates. With our experimentation, we expect to see an increased reproductive rate in individuals treated with astaxanthin, as well as higher population density in mass cultures. We also expect to see increased fluorescence in astaxanthin treated rotifers in comparison to non-treated rotifers.

.

CHAPTER II

BACKGROUND

In natural systems, copepods are the main feed of larval fish. One particularly important quality of copepods is that they naturally produce astaxanthin, an important antioxidant and carotenoid (Lotocka and Styczynska-Jurewicz 2001, Higuera-Ciapara et al. 2006). While copepods would be ideal for use in the aquaculture industry, they can be difficult culture, and as a result, rotifers are the main methods of feed for larval fish in aquaculture systems. Rotifers often do not provide complete nutrition to larval fish, and some aquaculturists enrich rotifers using different compounds and enrichment mixtures (Hamre 2016).

Notably, rotifers can be enriched specifically with astaxanthin and astaxanthin-like substances (Dominguez et al. 2005, Bergeijk et al. 2013). Bergeijk et al. used HPLC to determine the amount of lutein, an astaxanthin-like substance, accumulated in both the gut and tissues of rotifers when cultured in an enrichment over both short and long time spans (2013). Rotifers enriched over shorter time spans were shown to have higher concentrations of lutein in the gut in comparison to those enriched over longer time periods (Bergeijk et al. 2013). In addition, rotifers enriched with lutein did not incorporate the compound into their tissues (Bergeijk et al. 2013). A study similar to the Bergeijk et al. study was done by Dominguez et al. (2005). This study also used HPLC to determine the concentrations of astaxanthin, instead of lutein, in the gut and rotifer tissues after different enrichment times (Dominguez et al. 2005). This experiment found similar results to the lutein experiment – that astaxanthin accumulated to higher concentrations in the gut after shorter enrichment times in comparison to longer

enrichment times, and that astaxanthin is not being built up in rotifer tissues (Dominguez et al. 2005).

Other previous research involving astaxanthin and rotifers mainly focuses on lifespan extension and does not focus solely on astaxanthin itself. One study found that the lifespan of rotifers is only significantly extended by a small number of red algal extracts (Snare et al. 2013). This experiment was done using life tables where rotifers were placed in treatment and scored daily to create a life table (Snare et al. 2013). These life tables were then compared to controls to determine lifespan extension effects (Snare et al. 2013). A similar experiment was done using antioxidants and antioxidant combinations (Snell et al. 2012). In this study, no singular antioxidant was found to increase rotifer lifespan, but a few combinations of antioxidants did increase lifespan (Snell et al. 2012). Astaxanthin is both a red algal extract and a powerful antioxidant and could have been included in these papers, although it was not directly named. Since no difference was found in terms of lifespan extension, it is important to look at other possible effects of astaxanthin on rotifers.

Overall, the importance of understanding the impacts of astaxanthin on rotifer cultures does not lie on the rotifers themselves but rather their role as a food source to larval fish. Astaxanthin enrichment of rotifers is a method of transferring astaxanthin to larval fish. Astaxanthin plays a significant role on multiple characteristics of life in larval fish. Studies have shown that astaxanthin increases growth rate, improves coloration and positively impacts overall fish health (Christiansen et al. 1995, Nakano et al. 1995, Yagi et al. 2001).

Generally, most research looking at the interaction between astaxanthin and rotifers quantifies the accumulation of astaxanthin in the gut and tissues of the rotifers using HPLC specifically for the use of these rotifers as feed for larval fish (Dominguez et al. 2005, Bergeijk et al. 2013). While most studies surrounding aquaculture look at the impact of the astaxanthin on fish, the impact of astaxanthin on rotifers is the main focus of my study, specifically looking at the reproductive effects, stress resistance effects, and the population density effects of treating rotifers with astaxanthin. The methodology I will be using is more similar to the methods used for testing the lifespan extension effects where life tables were used in comparison to the HPLC generally used in experiments involving rotifer accumulation of astaxanthin.

CHAPTER III

METHODOLOGY

Rotifer Cultures and Astaxanthin Treatments

Each experiment was performed on rotifer cultures of the species *Brachionus manjavacas* (Russian strain). Population growth experiments were started from existing serial cultures of *B. manjavacas* maintained at Sustainable Aquatics. For all other experiments, neonates were hatched from *B. manjavacas* resting eggs prior to each experiment. Resting eggs were hatched in 15ppt ASW (artificial salt water) at 25°C under constant light.

Astaxanthin was extracted from *Haemotococcus* algae by Sustainable Aquatics using a proprietary milling process. In this experiment astaxanthin enrichments were created with three different types of astaxanthin products. The first was created with Sustainable Aquatics Astaxanthin, with a 3% make up of astaxanthin in the product. Two different types of stock solutions were created; the first in DMSO and the second in ASW with glass bead homogenization both at a concentration of 28 mg/mL. The second compound was 1.1% astaxanthin, and was a low concentration Extracted *Hp* Biomass. This Extracted *Hp* Biomass stock solution was created at a 28 mg/mL concentration in DMSO. Finally, the third compound was a 2.6% Astaxanthin Oleoresin product. Stock solutions with the Astaxanthin Oleoresin were created at 28 mg/mL in DMSO. Controls for experiments conducted with DMSO were treatments of equal volume of DMSO only.

Reproductive Range Finding Experiments

To determine the viable range through which rotifers are affected by astaxanthin enrichment, reproductive range finding experiments were done. Individual rotifers were

placed in every well of a 24-well plate filled with 1 mL of *Tetraselmis suecica* at a concentration of 2×10^5 cells/mL. Four wells were treated with each enrichment. This experiment used treatments of 0.5% DMSO, 1 µM, 2µM, 3.9 µM, 5.2 µM and 6.8 µM Sustainable Aquatics Astaxanthin. Each treatment was only applied on the first day of the range finding experiment, and the 0.5% DMSO enrichment is used as a control because the astaxanthin is in a DMSO solution. The number of offspring of each rotifer was counted and then removed from each well daily for 72 hours. The plates were kept at 22˚C in low light conditions.

A second reproductive range experiment was done to determine if it was possible to still treat rotifers with Sustainable Aquatics astaxanthin without the use of an organic solvent. The treatments were a DMSO control, astaxanthin in DMSO, and astaxanthin homogenized in artificial salt water (ASW) using glass beads. The concentration of astaxanthin in each of these enrichments was 4µM. Rotifers were placed in each well of a 24-well plate filled with 1 mL of *T. suecica* at a concentration of 2×10^5 cells/mL. Then each of these treatments was applied to 4 of the wells at the beginning of the experiment. Rotifer offspring were counted and removed from each well daily for 72 hours.

Finally, a third reproductive range finding experiment was done to determine the range at which rotifers should be treated with the Astaxanthin Oleoresin compound. For this range finding experiment, the treatments included a DMSO control and concentrations of 0.25 µM, 0.5 µM, 1 µM, 2 µM and 4 µM of Astaxanthin Oleoresin. Each treatment, similarly to the first range finding experiment, was only applied on the first day of the experiment, and the number of offspring were counted and then removed

from each well daily for 72 hours. Again, the plates were kept at 22˚C in low light conditions.

Population Growth Experiments

The cultures from each experiment were set up using rotifers from existing cultures that were removed, washed and inoculated in 10L and 173L cultures at an approximate density of 150 rotifers per milliliter. The cultures were created in 26.5 ppt saltwater at 20.5˚C. For this experiment, the population density of each culture was measured every 24 hours to determine population growth. To count population density, one milliliter was sampled from each treatment and the rotifers were counted in a hemocytometer using a compound microscope. Rotifers were fed from a mixture of 2 L seawater, 1 L of algae paste consisting of concentrated *Nanochloropsis*, 110g ClorAm-X powder (an ammonium and chlorine neutralizing reagent), and 6.2 g of Seachem marine pH buffer. The amount of this mixture fed to the rotifers was calculated using the formula:

$$\mathrm{Feed(mL)} = 0.0016\mathrm{V(R} + 2\mathrm{E)}$$

In this formula, V is the volume of the culture in L, R is the rotifer density in individuals/mL, and E is the egg density in eggs/mL. The rotifers were fed the calculated amount twice per day over 3 feedings, with 2/3 the full amount being fed in the morning, 1/3 the amount being fed in the afternoon and the full amount being fed in the evening.

In this experiment, population density was measured under three different enrichments – control, 0.3% DMSO, and 0.1 μM Astaxanthin Oleoresin. All of the treatments were incubated in variable temperature and lighting conditions. The room temperature was kept at approximately 25˚C for the 10L treatments and 20.5˚C for the

173L treatments. Lighting followed daily 12 hour light cycles and approximately 1/3 of the water was changed every two days.

The 10L cultures grew for 20 days and the 173L cultures grew for 24 days. The eight 10L cultures were split into the three treatments as follows – two control treatments, two 0.3% DMSO treatments and four 4μM Astaxanthin Oleoresin treatments. There were only three 173L cultures – one of each treatment. The 0.3% DMSO and Astaxanthin Oleoresin treatments were re-dosed daily to maintain a 4μM concentration.

Reproductive Assays

Four 24-well plates were filled with 1 mL of *T. suecica* in 15ppt ASW at a concentration of 2×10^5 cells/mL. Once the plates were filled, 1 rotifer was placed in each well. Each plate was then placed under one of the following enrichments: a control, 4μM Sustainable Aquatics astaxanthin, 4μM Stustainable Aquatics astaxanthin with a daily 4μM re-dose, and a 10μM Sustainable Aquatics astaxanthin. All astaxnathin treatments were prepared in 15ppt ASW. Each treatment was applied on the first day of the treatment, and the re-dose treatment received an additional dose every 24 hours. The rotifers were incubated under low light at 22˚C. Each day the number of offspring were counted and then removed from each well, and mortality was recorded daily until all animals were dead. On day 6, the individuals were transferred to new 24-well plates and with a re-dose of enrichment.

As a continuation of this experiment, plates were made using lower concentrations to determine if it was possible to reach the same difference using lower concentrations of treatments. The second experiment used control, 0.5μM astaxanthin, 1μM astaxanthin, and 2μM astaxanthin treatments. These treatments were also prepared

in 15ppt ASW. Offspring were counted and removed daily for 6 days. This low concentration experiment ran for only 6 days, as the results from the previous pointed to a significant difference between the enriched rotifers and the controls occurring before the first 6 days of experimentation.

Astaxanthin Uptake and Gut Passage Visualization

Astaxanthin is a naturally fluorescent compound, and as a result can be visualized under an Alexa filter. This ability can be used to visualize the uptake of astaxanthin into the rotifer gut. For the uptake experiment, rotifers were hatched and then put into 4μM SA astaxanthin in DMSO. The rotifers were removed from the treatments at the time points – 0 hours, 0.5 hours, 1 hour, 1.5 hours, 3 hours, 24 hours and 48 hours. These rotifers were anesthetized with 1 mL of soda water and fixed with 40 μL of 20% formalin. After being fixed, rotifers were placed on a slide and imaged using a Zeiss Imager Z_1 microscope at 20X magnification with an Alexa 568 nm excitation filter and 630 ms exposure. Images of 20 rotifers were collected for each treatment, and average pixel intensity was measured using ImageJ.

To measure the gut passage of astaxanthin, rotifers were hatched and then put into three treatments – a control, 0.3% DMSO and 4μM Sustainable Aquatics Astaxanthin in DMSO solution. The rotifers remained in the 3 treatments for 3 hours, a time previously discovered for the rotifers to reach saturation of astaxanthin. After 3 hours, the rotifers were removed from treatment, washed and placed in 15ppt ASW. At the time points, 0 hours, 0.5 hours, 1 hour, 2 hours, 4 hours, 6 hours and 24 hours, rotifers were removed, fixed, imaged and analyzed using the same methods for the uptake experiment.

Oxidative Stress Resistance

Juglone is a compound used to induce oxidative stress (Snell and Johnston 2014). Hatchlings were placed in the enrichments of 4μM astaxanthin and a control for 4 days in 6-well plates. These plates were fed $6x10^5$ cells/mL *T. suecica* and treated with 20uM 5-fluoro-2-deoxyuridine (FDU) to prevent asexual egg hatching (Snell et al. 2012). Animals were then transferred into 24-well plates with 0.1μM juglone in 15ppt ASW. Each well contained 10 animals. These treatments were placed in low light at 25˚C. The number of surviving animals was counted at both 24 and 48 hours.

Swimming Speed

Between 1 and 5 rotifers were pipetted onto a slide in 12 μL of ASW. The PixeLink M1C-SE camera and the "Capture OEM" software were used to capture video at a magnification of 1X. The video was captured at 14 frames per second for 30 seconds. Video was taken of at least 15 animals. The video then analyzed in the program Tracker to determine the swimming velocity of the rotifers. In this experiment, the rotifers were grown in a control treatment, a 4 μM Sustainable Aquatics Astaxanthin treatment and a 4 μM Sustainable Aquatics Astaxanthin treatment with a 2 μM daily redose. All treatments were fed $6x10^5$ cells/mL *T. suecica* and treated with 20uM FDU. Their swimming speeds were measured on day 0, 1, 6 and 10.

Statistical Analysis

Reproductive range finding experiments were analyzed using a student's t-test. Population growth experiments were analyzed using a matched pairs analysis. Reproductive life tables and stress tests were analyzed using a Dunnett's test with

control. For the imaging analysis experiments, trend lines were fit to each time series using a logarithmic regression.

CHAPTER IV

RESULTS

Reproductive Range Finding Experiments

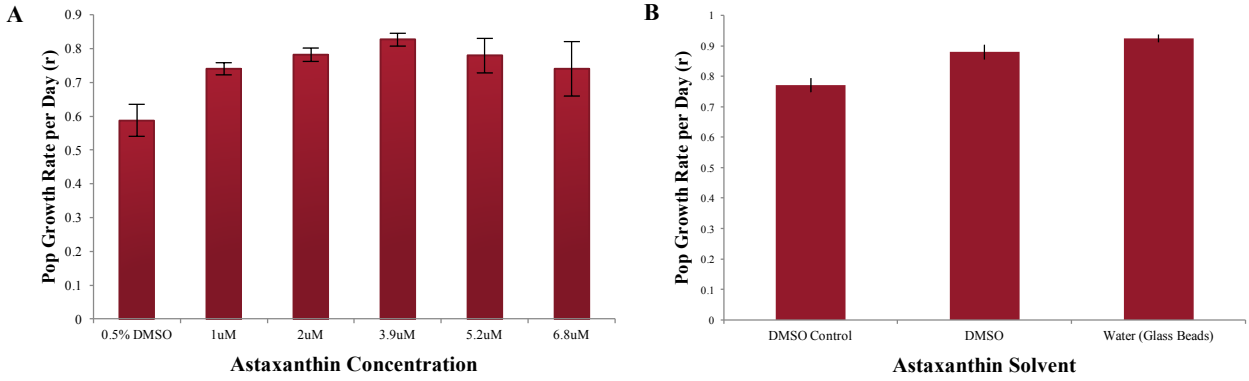

Figure 1. Population growth rate of rotifers at 72 hours in SA Astaxanthin treatments (A) at different concentrations and (B) at 4uM in different

During the first reproductive range finding experiment, where rotifers were only treated with SA Astaxanthin dissolved in DMSO, the population growth rate per day ranged from 0.7401 to 0.8266 rotifers per day and the 0.5% DMSO control showed growth of 0.5874 rotifers per day when specifically looking at day 3 reproductive data (Figure 1a). All the SA Astaxanthin enrichments were significantly different than the 0.5% DMSO enrichment, but the one which showed the most growth was the 3.9µM treatment (p=0.001). As a result, a 4µM SA Astaxanthin concentration was used throughout many of the rest of the experimentation done for this paper.

When testing the effects of dissolving SA Astaxanthin in solvents other than DMSO, there was significant

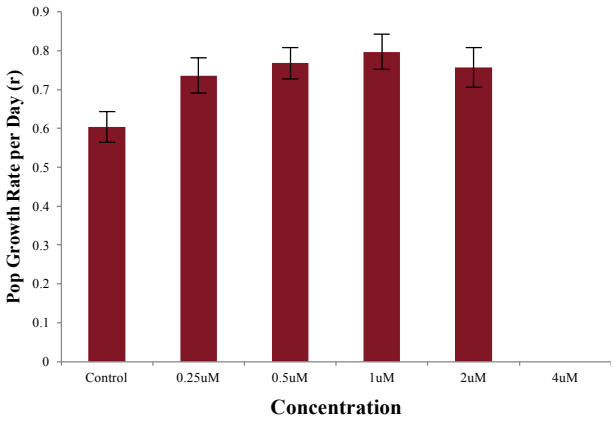

Figure 2. Population Growth rate of rotifers at 72 hours in various Astaxanthin Oleoresin conentrations.

increase in the growth rate from the control by both the SA Astaxanthin in DMSO solvent and water. The population growth rate per day for the SA Astaxanthin in DMSO solvent was 0.8799 rotifers per day, while the population growth rate for the SA Astaxanthin in water which was mixed using glass beads was 0.9212 rotifers per day (Figure 1b).

For the Astaxanthin Oleoresin range finding, there was a significant increase growth rate from control by the 0.25 µM (p=0.047), 0.5 µM (p=0.014), 1 µM (0.009) and 2 µM (p=0.038) treatments, and no difference from control in the 4 µM treatment. The largest difference was due to the 1 µM treatment, and resulted in this value being used for subsequent experimentation (Figure 2).

Population Growth Experiments

In the 173 L cultures, population growth was higher in astaxanthin enriched cultures than in cultures enriched with only DMSO as well as the control cultures. The maximum population density of the astaxanthin enriched culture reached 175% of the maximum population density of the DMSO only enriched culture and 157% of the maximum population density of the control culture (Figure 3).

In the 10 L cultures, similar results were obtained where the astaxanthin enriched cultures reached a higher population density and then maintained that density for a longer period (Figure 4). The mean rotifer density in the DMSO only enrichement was 121 rotifers, and the mean rotifer density in the astaxanthin

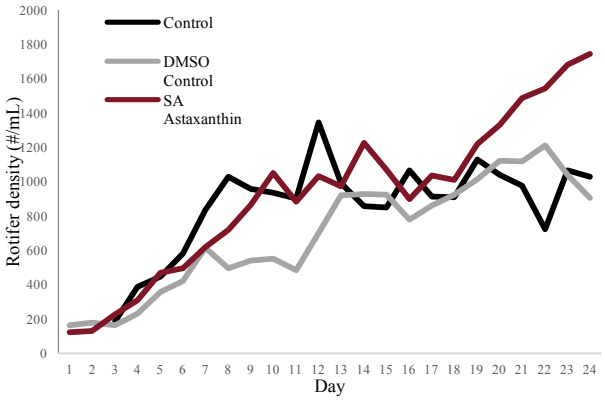

Figure 3. Rotifer Density in 173 L cultures through time in a control, DMSO control and 4uM astaxanthin enrichment treatment.

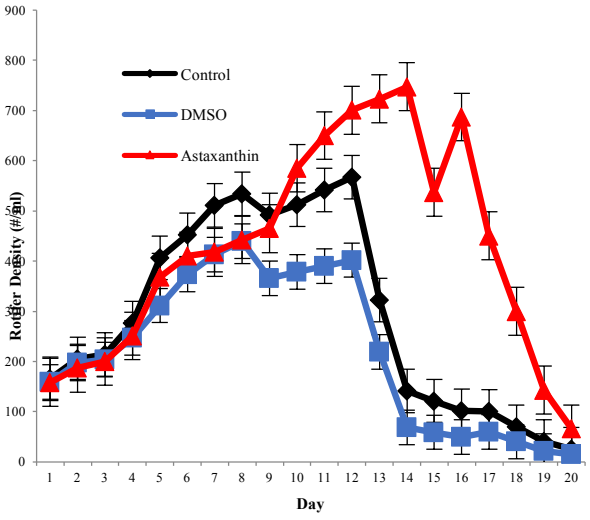

Figure 4. Rotifer density in 10 L cultures through time in control, DMSO control and 4uM astaxnathin treatments.

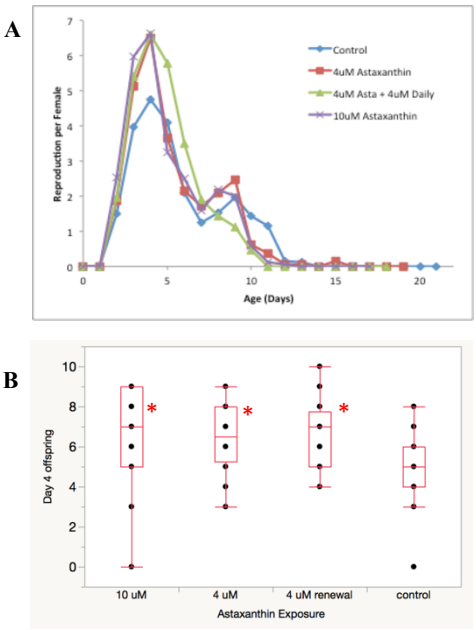

Figure 5. The reproduction per female (A) in a reproductive life table test with astaxanthin treatments and (B) the average number of offspring in each treatment on day 4.

enrichment was 424 rotifers. Using matched pairs analysis, these values are significantly different ($p<0.001$).

Reproductive Life Tables

The reproductive life tables measured reproduction as well as lifespan. There was no difference between the lifespan of rotifers treated with 0.3% DMSO and each of the astaxanthin enrichments. While there was no difference between the lifespans of each of the treatments, there was a difference between the reproduction of the rotifers under the astaxanthin treatments and the control (Figure 5a). This difference can specifically be seen in the early life stage. On day 4 of the experiment, where there was significant difference between the control and the 10µM astaxanthin ($p=0.004$), 4µM astaxanthin ($p=0.005$), and the 4µM astaxanthin with daily retreatment and the control ($p=0.008$). This difference can be represented using a box and whisker plot (Figure 5b).

The second reproductive experiment was conducted at lower concentrations. Offspring was counted daily, and total reproduction was analyzed on day 6 (Figure 6). The mean offspring for the 0.5µM astaxanthin concentration was 13.7 rotifers, and the mean offspring in 1uM astaxanthin was 14.4. Mean offspring for these two treatments was not significantly different than the mean offspring for the control at 12.7 rotifers. However, the mean offspring for the 2µM and 4µM concentrations are significantly different than the control mean offspring number, with the mean offspring being 20.3 ($p < 0.001$) and 19.3 respectively ($p<0.001$).

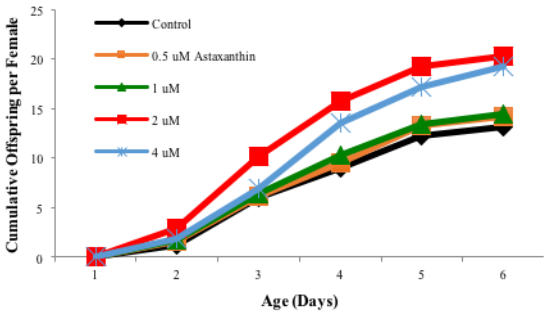

Figure 6. The cumulative offspring through day 6 at lower concentrations of astaxanthin. Both the 2µM and 4µM astaxanthin treatments significantly enhanced reprodution.

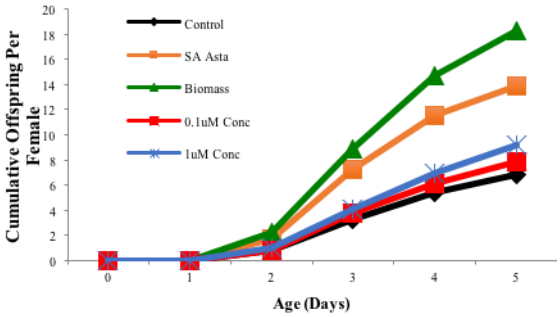

Figure 7. The cumulative offspring through day 5 using different concentrations of multiple different astaxanthin products. The Extracted *Hp* Biomass and SA Astaxanthin show significantly enhanced reproduction.

The third reproductive test was done with multiple astaxanthin types at their individual optimal concentrations and other concentrations used in previous experiments. These experiments were grown for 5 days and analyzed on the final day (Figure 7). The mean offspring from the 0.1 µM Astaxanthin Oleoresin and 1 µM Astaxanthin Oleoresin were 7.63 and 8.71 respectively, and were not significantly different than the control which had a mean offspring count of 6.63. On the other hand, the 2.6 µM Extracted *Hp* Biomass and the 4 µM SA Astaxanthin had mean offspring of 18.13 and 13.92, which were significantly different than the control ($p<0.001$).

Astaxanthin Uptake and Gut Passage Visualization

In the astaxanthin uptake experiment, the pixel intensity from the fluorescence of astaxanthin was plotted against the time (Figure 8a). The values followed a logarithmic pattern and were fitted with a logarithmic trend line (R=0.77). Pixel intensity, and therefore astaxanthin uptake, reaches its maximum at 3 hours, and further treatment provides no additional benefit.

The gut passage visualization follows a similar pattern, but rather than a logarithmic pattern, the gut passage data follows an exponential pattern and is fitted with an exponential trend line (Figure 8b). The trend line follows the equation, $y = 2.0008e^{-0.035x}$ which has an R^2 value of 0.63. After being removed from astaxnathin treatment, fluorescence begins dimishing within 30 minutes, and is almost completely back to control levels at 24 hours.

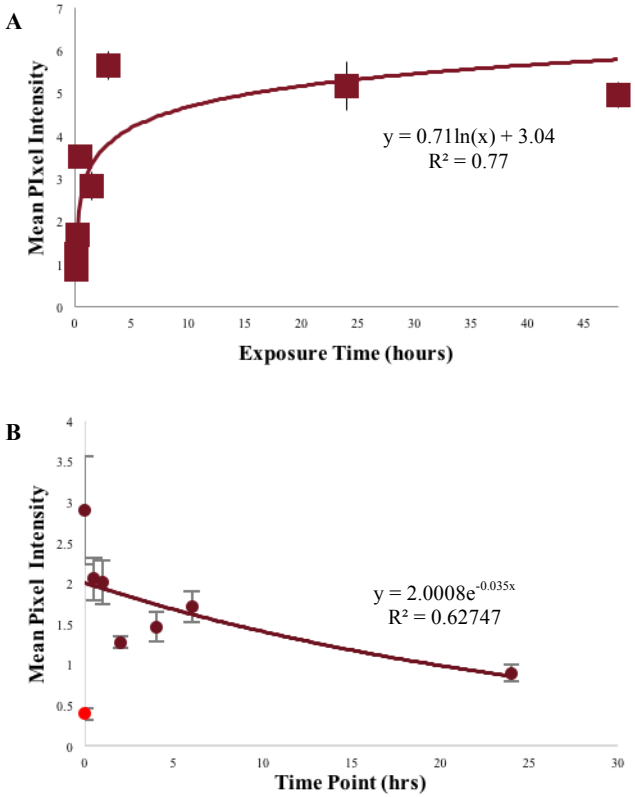

Figure 8. The mean pixel intensity of the rotifers measured under the Alexa filter after being exposed to astaxanthin for different lengths of time (A) and after being removed from astaxanthin exposure for different lengths of time (B).

Oxidative Stress Resistance

Astaxanthin showed the ability to overcome oxidative stress when the rotifers were treated with juglone at each time point measured. The percent of population surviving was significantly higher in rotifers treated with 4uM astaxanthin

before being treated by juglone than control animals treated with juglone (Figure 9). The percent of rotifers surviving decreased from 95 to 41.67% in the astaxanthin enrichment over the first 72 hours, while the percent of rotifers surviving decreased from 85 to 26.67% in the control treatment.

Swimming Speed

The largest difference in swimming speeds between treatments occurred on day 6. The 4µM astaxanthin treatment and the control were not significantly different on day 6, but the 4µM astaxanthin with a 2µM daily redose showed significantly increased swimming speed on day 6 ($p=0.000$). There were no significant differences between any treatments on days 1 and 10.

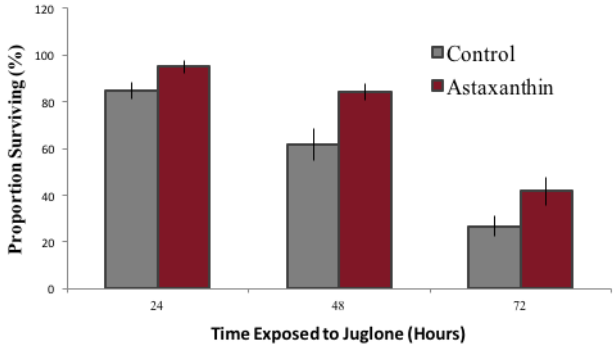

Figure 9. The proportion of individuals surviving at 24, 48 and 72 hours under 0.1µM juglone exposure of a control treatment and 4uM astaxanthin treatment. There is a higher proportion of individuals surviving in the astaxanthin treatment at all time points.

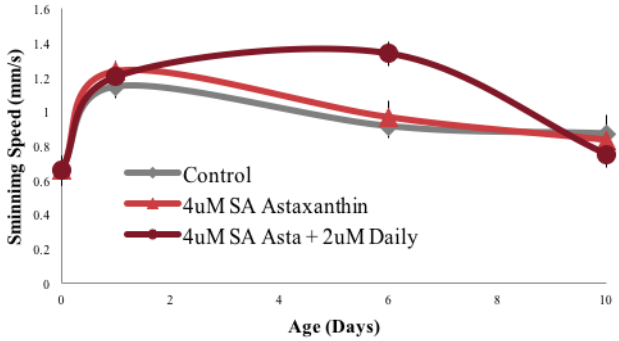

Figure 10. The swimming speed of rotifers as they age under two different astaxanthin treatments in comparison to a control treatment. The only significant difference in the swimming speed occurred on day 6 where the swimming speed of the rotifers under the 4µM astaxanthin with a 2µM daily redose treatment was increased.

CHAPTER V

DISCUSSION

Through these experiments, the use of astaxanthin as an enrichment to improve the growth and health of rotifers was analyzed. Within the range finding experiments, the population growth per day was found to be the highest at a concentration of 4 µM SA Astaxanthin, 1 µM Astaxanthin Oleoresin, 400 µg/mL Extracted *Hp* Biomass. This led to these concentrations being used throughout the rest of the experimental procedures. Along with this, SA Astaxanthin dissolved in both DMSO and artificial salt water (ASW) significantly increased the population growth rate in comparison to the control. This is notable because it shows that there is no difference in bioavailability or bioactivity between SA astaxanthin dissolved in DMSO or ASW.

One of the major results from this study is the effect of astaxanthin on mass cultures of rotifers and the ability of these rotifers to bio-encapsulate astaxanthin. Through this bio-encapsulation, commercially raised fish can have astaxanthin added to their diet at a younger age. Often in aquaculture, rotifers are kept in mass cultures. Both 10L and 173L mass cultures show that cultures treated with just 0.1µM astaxanthin reach higher densities and remain stable for longer. This suggests there is an increase in the ability of the rotifers to reproduce and their ability to overcome possible biological limitations including oxygen and nutrition limitations.

Oxygen limitation is commonly referred to as hypoxia and can cause a multitude of problems in an aquaculture setting. Hypoxia is an important factor to be aware of in aquaculture due to its numerous negative effects on aquatic species. In juvenile turbot, hypoxia leads to a decrease in feeding intensity and growth as well as temporary

reduction in nitrogen excretion and oxygen consumption (Pichavant et al. 2000). Hypoxia in aquaculture becomes a problem when tanks are overpopulated with any kind of species and the environments become nutrient limited (Ozaki et al. 2010).

In addition, nutrient limitations can occur when populations of organisms in aquaculture are not being fed enough to suffice for the whole population size (Ozaki et al. 2010). Nutrient limitation is an additional environmental stress that can result in nutrient deficiencies that can also have numerous effects on aquatic species. This nutrient limitation suppresses reproduction and also increases lifespan, resulting in a stable population size (Ozaki et al. 2010). In addition, the rotifer species *Brachionus plicatilis* caloric restriction enables them to better withstand hypoxic conditions (Ozaki et al. 2010). In addition, studies have found that adding oxygen specifically to rotifer cultures leads to higher population densities than adding air (Park et al. 2001).

The idea that astaxanthin increases tolerance of oxidative stress is supported by the observation that astaxanthin enriched rotifers are resistant to juglone, a compound used to experimentally induce oxidative stress. A greater fraction of hatchlings initially exposed to astaxanthin survived better than controls when removed from astaxanthin and then exposed to juglone. Together these results suggest that astaxanthin increases rotifer resistance to oxidative stress and could be beneficial for managing rotifer mass cultures in aquaculture. In addition, rotifers were resistant to starvation when treated with astaxanthin (Unpublished Data). This suggests that astaxanthin, in addition to increasing oxidative stress resistance, also increases tolerance to starvation.

In addition to looking at the effect of astaxanthin enrichment on the populations it is important to determine how astaxanthin effects the health of individual rotifers, as

having healthy rotifers is important for the rotifers to be available to the fish themselves. The reproductive life tables done using astaxanthin enrichments demonstrate a significant increase in the reproductive rate of rotifers in their early life. While reproduction in the first 4 days is significantly higher with astaxanthin, the individual reproduction per female over the entire lifespan is not significantly different from control. For the SA Astaxanthin the highest early life reproduction occurred under the 4 μM treatment, for the Astaxanthin Oleoresin at the 1 μM treatment, and for the Extracted *Hp* Biomass at a 400 μg/mL treatment. For the SA Astaxanthin no significant difference was found between 2 μM and 4 μM dosages. The highest overall reproduction was found in the Extracted *Hp* Biomass treatment. This suggests that there is a benefit to the biological compounds in *Haematococcus pluvialis* and not the astaxanthin alone.

Astaxanthin is a hydrophobic molecule, and tends to form an aggregate and fall out of solution in water (Lannibois et al. 1997). With an increased size, it is logical that it is more difficult for these aggregates to pass through membranes. Our results suggest that the Extracted *Hp* Biomass may counteract astaxanthin's hydrophobic qualities, and allow it to be more biologically available. This is the reason Extracted *Hp* Biomass would be more beneficial in an aquaculture because it would optimize the balance between cost and beneficial health effects.

The data also suggests astaxanthin uptake in rotifers saturates after 3 hours of exposure. As a result, only a 3 hour enrichment period is needed to maximize astaxanthin content in rotifers for larval feeding. Furthermore, astaxanthin is only retained in the rotifer tissues for a limited period of time. At 24 hours, the amount of astaxanthin built up in the rotifer body decreases back to near control values. Based on these results, rotifers

need to be fed to larval fish after approximately three hours of treatment to receive optimum astaxanthin treatment amount to larval fish. It is also possible that as astaxanthin is incorporated into rotifer tissues it is immediately broken down into other compounds which are not visualized easily. This differential sequestration of astaxanthin is also found in lobsters, where different methods of sequestering astaxanthin change the shell color of lobsters with the same physical amounts of astaxanthin (Wade et al. 2005).

Another measure of individual rotifer health is swimming speed. As rotifers age, swimming speed slows (Snell et al. 2012). After treatment under astaxanthin, this swimming speed does not decrease to the same level as control. Their smooth swimming behavior and moderate swimming speed of rotifers is possibly what makes them an ideal prey for larval fish (Buskey et al. 1993). At the same time, Buskey et al. include data which show that rotifers have higher swimming speeds than multiple species of copepods nauplii, ciliates and dianoflagellates and lower speeds than branchiopod nauplii (1993). As rotifers already have a higher swimming speed, an increase in swimming speed with astaxanthin treatment, could make rotifers more visible to fish and would maintain the rotifers at a relatively "moderate" speed. This would make the rotifers easier to consume while also helping the process of the rotifers vectoring the astaxanthin to the larval fish.

Altogether, the results from the range finding, mass culture, reproductive life tables, uptake, gut passage and swimming speed experiments suggest an increased fitness in rotifers treated with astaxanthin. Of all the types tested, the astaxanthin in Extracted *Hp* Biomass seems to have the highest positive effect on the rotifers and would be recommended as the best astaxanthin treatment option for rotifers in aquaculture.

While this experimentation has significant implications in treatment of rotifers with astaxanthin and the effect that astaxanthin can have on the rotifer, there are additional experiments that would help us to better understand the effects of astaxanthin enrichment in the aquaculture industry. An important experiment to be done would be to feed astaxanthin-enriched rotifers to larval fish. Observing the survival, growth and coloring of these fish will help us to understand the efficiency of the rotifers acting as vectors of astaxanthin to larval fish. Additionally, understanding the amount of astaxanthin incorporated into rotifer tissue is not fully tested through the visualization experimentation done in this methodology. To corroborate the results found here, future studies will use HPLC to determine the exact amount of astaxanthin incorporated into rotifer tissues. Finally, since the population growth experiments were done with the astaxanthin oleoresin, it is important do a similar population growth experiment using the Extracted *Hp* Biomass, as it seems that this compound makes astaxanthin more bioavailable. An increase in bioavailability of astaxanthin could have an impact on the maximum population density as well as the length at which these high population densities can be maintained.

One part of the rotifer life cycle that was not studied here includes sexual reproduction of rotifers. Males and sexual reproduction in rotifers is induced in high stress situations, including but not limited to high population crowding, age, salinity and diet (Pourriot and Snell 1983, Gilbert 2004). Female rotifers can either be mictic or amictic; males are produced when mictic females produce unfertilized haploid eggs which hatch into males (Allan 1976). When the haploid eggs produced by mictic female rotifers are fertlizied by haploid males, resting eggs are produced (Allan 1976). Resting

eggs have an evolutionarily important role both in their production and their hatching. As resting eggs are the product of sexual reproduction, they allow for a higher amount of genetic variability in unfavorable environmental conditions along with the ability to escape these conditions (Pourriot and Snell 1983). As a result, understanding the impact of astaxanthin on resting eggs and resting egg production would help create a full understanding of how astaxanthin effects the rotifer life cycle.

CHAPTER VI

CONCLUSION

Astaxanthin is an important antioxidant and carotenoid in aquatic systems, and must be artificially given to fish in aquaculture facilities. Rotifers play a large role in the aquaculture industry as they are easy to culture and can be fed astaxanthin, and hopefully can act as a vector to transfer astaxanthin to fish during their larval stage. On top of this, it is important to understand the best astaxanthin compound which can be used to treat rotifers as well as the optimal concentration at which the treatment has an effect. This study found that astaxanthin has a positive effect overall on the populations density, reproductive output and health of rotifers. After all experimentation, the best compound to use for treatment of rotifers with astaxanthin was found to be the Extracted *Hp* Biomass sample, which is 1.1% astaxanthin, at a concentration of 400 μg/mL. One possible explanation for this being the best method of vectoring astaxanthin is that when astaxanthin is bonded with other molecules it is more bioavailable. Future studies will be important to determine if treating rotifers with astaxanthin is a possibility for vectoring astaxanthin to fish at a younger age and to continue to determine the effects of astaxanthin enrichment on the aquaculture industry.

REFERENCES

Allan, J. D. 1976. Life history patterns in zooplankton. The American Naturalist **110**:165-180.

Andersson, M., L. Van Nieuwerburgh, and P. Snoeijs. 2003. Pigment transfer from phytoplankton to zooplankton with emphasis on astaxanthin production in the Baltic Sea food web. Marine Ecology Progress Series **254**:213-224.

Bergeijk, S. A., J. A. Paullada Salmerón, A. I. López Pérez, J. Moreno, J. P. Canavate, and M. García-González. 2013. Lutein enrichment of the rotifer Brachionus sp. using freeze-dried Muriellopsis sp. cells. Aquaculture Research **44**:1724-1730.

Buskey, E. J., C. Coulter, and S. Strom. 1993. Locomotory patterns of microzooplankton: potential effects on food selectivity of larval fish. Bulletin of Marine Science **53**:29-43.

Christiansen, R., O. Lie, and O. Torrissen. 1995. Growth and survival of Atlantic salmon, Salmo salar L., fed different dietary levels of astaxanthin. First-feeding fry. Aquaculture Nutrition **1**:189-198.

Dominguez, A., M. Ferreira, P. Coutinho, J. Fábregas, and A. Otero. 2005. Delivery of astaxanthin from Haematocuccus pluvialis to the aquaculture food chain. Aquaculture **250**:424-430.

Gilbert, J. J. 2004. Population density, sexual reproduction and diapause in monogonont rotifers: new data for Brachionus and a review. Journal of Limnology **63**:32-36.

Hamre, K. 2016. Nutrient profiles of rotifers (Brachionus sp.) and rotifer diets from four different marine fish hatcheries. Aquaculture **450**:136-142.

Higuera-Ciapara, I., L. Felix-Valenzuela, and F. Goycoolea. 2006. Astaxanthin: a review of its chemistry and applications. Critical reviews in food science and nutrition **46**:185-196.

Lannibois, H., A. Hasmy, R. Botet, O. A. Chariol, and B. Cabane. 1997. Surfactant limited aggregation of hydrophobic molecules in water. Journal de Physique II **7**:319-342.

Lorenz, R. T., and G. R. Cysewski. 2000. Commercial potential for Haematococcus microalgae as a natural source of astaxanthin. Trends in biotechnology **18**:160-167.

Lotocka, M., and E. Styczynska-Jurewicz. 2001. Astaxanthin, canthaxanthin and astaxanthin esters in the copepod Acartia bifilosa (Copepoda, Calanoida) during ontogenetic development. Oceanologia **43**.

Nakano, T., M. Tosa, and M. Takeuchi. 1995. Improvement of biochemical features in fish health by red yeast and synthetic astaxanthin. Journal of Agricultural and Food Chemistry **43**:1570-1573.

Ozaki, Y., G. Kaneko, Y. Yanagawa, and S. Watabe. 2010. Calorie restriction in the rotifer Brachionus plicatilis enhances hypoxia tolerance in association with the increased mRNA levels of glycolytic enzymes. Hydrobiologia **649**:267-277.

Park, H. G., K. W. Lee, S. H. Cho, H. S. Kim, M.-M. Jung, and H.-S. Kim. 2001. High density culture of the freshwater rotifer, Brachionus calyciflorus. Hydrobiologia **446**:369-374.

Pichavant, K., J. Person-Le-Ruyet, N. Le Bayon, A. Sévère, A. Le Roux, L. Quéméner, V. Maxime, G. Nonnotte, and G. Boeuf. 2000. Effects of hypoxia on growth and metabolism of juvenile turbot. Aquaculture **188**:103-114.

Pourriot, R., and T. W. Snell. 1983. Resting eggs in rotifers. Hydrobiologia **104**:213-224.

Snare, D. J., A. M. Fields, T. W. Snell, and J. Kubanek. 2013. Lifespan extension of rotifers by treatment with red algal extracts. Experimental gerontology **48**:1420-1427.

Snell, T. W., A. M. Fields, and R. K. Johnston. 2012. Antioxidants can extend lifespan of Brachionus manjavacas (Rotifera), but only in a few combinations. Biogerontology **13**:261-275.

Snell, T. W., and R. K. Johnston. 2014. Glycerol extends lifespan of Brachionus manjavacas (Rotifera) and protects against stressors. Experimental gerontology **57**:47-56.

Wade, N., K. C. Goulter, K. J. Wilson, M. R. Hall, and B. M. Degnan. 2005. Esterified astaxanthin levels in lobster epithelia correlate with shell colour intensity: potential role in crustacean shell colour formation. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology **141**:307-313.

Yagi, M., K. Tachibana, T. Mishima, K. Hara, and M. Tsuchimoto. 2001. Effects of feeding of carotenoids supplement rotifer on splenocyte proliferation reaction of red sea bream larvae (Pagrus major) and tiger puffer (Takifugu rubripes). Suisanzoshoku (Japan).