Development of Yellowtail Kingfish Aquaculture in Western Australia: Removal of Barriers to Profitable Production

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AUSTRALIAN SEAFOOD COOPERATIVE RESEARCH CENTRE

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2011/754 Development of Yellowtail Kingfish Aquaculture in Western Australia: Removal of Barriers to Profitable Production.

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This project aimed to remove some key barriers and optimise some key inputs to yellowtail kingfish (YTK) production and improving the commercial viability of the development of a larger scale industry in Western Australia.

Objectives of Sub-Project 1 - Genetics:

1. To analyse genetic variation and pedigrees in existing broodstock, investigate options and make recommendations for how to best capture, maintain and effectively utilise genetic variation among cultured YTK in WA.
2. To undertake a more detailed population genetics study of Western Australian stock and existing captive broodstock.

Objectives of Sub-Project 2 – Larviculture:

3. To compare the differences between continuous and pulse feeding strategies on the cost of production and performance of YTK larvae.
4. To compare the performance of those YTK larvae reared on live food enriched on current best-practice enrichment products with those reared on an experimental diet that has been demonstrated through laboratory analyses to have superior fatty acid profiles and concentrations of other essential nutrients.

5. To compare the effect of new bacterial management approaches within larval rearing tanks in reducing malformation rates.
6. To exchange larviculture techniques between WA, NSW and SA.

All project objectives were achieved with the exception of 2 and 6. Whilst many samples of wild caught YTK were collected from the Perth and Mid-West regions of WA, an insufficient number from the south coast were collected in order to complete Objective 2 and the funds allocated to this objective were subsequently invested into expanding and improving the genetic database. Objective 6 could not be achieved as no suitable time could be found for all parties to conduct the proposed staff exchanges. A milestone variation was therefore approved in which ACAAR utilised the allocated funds to conduct an additional taurine dose-response trial and a larval rearing trial comparing the effect of rotifers enriched with different taurine concentrations.

OUTCOMES ACHIEVED

Sub-Project 1 – Genetics:

The outcome of this sub-project is the WA industry having a clear understanding of the relatedness of their limited F1 broodstock pool and a management tool and mating strategy to avoid inbreeding amongst these fish.

Sub-Project 2 – Larviculture:

Following this research, Australian hatcheries can now produce YTK larvae with significantly fewer rotifers (up to 65% less) without adverse effects on larval survival or growth, resulting in significant savings in hatchery operating costs.

Biochemical analyses demonstrating some key differences in the nutritional profiles of live foods enriched on different products demonstrated that the current industry standard diet Spresso is the best choice of the diets investigated, particularly in terms of its total and phospholipid profile.

Based on the significantly better phospholipid profiles in rotifers compared with *Artemia*, we recommend that industry investigate the potential benefits of extending

the rotifer feeding phase.

Whilst the successful supplementation of selenium in rotifers to copepod levels did not improve growth or survival, supplementation of this important element using the methods described in this study is likely to improve the oxidative status of YTK larvae and is therefore recommended.

We have provided the first indication that high taurine concentrations may be detrimental to the survival of YTK larvae and identified potential interactive effects between diet type, dose rate and enrichment time on live feed taurine levels that require further investigation. Our findings suggest that the requirement for taurine by pre-metamorphic larval fish is much lower than post-metamorphic juveniles and enrichment of live feeds to levels seen in juvenile fish diets can lead to significant reductions in survival. The findings can be utilised by industry or researchers to further refine and optimise the live food enrichment protocols for taurine and subsequently quantify the exact taurine requirements for YTK. The supplementation of *Artemia* with taurine produced equivocal results in terms of malformation reductions and the interpretation of these data was complicated by differences in larval survival.

The use of inorganic clay was effective in reducing bacterial concentrations in both cobia and YTK larval rearing tanks. Whilst there was no negative impact of using clay on the former species, this addition resulted in significant impairments to feeding, swim bladder inflation, growth and survival in YTK larvae. The outcome of this research is the recommendation to industry that clay not be utilised in the larval rearing of YTK until further research is conducted to determine whether the negative impacts of clay seen in the current study can be overcome by changes to lighting and/or tank design.

LIST OF OUTPUTS PRODUCED

Sub-Project 1 – Genetics:

1. Recommendations and protocols for the best use of existing YTK broodstock in Western Australia to minimise inbreeding.
2. A database for recording details about YTK broodstock developed and in use to track and assist the genetic management of the YTK stocks

Sub-Project 2 – Larviculture:

3. Recommendation to industry on the optimum rotifer feeding strategy for YTK larvae.
4. Protocols for increasing the concentration of deficient selenium in rotifers to match those present in copepods.
5. Protocols for increasing the concentration of deficient taurine in both rotifers & *Artemia*.
6. Protocols for increasing the vitamin C and vitamin E concentrations in enriched rotifers.
7. Evidence that excessive taurine concentrations in live foods may be toxic to YTK larvae and recommendations for further investigation.
8. Protocols for the delivery of clay particles to larval rearing tanks.
9. Evidence of sensitivity of YTK to the use of clay particles in larval rearing tanks and recommendations that clay particles should not be used in YTK larvae rearing without further evidence of benefits.

Sub-Project 1: Genetics

DNA analyses were used to develop a mating strategy to avoid inbreeding among the limited number of existing captive broodstock in WA. A genetic management workshop was carried out in with ACAAR and WA Industry and used to inform attendees about basic genetic management principles and to collect information relevant to the formulation of the strategy. A genetic management database for tracking fish, collection of water quality data, recording treatments, pedigrees, relatedness and fish traits was developed and tested. The same database has been further enhanced and rolled out to ACAAR and two barramundi hatcheries (Project 2009/730) offering a simple but effective genetic management tool for both of these species.

Sub-Project 2: Larviculture

Our study comparing different rotifer feeding strategies demonstrated that up to 65% fewer rotifers can be used in the production of juvenile YTK than are currently being used in commercial hatcheries, without any negative impact on growth or survival. This is the result of efficient prey capture at low prey densities, even from a young age. This reduction in rotifer usage translates directly to the same percentage reduction in rotifer operating costs. We were unable to determine whether these different strategies had an effect on larval malformation rates, due to the small size of the larvae at the end of the rotifer feeding phase. The results of this research are now being implemented at ACAAR and a manuscript describing this research has been submitted to the ISI Journal, Aquaculture Research.

Detailed biochemical composition analyses were performed on rotifers and *Artemia* enriched on various diets and a blend of diets used in the commercial hatchery production of cobia. These analyses revealed some significant differences in certain aspects of the nutritional composition of both live feed types.

Rotifers enriched on all diets were deficient in taurine and selenium relative to wild zooplankton and we were able to increase the level of both of these nutrients via supplementation. *Artemia* contained sufficient levels of selenium and further supplementation of this element was not required. Whilst the levels of taurine in *Artemia* were higher than rotifers, they were still deficient relative to wild zooplankton.

Spresso (the current YTK industry standard enrichment diet) typically resulted in the best lipid profile in both rotifers and *Artemia*. Spresso-enriched rotifers and *Artemia* typically had the highest total lipid content, the highest absolute amount of DHA in both the total and phospholipid fractions and the best DHA:EPA ratio in both fractions. The two UM diets (cobia enrichment diets) yielded similar levels of these factors to those live foods enriched on Spresso, whilst those enriched with N-Rich PL Plus and Ori-Green (tested only with rotifers) were typically worse. Furthermore, the levels of astaxanthin and vitamins in Spresso-enriched live foods were equal to or better than those in other enriched live foods.

Of potential importance were the differences seen in phospholipid DHA between enriched rotifers and *Artemia*. All enriched rotifers had at least double the quantity of DHA in the phospholipid fraction than *Artemia* enriched on the same diets. Furthermore, most enriched rotifers had a DHA:EPA ratio in the phospholipid fraction close to the optimum value of 2, whilst *Artemia* had ratios in this fraction of 0.3 at best. Whilst beyond the scope of this study, these data suggest that extending the rotifer feeding period may be beneficial to the larval development of YTK.

Larval rearing trials subsequently compared the performance of YTK larvae fed rotifers and *Artemia* enriched on different products. The trial comparing Spresso, UM Standard and UM Plus (boosted with taurine, selenium and vitamins) during the rotifer feeding stage revealed no significant differences in growth or survival between treatments. Again, malformation rates could not be assessed at the completion of this trial due to the larvae's size. A second trial comparing the performance of larvae during both the rotifer and *Artemia*

feeding stages found that whilst those larvae receiving taurine supplemented *Artemia* grew faster and had fewer malformations, their survival was significantly worse than those not receiving taurine supplementation – thereby complicating interpretation of the growth and malformation data. A subsequent dose-response study of taurine enriched *Artemia* yielded similar findings to the first in terms of growth and survival, however in this trial there was no effect of taurine dose rate on malformation rates. Given that species closely related to YTK have been demonstrated to be lacking in the enzyme responsible for taurine synthesis, these results showing no improvement or a negative impact of taurine enrichment were unexpected. A comparison of the taurine content of the enriched live foods and the larvae's whole-body taurine content together with results from the literature suggested that the level of taurine in the enriched live foods may have been too high (even in those *Artemia* enriched at the lowest dose of taurine). This was despite utilising published taurine enrichment dose rates. During these trials we accumulated evidence suggesting a strong interactive effect of enrichment diet, taurine dose rate and enrichment time on the taurine level of enriched live foods. Whilst the research conducted to date has been very valuable in gaining an understanding of the effects of high taurine concentrations and identifying these potentially important interactive effects, the optimum level of taurine in rotifers and *Artemia* for YTK larvae remains to be determined. To capitilise on the investment to date, further research is required to quantify these interactive effects, thereby allowing the development of a reliable protocol from which to deliver consistent target taurine levels in enriched live feeds. Further larval rearing studies can then be undertaken to determine the optimum levels of taurine for rotifers and *Artemia*.

In a preliminary collaborative study with the University of Miami it was demonstrated that inorganic clay significantly reduced the concentration of Vibrios in cobia larval rearing tank and could successfully replace microalgae without any negative impacts on growth and survival. Such a replacement results in significant savings due to the high cost of microalgal paste. Whilst the same study conducted with YTK larvae also showed reductions in bacteria within the larval tanks containing clay, this addition resulted in a low feeding

incidence, poor growth, no swim bladder inflation and poor survival in this species. Based on the behaviour of the larvae and subsequent lighting tests, we hypothesised that the scattering or internal reflection of light caused by the clay particles resulted in disorientation and stress to the larvae in the shallow, white-based tanks used in the study. Further studies are therefore required to determine if these issues can be overcome through the use of different lighting or tank colours and/or sizes.

6. Acknowledgements

We thank the Marine Fishfarmers Association and Indian Ocean Fresh for supporting this project.

7. Project Introduction

Western Australia's extensive coastline is currently under-utilised for marine finfish production, yet a recent Aquaculture Development Council discussion paper pointed out that aquaculture sites along this coastline can collectively host between 25,000 and 100,000 tons of annual finfish production. An industry of this size could directly contribute between $200 and $800 million to the economy, plus multiplier effects. The main species of interest in Western Australia are yellowtail kingfish (YTK), mulloway and yellowfin tuna.

The Marine Fishfarmers Association (MFA) of WA has a long-term objective of supporting the growth of marine finfish aquaculture in this state. The MFA have been managing collaborative trials in Geraldton to assess the opportunities and limitations of commercial seacage farming of YTK in this state. These projects represent the first steps in developing aquaculture in the Midwest area of WA and are linked to the development of the Abrolhos Islands Aquaculture site of 800 ha, as approved in 2004.

This project comprised sub-projects aimed at optimising some key inputs to production and improving the commercial viability of the development of a larger scale industry. These projects were:

Sub-Project 1:	Genetic management strategy for cultured YTK in Western Australia.
Sub-Project 2:	Larviculture of YTK.

8. Part 1: Genetic Management Strategy

8.1. Need

There is a need for a genetic management strategy to ensure that the captive broodstock comprise a genetically diverse population in order to avoid inbreeding and to provide good opportunities for future genetic selection. There is also the need to more fully understand what genetic variation exists in wild YTK from within WA to both ensure that broodstock populations are representative of this diversity and to make management decisions regarding integration into national breeding programs.

8.2. Objectives

Objective 1 To analyse genetic variation and pedigrees in existing broodstock, investigate options and make recommendations for how to best capture, maintain and effectively utilise genetic variation among cultured YTK in WA.

Objective 2 To undertake a more detailed population genetics study of Western Australian stock and existing captive broodstock.

8.3. Methods

Objective 1:

Samples were obtained from all domesticated broodstock held in WA. All of these broodstock were genotyped and the results used to recommend how to best make use of the limited number broodstock in WA. A genetic management database was created, tested and implemented at the ACAAR hatchery.

Objective 2

All existing captive broodstock in WA were sampled and genotyped, but it was difficult to obtain samples from wild fish representing a broad range of areas along the coast (particularly in south west WA). The project managed to collect ca. 20 samples from an area between Perth and Geraldton and ca. 8 samples from the Abrolhos Islands, however none from the south coast. This was not enough to be able to assess population genetic structure and diversity. This information would have been used to guide the hatchery so that a broad genetic base of broodstock were continually sourced and utilised. In the absence of this information, and given the difficulties with sourcing stock from many of these areas anyhow, the safest strategy for now is to source fresh broodstock from as many different widespread localities as is possible. A decision was made not to genotype this small number of collected wild samples for the population genetic analysis (although DNA had already been extracted for all of these samples).

8.4. Results & Discussion

Genetic management strategy

A workshop was held in Fremantle to discuss the needs for a genetic management strategy for YTK in Western Australia. Some basic genetic management principles were presented and it was decided that:

- A simple database should be created to assist Challenger Institute of Technology with recording, storing and accessing information.
- That all existing broodstock and potential broodstock from other sources, should be pit tagged and DNA tested to check for common ancestry to help avoid inbreeding.

An Access database was created to allow the participants in the project to quickly and simply record data required for genetic management and to generate useful reports (Figure 8-1). The following information can be entered into the database at the following stages in the process:

- Broodstock collection: Pit tag, sex, wild location, GPS, date collected, collector and weight at collection
- Quarantine: Tank ID, date of entry, current tank, feed type, feed weight, broodstock tank conditions, treatment type, treatment dose and treatment duration. For each fish within each tank, information about the PIT tag, transfers from and to tanks, fish weight and other observations can be recorded.
- Spawning events: Date of spawn, time, tank, total number of eggs, number of viable eggs, plate assessment data and egg incubation data.
- Grow out: Date recorded, transfers between tanks, number of fish, total weight, feed, feed intake, number of mortalities, temperature, flow rate and other comments can be entered.
- Tagging: PIT tag, date, spawn batch (F1 source or date collected if sourced from other localities)
- Mortalities: PIT tag, date found, cause, weight, other observations
- Sample for DNA testing: Date sampled, tube number, PIT, Facility, Wild location and comments
- Allocation of parentage from genotypes: PIT tag, dam PIT tag, sire PIT tag

All the above information exists in a series of tables in the background of the database. Data entry is done by pressing appropriate buttons and using drop down lists where appropriate. The drop down lists enforce adherence to use of set terms and descriptions so that the data can be simply and accurately categorised for reporting purposes. Drop down list categories include:

- Maturation terms
- Facility/tank names
- Disease status
- Wild source locations
- Cause of death terms
- Feed type
- Hormone type
- Treatment type

All data is linked and can be queried, and a number of set queries and reports have been created:

- Fish can be tracked and found
- Checks can be made on the biomass existing in tanks
- Transfers between tanks can be checked
- PIT tags by dates and current tank can be checked

The database was tested and improved in April 2013. More reports were created for different purposes. Data on existing broodstock was entered into the database. Nick Robinson has rolled out the same database shell to the Darwin Aquaculture Centre (DAC) and Good Fortune Bay (GFB) hatcheries as part of a related CRC project on barramundi (2009/730). The database has been made freely available under agreement that the creator takes no responsibility for tailoring it to suit specific hatchery needs, or for any errors etc. that may exist, or may be introduced, with changes to the database. Background tables in the database could be transferred to another database system in the future if that is required (eg. Microsoft SQL server). Nick Robinson has implemented a similar database transfer for another project. After transfer, the original Access database, containing queries, forms and reports, acts as a user-friendly “front end” and links to tables containing data on the Microsoft SQL server. The server is secure, accessed seamlessly over the internet by the front-end, and contains all the raw data. In this way it is possible to reliably coordinate multiple users of the same database and to securely maintain and backup the data on a central server. It is also possible to restrict access to the raw data and raw database structure while giving users the freedom to create their own queries and reports based on the data. When the database was rolled out to DAC and GFB a number of improvements were made and incorporated into the original database (in response to suggestions and requirements of the new users):

- A simple guide to use of the database was produced and distributed (Appendix 1).
- The entry of data regarding broodstock within tanks (date of entry, PIT, tank, weight, stage of maturation, oocyte diameter, external markings, hormone type, hormone dose and other observations) was split from the entry of information regarding tank treatments and conditions.
- A much more comprehensive set of data entry fields was added regarding tank conditions and treatments. This includes selection of multiple feed types and quantities fed, treatment types, doses and durations, lighting and photoperiod type and duration, data reporter name, days old, salinity, dissolved oxygen, pH, Temperature, ammonia, nitrite, algal density, algal/rotifer/*Artemia* density etc, weight fed by feeders vs hand fed, clinical observations, mortalities and appetite etc.

- A summary report of animals sampled for DNA testing and tube numbers that can be sent to the laboratory with the samples was produced.
- A new facility was created so that DNA data on relatedness between animals can be entered, and four new reports can be made which order pair combinations of animals according to their relatedness (every animal with every other, all males with all females, all males with all other males and all females with all other females). This is useful for picking unrelated combinations of animals for group spawning.
- A table summarising the total food usage per batch can be easily generated. This is useful when comparing the performance of different batches under different diets.
- A batch delivery report which can be generated to be sent with larvae or fingerlings to customers (detailing and recording date delivered, batch number, company supplied, number delivered, average weight, weight range, average length, length range, date spawned, parentage, antibiotic use, deformities, feed type and pathology references) was created.
- A new report giving a general overview of PIT, generation, sex, weight at collection and current weight was also been created.
- Additional forms for entering customer details, staff details, and generation details were also created.

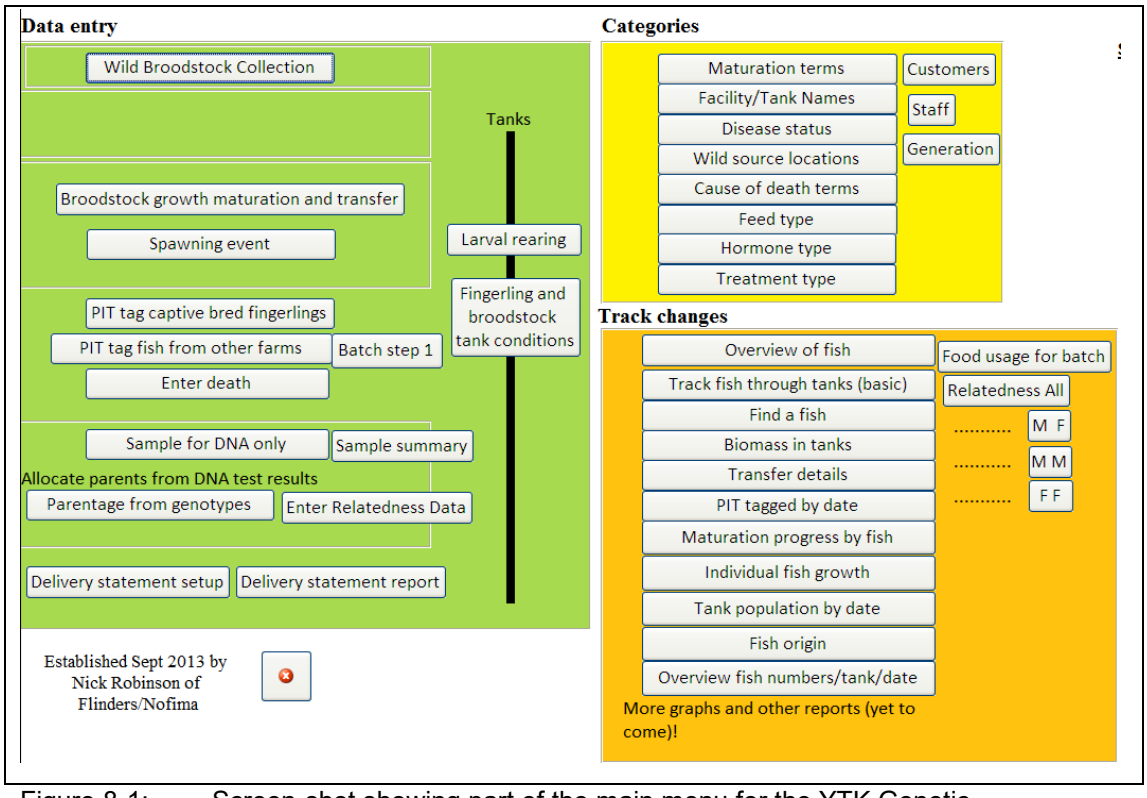

Figure 8-1: Screen-shot showing part of the main menu for the YTK Genetic Management Database

Genetics of existing domesticated YTK stock in WA

Samples were obtained from all domesticated broodstock held in WA. All domesticated broodstock were genotyped using a panel of 6 microsatellite markers. The number of alleles for both groups was quite high. Two individuals at BCMI (tag# A9E5 & 51C0) exhibited unique or rare alleles that were not found at ACAAR, so these fish could make likely candidates for inclusion into a breeding program but this was only found at one locus whereas alleles at the other loci were common across groups. Tag# 83C7 from ACAAR has a rare allele not possessed by other fish but again only at one locus.

Analysis has revealed that BCMI and ACAAR broodstock are not significantly genetically differentiated (no significant difference between loci or across all loci). Within the BCMI group there is an average level of 33% relatedness, ACAAR 29% (-4%). If the two groups were combined they would exhibit on average 37% relatedness. This result has led to the recommendation that transport of BCMI animals to ACAAR for use as broodstock is not warranted. A genetic relatedness matrix for all existing broodstock at ACAAR has been developed and this was used to plan how to best group the broodstock for future spawning. All the remaining broodstock are F1 animals derived from a single spawning run. The genotype data has shown that all F1 fish from this spawning run are offspring from a single female (A75A- wild Abrolhos fish) and 6 males. One of the 6 males (47B1 – wild Abrolhos) sired 72% of the batch from that spawn. At least one offspring from each of these contributing males has been kept, except that there are no offspring from male M66. So all existing broodstock are closely related (at least half-siblings), and some even have full-sibling relationships. Therefore to avoid inbreeding (mating of half- or full-siblings) for the next spawning, remaining males should be separated from females into separate spawning tanks, and fresh (supposedly unrelated) females and males found as mates for the spawning. As all the offspring from this next spawning will share a common grand dam it is recommended that either the same procedure is applied again (separating males and females and finding fresh unrelated mates) or that an entirely new population of fish is

used for subsequent spawning. Broodstock fishing by ACCAR is continuing in an effort to bring in unrelated stock.

Samples from wild populations

It has been difficult to obtain samples for the wild population genetic component of the project (particularly from the south west of WA). Only samples from an area between Perth and Geraldton (20 individuals) and ca. 8 individuals from Abrolhos Islands were collected. Table 8-1 shows the samples received by the lab at Flinders.

DATE	TUBE #	SOURCE	extracted	COMMENTS
30/11/11	W725	Rottnest Island	15/08/2012	wild collection
30/11/11	WA63	Rottnest Island	15/08/2012	wild collection
30/11/11	W75E	Rottnest Island	15/08/2012	wild collection
24/08/10	297	Rat Island KF1	15/08/2012	wild collection
11/08/10	298	Rat Island KF1	15/08/2012	wild collection
24/08/10	299	Rat Island KF2	15/08/2012	wild collection
11/08/10	300	Rat Island KF2	15/08/2012	wild collection
24/08/10	301	Rat Island KF3	15/08/2012	wild collection
24/08/10	302	Rat Island KF4	15/08/2012	wild collection
12/06/10	303	WKF Cohort 2 Fish 1	15/08/2012	wild collection
12/06/10	304	WKF Cohort 2 Fish 2	15/08/2012	wild collection
12/06/10	305	WKF Cohort 2 Fish 3	15/08/2012	wild collection
12/06/10	306	WKF Cohort 2 Fish 4	15/08/2012	wild collection
12/06/10	307	WKF Cohort 2 Fish 5	15/08/2012	wild collection
09/08/10	308	Kalbarri Steep Point KF1	15/08/2012	wild collection
09/08/10	309	Kalbarri Steep Point	15/08/2012	wild

		KF2		collection
09/08/10	310	Kalbarri Steep Point KF3	15/08/2012	wild collection
02/09/10	311	Albrohos KF1	15/08/2012	wild collection
18/11/10	FH10-144-11	Turtle Dove Shoal	15/08/2012	wild collection
18/11/10	FH10-144-12	Turtle Dove Shoal	15/08/2012	wild collection
24/04/12	022	Abrolhos	15/08/2012	wild collection
24/04/12	032	Abrolhos	15/08/2012	wild collection
24/04/12	036	Abrolhos	15/08/2012	wild collection
22/05/12	038	Kalbarri	15/08/2012	wild collection
5/04/12	040	Abrolhos	15/08/2012	*Seriola dumerili*
3/06/12	102	Rottnest Island	15/08/2012	wild collection
2/06/12	119	Rottnest Island	15/08/2012	wild collection
4/03/12	150	Direction Bank Mindarie	15/08/2012	wild collection
4/03/12	153	Seaward Ledge Jurien	15/08/2012	wild collection

Table 8-1: YTK samples received for genotyping.

The effort to obtain samples from the south-west region over summer failed because the worst YTK catches on record were experienced during the project period (possibly because of the strong Leeuwin current) and samples could therefore not be obtained. This information would have been used to guide the hatchery so that a broad genetic base of broodstock were continually sourced and utilised. In the absence of this information, and given the difficulties with sourcing stock from many of these areas anyhow, the safest strategy for now is to source fresh broodstock from as many different widespread localities as is possible. A decision has been made not to genotype this small number of collected wild samples for the population genetic analysis (although DNA has already been extracted for all these samples).

Appendix 1. Simple user guide for the Genetic Management Database

Easy Guide to the Barramundi Genetic Management Database

Nick Oct 2013

A. Everything can be accessed by using the buttons on the main page!
B. Moving around when entering information. Normally when you hit a button to enter some new information the cursor will land in the first field for a "new" record. Some fields you need to pick from an existing list of items. These lists need to be made up first before you start to enter data (see all the buttons under the "*Categories*" heading. i.e. enter all the *facility* or *tank names* you want to use, the *feed types*, *disease status*, *staff names*, *wild locations*, *maturation terms* (make sure to include terms "Male" and "Female" for mature males and mature females), *hormone types*, *treatment types*, *generation* terms etc BEFORE YOU START. This forces some consistency to the data so that it is easy to retrieve groups of information etc later. Type information, tab to the next field. You can move between rows or screens using the arrows at the bottom of the window. Normally you cannot enter info in the ID fields, these fields provide a unique row number for the data tables in the background. Access will beep and give errors if you enter the wrong type of information in a field. Close the window when you are finished entering info. Info is saved whenever you tab to the next field.
C. Entering details about newly acquired or newly PIT tagged fish. There are 3 ways to enter information about these fish and their PIT tags. All three methods populate the same tables with data, but each has different fields depending on where these animals are coming from:
    a. *Wild broodstock collection* (or *Broodstock collection*). Use this button when you catch fish from the wild. Enter information about the source location, person collecting the fish, date, PIT tag number etc.
    b. Captive bred fingerlings from a spawning run on your farm to be PIT tagged. Before you enter the PIT tag details for these fish, you need to enter details about the spawning run (under the "*Spawning event*" button). Once you have entered information about the spawning run, hit the "*PIT tag captive bred fingerlings*" button and enter all the newly PIT tagged fish and assign them to the spawning run (date, time and tank).
    c. PIT tag fish from other farms. Use this button when captive bred fish come from other farms. Enter details about those farms, PIT tag number and date collected etc.

D. Entering details about potential or actual broodstock. Whenever you capture your broodstock record the fishes weight, the tank it is in (is transferred to) and other details such as hormone manipulation, maturity, oocyte diameter etc. by using the "*Broodstock growth maturation and transfer"* button.
E. Enter details about tank conditions. Down the right hand side of the Data Entry green area are two buttons for entering data about larval rearing tank conditions ("*Larval rearing"*) and "*Fingerling and broodstock tank conditions*". Record tank, DO, temp, feed etc. here.
F. Use the "*Enter death*" button to record lost broodstock due to death or culling.
G. To generate a list of tube numbers and corresponding PIT tags use the "*Sample for DNA only"* button. Check and print the list (for the farm and the lab) using the "*Sample summary"* button.
H. Enter parentage results ("*Parentage from genotypes"*) and relatedness data (*"Enter relatedness data"*) once you have your DNA test results back.
I. Set up a larvae or fingerling delivery statement ("*Delivery statement setup"*) and print a report for your records and for your customers using the "*Delivery statement report*" button.
J. Under the yellow "*Track changes*" heading are a number of reports that you can generate at any time. These reports will be updated after you enter new information, close all the information and report windows, and press these report buttons again. If relevant information is not entered, the reports will not show data in some instances (eg. if Tank data or "*Transfer to"* data is not entered, the tank reports for those animals will not appear. You should be able to just print out the reports produced. Other simple reports can be provided on request.
K. IMPORTANT: DO NOT ALTER INFORMATION IN THE NAVIGATION PANE (ALL ACCESS TABLES, QUERIES AND OTHER OBJECTS) DOWN THE LEFT HAND SIDE OF THE SCREEN! This can break the database.
L. Every now and then you should repair and compact the database. First make a backup. Then choose the compact and repair option in Access. Do this every 6 months or so to ensure the database operates smoothly.

8.5. Benefits and Adoption

All results from the analysis have been communicated to ACAAR and Erica Starling. The results have helped ACAAR decide on which broodstock to use for spawning and to source more wild fish to increase the diversity of their stocks

9. Part 2: Larviculture

9.1. Need

Production of juvenile YTK is somewhat constrained by low survival and juvenile quality. High incidences of malformations continue to impact heavily on the production cost of juvenile YTK. There is a need therefore, to reduce the incidence of such malformations to reduce the cost of juvenile production and improve the quality of fish being put to sea. This project addressed a number of potential causes of low survival, high malformation rates and cost of production. The project objectives specific to larval rearing were:

Objective 1: To compare the differences between continuous and pulse feeding strategies on the cost of production and performance of YTK larvae.

Objective 2: To compare the performance of those YTK larvae reared on live food enriched on current best-practice enrichment products with those reared on an experimental diet that has been demonstrated through laboratory analyses to have superior fatty acid profiles and concentrations of other essential nutrients.

Objective 3: To compare the effect of new bacterial management approaches within larval rearing tanks in reducing malformation rates.

Objective 4: To exchange larviculture techniques between WA, NSW and SA.

9.2. Objective 1 – Rotifer Feeding Regimes

9.2.1. Introduction

With the exception of Japan, YTK growout is reliant upon closed-cycle hatchery production for the provision of juveniles (Fowler et al., 2003; Nakada, 2002). Hatchery production of this species is typical of most marine fish and involves feeding rotifers *Brachionus plicatilis* from first feeding (three days post hatch (dph)) to *ca*. 12 dph before transitioning onto *Artemia* then weaning onto a dry diet from *ca*. 18 dph (Fielder, 2013). Despite a fairly large body of published knowledge on hatchery production of this species, there is little consensus on the best rotifer feeding strategy that should be employed for optimising growth, survival and production costs (Ma et al., 2013).

The most common rotifer feeding strategy for YTK is a 'high density' approach similar to that used for many marine fish larvae (Fielder, 2013). Under this approach, rotifers are maintained in the rearing tanks at a relatively high density with the premise of ensuring that the larvae are never food-limited. Disadvantages of this practice, however, include a potential degradation of the rotifer nutritional value with increasing rotifer residence times and the increased potential for bacterial blooms and deterioration of water quality as a result of continued high rotifer densities (Dhert, 2001; Valleś, 2013). Furthermore, this approach may lead to higher live feed production costs as larvae are offered more prey than they can consume, with most being flushed from the rearing tank (Rabe and Brown, 2000).

An alternative feeding strategy is to 'pulse feed' the larvae. Under this strategy, lower densities of rotifers is offered and are allowed to be heavily depleted before being replaced (Rabe and Brown, 2000). There are several advantages to this technique including improved prey digestion as a result of a longer gut retention time (Canino and Bailey, 1995); improved nutritional value of live feeds as a result of a shorter residence time in the rearing tank (Dhert et al., 2001; Curnow et al. 2006b), improvements in early weaning

success (King et al., 2011) and lower rotifer production costs resulting from a requirement for fewer rotifers. This feeding technique also has the potential to reduce the amount of bacteria transferred from the live feed cultures to the larval rearing tanks (Prol-Garcia et al., 2010; Curnow et al., 2006a). One potential disadvantage of pulse feeding is that lower live prey densities may reduce prey encounter rates, which may impair ingestion rates and subsequently lead to lower growth or survival. This is of particular concern for younger larvae, which are more prone to these effects due to their limited visual acuity and smaller search volumes relative to older and more morphologically advanced larvae (Boeuf and Le Bail, 1999; Houde and Schekter, 1980).

This trial compared the effects of three rotifer feeding regimes on ingestion rates by YTK larvae and their subsequent performance in growth and survival. A high density regime, typical of that used in Australian YTK hatcheries, was compared against a low density feeding regime and a 'hybrid' regime in which larvae were offered a high density of rotifers during the first five days post hatch before switching to the low density feeding regime in order to test the hypothesis that early larvae may benefit from a higher prey density.

9.2.2. Materials and Methods

Larval Fish Rearing System

Fertilised YTK eggs were sourced from captive broodstock held at Clean Seas Tuna Ltd., Arno Bay (South Australia) and transported to the Australian Centre for Applied Aquaculture Research (Western Australia). The eggs were hatched in a 300 L incubator at 20°C. After hatching, one day post hatch larvae were randomly stocked into twelve 300 L tanks at 60 larvae/L. The rearing tanks were part of a flow-through system supplied with seawater (34‰) with an exchange rate of 54 L/h (430% daily water exchange = 18%/hour) in each tank. Four rearing tanks were floated in each of three 5000 L tanks to maintain a stable water temperature. Upon transferring larvae

from the incubator, water temperature was increased from 20°C to 24°C over 48 hours and then maintained at this temperature throughout the experiment. Treatments were randomly allocated within and between each bath tank. Two airstones were placed in each rearing tank to maintain the dissolved oxygen levels close to saturation. A diffused metal halide light (400 W) above each 5000 L tank provided a surface light intensity of 4700 ± 1300 lux at the center of each rearing tank for a photoperiod of 12 h light (0900 to 2100 h) and 12 h dark. Microalgal paste (*Nannochloropsis* sp. (80%) and *Isochrysis* sp. (20%), Reed Mariculture Inc., USA) was automatically dosed into the rearing tanks during daylight hours to maintain turbidity within the range of 1.7 to 1.9 NTU, equivalent to a Secchi disk depth of 55 to 60 cm.

Experimental Design

Larvae were fed rotifers under one of three feeding regimes from 3 to 12 dph (Table 9-1). The first regime was a high density regime typical of that used in Australian YTK hatcheries (Fielder, 2013) and involved increasing rotifer densities from 10 to 30 /mL throughout this period. Under the second regime rotifer densities were increased from 5 to 8 /mL. Under this regime, the lower density of rotifers offered became heavily depleted before the next feed. The third regime was a hybrid of the two aforementioned regimes whereby rotifer densities followed that of the high density regime from 3 to 5 dph and then decreased to match the low density feeding regime from 6 to 12 dph. The three regimes were compared with four replicate rearing tanks. Rotifers were enriched with Spresso (INVE Aquaculture, Belgium) according to the manufacturer's instructions. Larvae were fed rotifers three times per day (0830, 1230 and 1530 h). Half an hour prior to each afternoon feed, rotifers were counted in three x 5 mL depth-integrated samples from each tank. Sufficient rotifers were then added to each tank to maintain the target densities shown in Table 9-1. Random checks of rotifer densities at 0800 h confirmed that rotifer residuals were at zero prior to the 0830 h feed.

Rotifer treatment	High density (rotifers mL^{-1})			Low density (rotifers mL^{-1})			Hybrid (rotifers mL^{-1})		
Feeding time/ Age (dph)	0830	1230	1530	0830	1230	1530	0830	1230	1530
3	10	10	10	5	3	3	10	10	10
4	10	10	10	5	3	3	10	10	10
5	15	15	15	5	5	5	15	15	15
6	15	15	15	5	5	5	5	5	5
7	15	15	15	6	6	6	6	6	6
8	20	20	20	6	6	6	6	6	6
9	20	20	20	7	7	7	7	7	7
10	30	30	30	7	7	7	7	7	7
11	30	30	30	8	8	8	8	8	8
12	30	30	30	8	8	8	8	8	8

1

Table 9-1: Rotifer feeding treatments.

Sampling Protocol

Larval standard length and dry weight were assessed on 4, 6, 8, and 12 dph on 20 randomly selected larvae per tank. Individual rotifers were quantified in the larval guts (5 per tank) by counting undigested mastaxes under a stereomicroscope two hours after the first feed on 4, 5, 7, 9 and 11 dph in squash-mounted larvae (Ma et al. 2013). The culture water from each tank was sampled for total bacterial counts on 12 dph and analysed for total bacterial counts and Vibrio sp. counts by the Department of Agriculture and Food, Western Australia according to standard methods described by Buller (2004). The trial was terminated on 13 dph, the end of the rotifer feeding stage, and larvae from each tank were hand counted to determine the survival. Sub-samples of 100 larvae per tank were lightly anaesthetised (AQUI-S), fixed in 10% seawater formalin and sent to CST for malformation assessment.

Statistics

A repeated measures ANOVA was used to determine the effect of larval age and rotifer feeding treatment on the growth of larvae (i.e. length and dry weight) and number of rotifers consumed (i.e. mastaxes per larval gut converted to a rate per hour). For all repeated measures ANOVA tests, larval age (dph) was selected as the within-subject factor and feeding treatment as the between-subject factor and each tank was used as the replication unit. One-way ANOVA was used to determine difference between treatments and parameters of survival, numbers of rotifers added to each tank and culture water bacteria counts. Data was arcsine transformed where necessary to ensure homogeneity of variance. Significance was set at $P < 0.05$ and values are presented as mean ± SD. All statistical analyses were performed using PASW Statistics 18 (Release 18.0.2, Chicago, IL, USA).

9.2.3. Results

Larval survival to 13 dph was 28 ± 7% in the hybrid treatment compared with 17 ± 5% and 17 ± 9% in the low and high density treatments, respectively, however these differences were not significant (P = 0.08; Figure 9-1).

Larval growth, measured as standard length and dry weight was not significantly affected by the feeding regime (P = 0.85 and P = 0.71, respectively; Figure 9-2). The number of rotifers ingested by larvae increased significantly with larval age (P < 0.01) but was not affected by the feeding regime (P = 0.62; Figure 9-3). The number of rotifers actually used (i.e. taking into account residual values) in the low density and hybrid feeding method was significantly less than the high density feeding regime, 65 ± 2% and 53 ± 2% respectively (P < 0.001). The mean total *Vibrio* spp. (5452 ± 3989 CFU/mL) and total bacterial (53 × 10^4 ± 2 × 10^4 CFU/mL) counts on 12 dph were not significantly affected by treatment (P = 0.11 and P = 0.95, respectively).

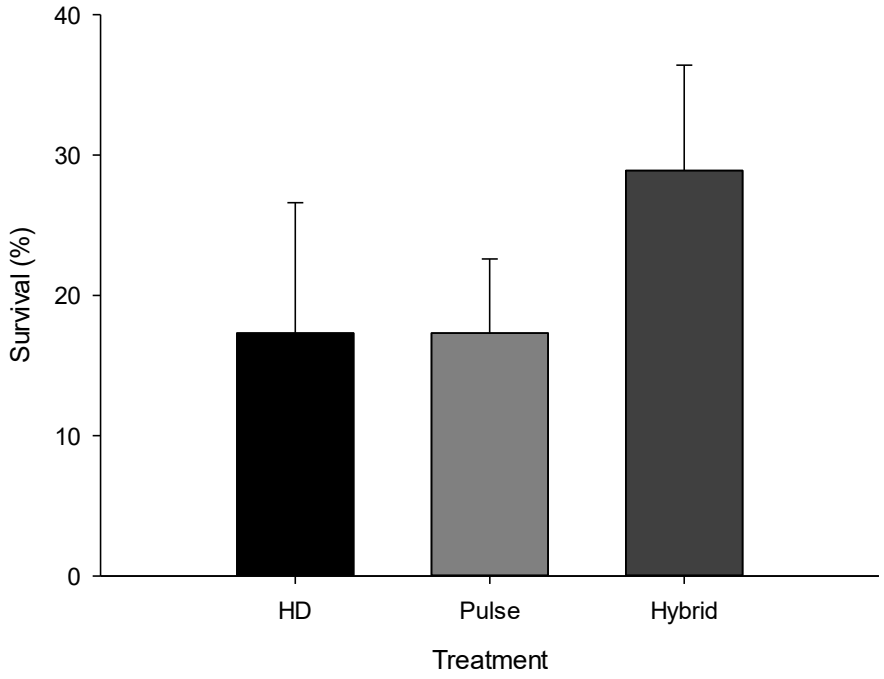

Figure 9-1: Survival (% mean ± SD, n = 4) of YTK larvae fed three rotifer feeding treatments.

Using the exchange rate of new water of 18% per hour and the rotifer consumption rates presented in Figure 9-3, we have calculated the theoretical decline in rotifer numbers under the high and low density feeding regimes for larvae of 4 and 9 dph in addition to showing the actual rotifer residuals measured in each of these treatments (Figure 9-4). These figures assume a survival rate of 100% and 40% of larvae at 4 and 9 dph, respectively and demonstrate that the actual rotifer residual values obtained were similar to those expected based on the flushing rates and rotifer consumption rates. Based on the theoretical residual values and target densities, we have calculated that following top-up, only 59% of rotifers in the high density tanks on 4 dph would be fresh rotifers compared with 75% in the low density feeding regime. On 9 dph these values were calculated to be 53% and 72%, respectively.

CST were unable to determine malformation rates as malformations could not been seen under the microscope in these larvae due to their size.

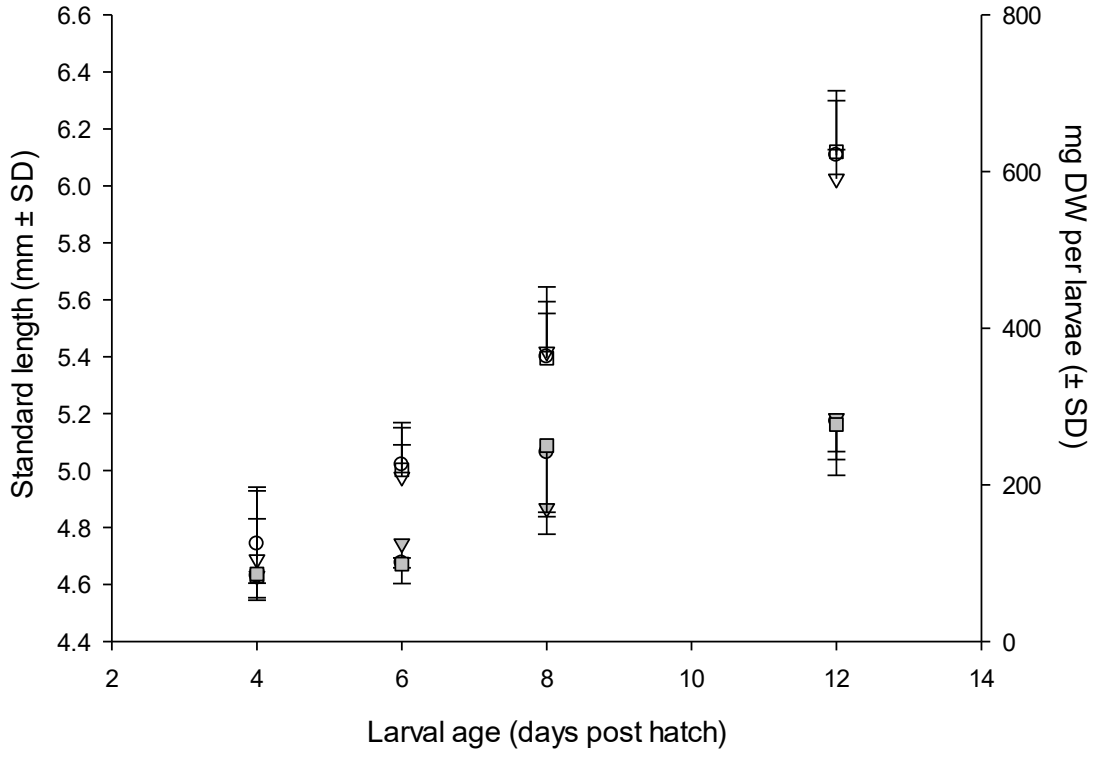

Figure 9-2: Growth performance of YTK larvae over the first 12 dph reared under three rotifer feeding treatments. Standard length (⬭ high density; □ low density;

hybrid) and dry weight (high density; low density; hybrid). Values are mean ± SD (n =4).

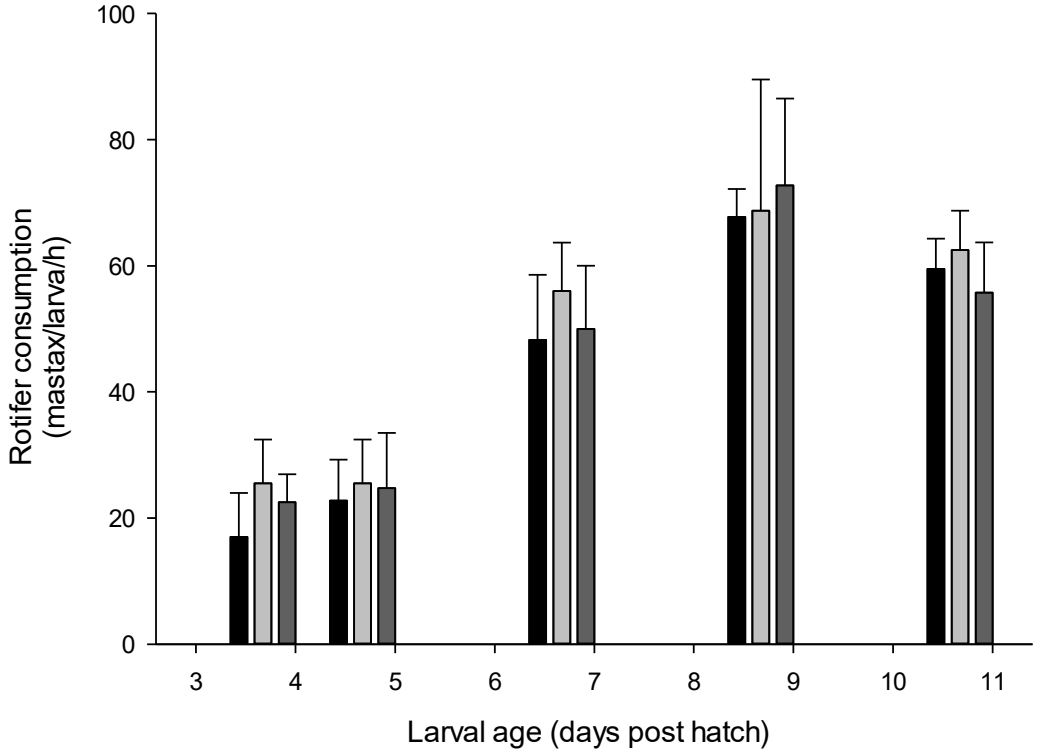

Figure 9-3: Rotifer consumption (measured as the number of rotifer mastaxes per larva gut) of YTK larvae from 4 to 11 dph. (high density; low density; hybrid), values are mean ± SD (n = 4).

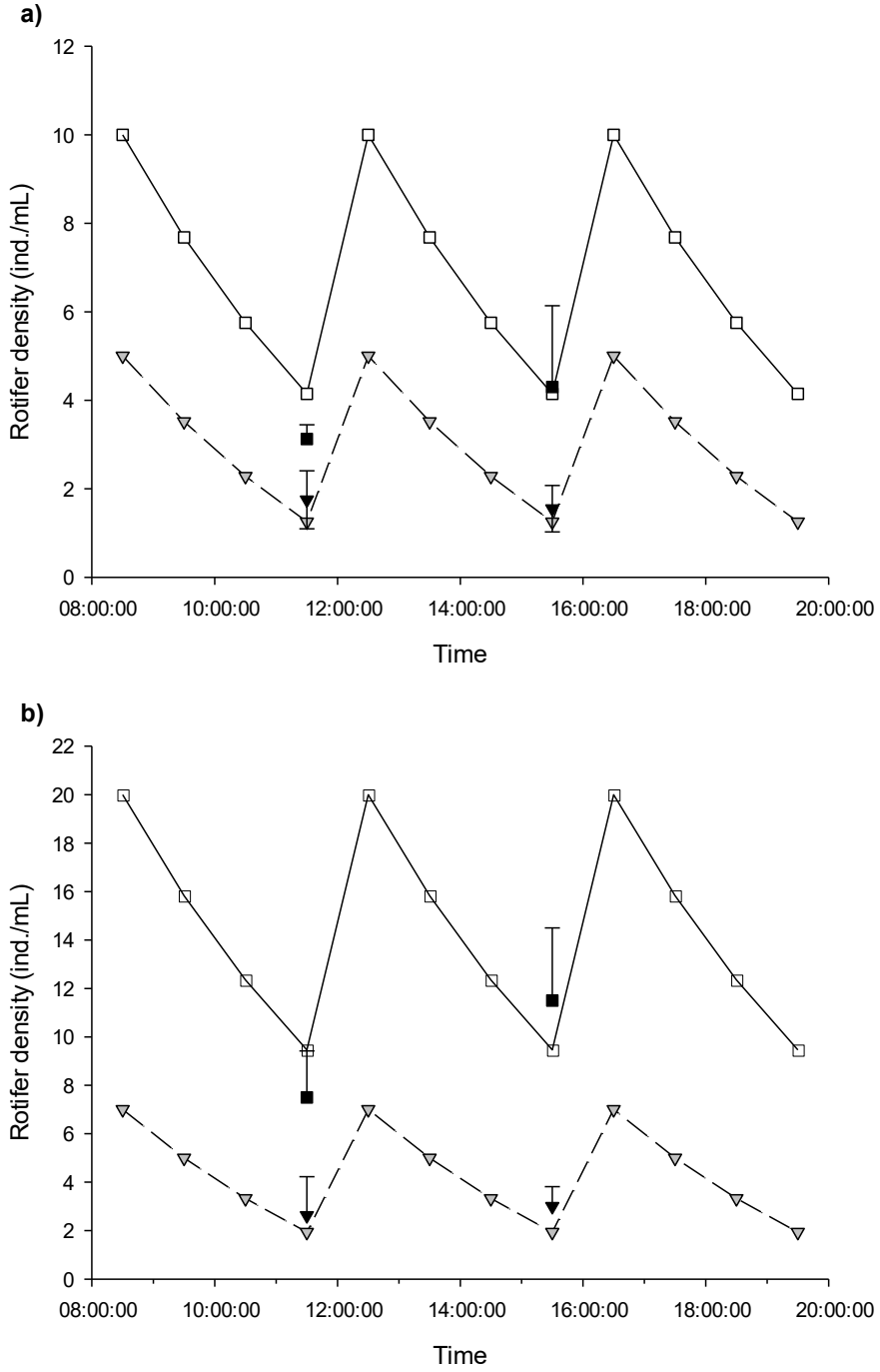

Figure 9-4: Loss model of the predicted decline and measured residual rotifer density (individuals per mL) under the high density (predicted decline and measured residual) and low density (predicted decline and measured residual) feeding regimes for larvae at a) 4 and b) 9 dph. Values are mean ± SD (n = 4).

9.2.4. Discussion

The results of this study demonstrate that YTK larvae are efficient predators and can feed effectively across a wide range of prey densities, regardless of age, and that low prey densities do not negatively impact on growth or survival under the conditions of this study. Whilst optimising prey density and feeding regimes is of great importance to optimising survival and growth of marine fish larvae in general, such optimisation is of particular importance at first feeding, as inadequate regimes at this critical transition from endogenous to exogenous feeding can result in a failure to commence feeding and subsequently lead to high rates of mortality (Chen et al., 2007; Cox and Pankhurst, 2000; Fushimi, 2001). Like most marine fish larvae, YTK are visual predators whose visual acuity and effective search volume increase with age (Carton, 2005; Georgalas et al., 2007). In order to be appropriate, prey density must be maintained at or above a critical threshold; a level which appears to be species and age specific and dependent upon abiotic factors such as light intensity and turbidity (Puvanendran and Brown, 1999). The light and algal-induced turbidity levels used in this trial were above and below, respectively, the levels that limit prey ingestion by YTK larvae (<8 μmol/s/m^2 and >16 × 10^4 cells/mL, respectively) (Carton, 2005).

The results from this study demonstrating an equal ingestion rate of rotifers and equal growth and survival rates across treatments suggest that the lowest first feeding rotifer density of 5 /mL is above the threshold density. Working with the same species, Ma et al. (2013) found that 5 dph larvae offered 1 rotifer/mL ate significantly fewer rotifers during the first meal of the day compared to those offered ≥ 10 /mL, but by 8 dph rotifer ingestion was equal across all rotifer densities. The early feed limitation resulted in significantly lower growth of the former larvae. Combined, these data suggest that the threshold rotifer density for early feeding of YTK under non-limiting light conditions is > 1 but ≤ 5 /mL. Whilst Shaw et al. (2006) suggested that larvae may spend more time searching out prey at lower densities, which may lead to slower growth, the fact that we observed equal growth across all regimes

suggests that any additional energy expended by YTK larvae on searching for prey at the lowest densities did not impact on their growth.

Sawada et al. (2000) determined that rotifer densities considered optimal for first feeding in Pacific northern bluefin tuna *Thynnus thynnus* were within a wide range between 10 and 30 /mL. These authors demonstrated that the consumption of rotifers was lower at 5 /mL than at densities of 10 /mL and above on the day of first feeding, i.e. 3 dph. Previous studies on snapper *Lutjanus analis* and grouper *Epinephelus suillus* larvae also suggest a much higher critical threshold compared to YTK larvae of 20 and 18 /mL, respectively (Duray et al., 1996; Watanabe et al., 1998). The lower critical threshold demonstrated in this study suggest YTK are more efficient predators and have a quicker learning process and/or feeding ability at the start of exogenous feeding (Yúfera and Darias, 2007).

Larvae need to consume more live prey as they grow in order to meet their increasing energy demands (Puvanendran et al., 2006). In this study the number of rotifers consumed increased with larval growth regardless of the rotifer feeding regime. This is similar to results by Hamasaki et al. (2009), who described an increase in the number of rotifers ingested by Japanese yellowtail *S. dumerili* with an increase in the body length. Although rotifer ingestion rates increased significantly with time, this does not imply that the number of rotifers offered also needs to increase at the same rate. This is due to the fact that as larvae grow and develop they become more efficient predators as a result of improved visual acuity, increased search volume (Rabe and Brown, 2000) and swimming ability (Fisher et al., 2000; Hunt von Herbing et al., 2001). Our observation of equal rotifer ingestion rates across the different feeding regimes with time and equal larval growth rates demonstrates that our rate of increase in rotifer density in the low density regime was sufficient to keep pace with the larvae's increasing energetic demands. Whilst we cannot rule out that further reductions in rotifer use may be possible by either maintaining the same low density or reducing the rate of increase with time, our loss modeling does suggest that had survival remained high, that rotifer numbers may have become limiting by 7 dph or,

conversely, that such a limitation may have caused lower survival. We believe however, that the latter is unlikely and if rotifers did become limiting that a reduction in larval growth would be the more likely outcome, as the same ration of rotifers is shared amongst a greater number of fish.

Rotifers metabolise the nutrients they acquire during enrichment, resulting in deterioration in their nutritional quality over time (Yamamoto et al., 2009). Although our loss modeling demonstrates the greater potential for nutritional depletion of enriched rotifers in the high density treatment via their longer retention time in the larval tanks and a smaller percentage of 'fresh' rotifers being added at each feed, the fact that we witnessed no difference in growth or survival suggests that this is not a significant determinant in the larvae's performance. Alternatively, survival and growth may be more strongly influenced by a factor other than nutrition.

There was a significant reduction in the numbers of rotifers used in the low density and hybrid feeding techniques compared to the high density feeding regime. With live feed production costs being a major component of the cost of producing juvenile marine fish (Ballagh et al., 2010), such reductions translate into significant savings in production and also ease production demands on the live feed cultures. The reductions in rotifer usage of 65% and 53% in the low density and hybrid feeding treatments, respectively, would directly translate into the same percentage reduction in the costs of production consumables, such as culture and enrichment diets.

Previous studies have indicated that many marine finfish larvae do not display constant ingestion but have diel feeding patterns (Kotani and Fushimi, 2011) and a pulse feeding strategy fits with this natural behaviour. YTK larvae are 'cruise searchers', spending most of their time swimming and foraging for food, however their swimming efforts decrease as they become satiated, similar to the behaviour observed in yellowtail flounder *Pleuronectes ferrugineus* by Rabe and Brown (2000). YTK slow down their predatory behaviour once they are satiated so there is no benefit in providing constant excess numbers of rotifers within the rearing tanks. This is supported by our

finding that the residual rotifer numbers in each tank were generally higher at 3 pm than at 12 pm; suggesting that the larvae ate more in the first feed of the day. The hybrid and low density feeding regimes are also likely to provide more suitable platforms for early weaning. The direct early weaning from rotifers to artificial diets has been achieved for barramundi *Lates calcarifer* (Curnow et al., 2006a, b) and more recently in several commercial hatcheries for sea bass *Dicentrachus labrax* (O'Brien, 2013) and sea bream *Sparus auratus* (King et al., 2011). Reducing the reliance on *Artemia* has many economic and technical benefits and has been a goal of commercial hatcheries for many years (Callan et al., 2003). The success of such early weaning in the aforementioned species is believed to be attributable (at least in part) to the use of a low density rotifer feeding strategy, whereby rotifer densities drop below the critical threshold level for a significant period of time between live prey feeds. This approach results in periods of time when the larvae are not fully satiated on rotifers and subsequently more willing to accept the artificial diet (Rosenlund et al., 1997). This is supported by a previous study on turbot *Scophthalmus maximus* larvae which demonstrated that larvae offered live prey at higher rations during weaning preferentially feed on the live prey, thereby delaying weaning (Bromley, 1978).

This study demonstrated that optimal rotifer densities thresholds are lower for YTK than previously described species. YTK are efficient at capturing prey at low densities and have a rapid learning process and/or feeding ability at the start of exogenous feeding, requiring feeding densities of >1 but ≤ 5 rotifers/mL.

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9.3. Objective 2 – Live Food Enrichment

9.3.1. Introduction

Many different products are commercially available for enrichment of the rotifers and *Artemia* used as live foods in marine fish culture. The primary objective of such products is to increase the concentration of essential fatty acids to more closely match the profiles seen in the zooplankton on which marine fish larvae naturally feed. In addition to fatty acids, however, there are many other nutrient and trace elements that are lacking in both rotifers and *Artemia*, relative to zooplankton. Some noteworthy elements which have been shown to be lacking include selenium, taurine, vitamins C and E and carotenoids such as astaxanthin.

Selenium is an essential nutrient for fish and deficiencies can lead to oxidative stress, reduced growth and increased mortality (Penglase et al., 2010). Whilst *Artemia* contain similar levels of selenium to copepods (ca. 3 to 5 mg/kg), rotifers contain only trace levels and the addition of selenium to rotifers has been proven to have some benefits to the larvae of other marine larval fish (Penglase et al., 2010).

Taurine is a neutral β-amino acid and the most abundant free amino acid in animal tissues, accounting for up to 50% of the amino acid pool. It is involved in many physiological processes and is an essential dietary component for those species which lack the enzyme cysteinesulfinic acid decarboxylase (CSD) required to produce it from its precursors, L-cysteine and methionine (Ripps and Shen, 2012). Many pelagic marine species fall into this category and whilst the CSD activity of YTK has not been studied, Japanese yellowtail *Seriola quinqueradiata* has been demonstrated to be completely lacking in this enzyme (Yokoyama et al., 2001). The rotifers and *Artemia* used to culture YTK contain lower concentrations of taurine than the zooplankton on which they would naturally feed. The taurine content of wild zooplankton, for example, ranges from the 0.58 to 1.77%DW, whereas rotifers contain only

0.04 to 0.19% and *Artemia* 0.69 to 0.83%DW (van der Meeren et al., 2008; Yamamoto et al., 2008). Furthermore, it has been hypothesised that larval fish may have a higher requirement for taurine than older fish due to the development of their organ systems and based on the fact that egg and yolk-sac larvae contain high levels of taurine (Pinto et al., 2013a; Pinto et al., 2010).

Vitamins C and E play importance anti-oxidant roles and assist in stress prevention and disease resistance. Fish lack the enzyme gulonolactone oxidase required to convert glucuronic acid into ascorbic acid (vitamin C) and therefore require a dietary source of this vitamin. Vitamin E also provides protection against oxidation of free radicals and is vital for normal development of various tissues. Vitamin E is therefore of great importance in live foods containing high levels of lipid. Vitamins C & E levels in copepods can be highly variable and dependent on their food supply. van der Meeren et al. (2008) reported ranges of 38-1232 and 23 to 206 µg/g DW, respectively in copepods.

Astaxanthin is another powerful antioxidant that is abundant in copepods (413 to 1422 µg/g DW) and naturally lacking in rotifers and *Artemia*. Rotifers cultured on RotiMac and Tahitian *Isochrysis* by van der Meeren et al. (van der Meeren et al., 2008) for example, were found to contain only 24 µg/g of astaxanthin. Whilst *Artemia* nauplii contain high concentrations of carotenoids, these are mainly in the form of canthaxanthin and very little astaxanthin. These other carotenoids may still play important roles as precursors for vitamin A production within fish larvae.

Whilst there have been many studies which have reported the fatty acid profile of enriched live foods, the majority of these report only the total fatty acid content, and do not report on the relative amounts of the fatty acids within the different lipid fractions. It has been demonstrated that the polar lipid fraction is the most important for larval development, with the neutral faction being used primarily for energy (Coutteau, 1997; Seoka et al., 2008). As such, it is the fatty acid profile in the polar lipid fraction that is of greatest

importance. Copepods have been demonstrated to contain between 56 and 62% of their lipids with the polar fraction (van der Meeren et al., 2008) with enriched rotifers containing 39%, and *Artemia* containing between 25 and 35% (Coutteau, 1997).

Prior to commencing larval rearing trials, the biochemical composition of rotifers and *Artemia* enriched on a number of commercially available enrichment products was determined to quantify a number of key nutrients and trace elements and to compare the fatty acid profiles both within the total lipid and within the polar and neutral fractions. Parts 1 and 3 below report on these analyses in rotifers and *Artemia*, respectively, whilst Parts 2 and 4 report on larval rearing trials testing different enrichment products.

9.3.2. Part 1: The effect of various enrichment products on the biochemical composition of enriched rotifers

9.3.2.1. Materials and Methods

Rotifers enriched on Spresso were enriched at 1000/mL according to the manufacturer's 'Overnight long term enrichment' in which a background of 100 ppm of Spresso was initially dosed into the enrichment tank followed by a 18 hour enrichment period during which two pulse doses of 175 ppm each were administered at 19:30 and 01:30.

Those rotifers enriched on Ori-Green were enriched following the manufacturer's directions by feeding 0.25 grams per million rotifers at 1000 rotifers/mL for two hours.

For N-Rich PL Plus, a number of different enrichment protocols are provided by the manufacturer. The method used in the current trial was the 'strong' enrichment in which rotifers were continuously fed at the rate of 0.2 mL/L/hour for 8 hours at a stocking density of 1000 rotifers/mL.

The UM Standard enrichment diet is a blend of various commercially available products described by Benetti et al. (2008) and summarised in Table 9-2. It was included in this study as juvenile cobia produced on this diet exhibit a low level of malformations. These rotifers were enriched at 1000/mL and fed at the rate of 0.3 g/million for 18 hours with a 100 ppm background.

UM Plus was prepared by the addition of organic selenium (Sel-Plex®, Alltech), taurine, soy lecithin, ascorbyl palmitate (vitamin C) and alpha tocopherol (vitamin E) to the standard UM diet according to Table 9-2. Selenium was added to match the level found in copepods (ca. 3 mg/kg) following methods described by Penglase et al. (2011). Taurine was added following the methods of Salze et al. (2011) (4 g/L for 18 hours). Vitamins C

and E were added following preliminary analyses demonstrating both to be lacking in UM Standard diet relative to other commercial enrichment diets.

Ingredient	UM Std	UM Plus
Dry yeast	45.0%	27.2%
Algamac 3050	42.5%	42.5%
Protein Plus	2.5%	2.5%
Sel-Plex (organic selenium)	0.0%	0.43%
Instant Algae Rotigrow Nanno	2.5%	2.5%
AstaRose (astaxanthin)	2.5%	2.5%
Ascorbyl palmitate	0.0%	10.0%
Soy lecithin	0.0%	5.0%
Alpha tocopherol	0.0%	2.4%
ARA	5.0%	5.0%
TOTAL	100%	100%

Table 9-2: Composition of UM diets used for enriching rotifers (from Benetti et al. 2008).

These samples were submitted to the ChemCentre for analysis of total nitrogen, amino acid profile (including taurine), iodine, total lipid, lipid class, total FAME, carbohydrates, ash, minerals (including selenium) and carotenoids. Sub-samples were also sent to the National Measurements Institute (NMI) for analysis of vitamins C and E. These samples were not prepared nor analysed with replication due to budget constraints and due to the fact they were solely for the purpose of general comparisons. Some of the results produced by these laboratories were highly erroneous and unreliable and further duplicate samples were subsequently submitted to CSIRO and the WA Department of Agriculture and Food (DAFWA) (for taurine analyses). These samples were of the actual enriched rotifers used in the larval rearing trial described in Section 9.3.3 below. Ori-Green samples were only analysed by CSIRO and DAFWA and not the ChemCentre.

9.3.2.2. Results and Discussion

Rotifer Protein & Amino Acid Profile (including taurine)

As rotifers contain high levels of non-protein nitrogen, their protein content is expressed as N x 4.48, rather than the usual N x 6.25 used for normal animal protein. The protein content (42-43%) and amino acid profile of all enriched rotifers in the preliminary ChemCentre analyses were very similar. This was the expected outcome as it has been previously shown that the protein and amino acid content of rotifer tissue cannot be manipulated via enrichment as these levels are genetically determined (Hamre et al. 2011). Most rotifer strains contain only 36-38% protein, but two other strains have previously been measured with levels of 42 and 48% protein. It therefore appears that the strain used at ACAAR in the production of YTK is similar to one of the latter, higher protein strains which is likely to be of benefit to fast growing YTK larvae.

The taurine content of all enriched rotifers (other than UM Plus) was very low and similar between enrichment diets (Figure 9-5). The level in Ori-Green enriched rotifers analysed by DAFWA was below the detectable limit of 0.01%DW, whilst those enriched on N-rich, Spresso and UM and analysed by the ChemCentre contained between 0.02% and 0.04%DW. This is consistent with other studies on rotifers and highlights that rotifers are highly deficient in taurine relative to copepods, which range in taurine content from 0.58% to 1.77%DW (Mæhre et al., 2013; Matsunari et al., 2005; van der Meeren et al., 2008). Given that most carnivorous pelagic marine fish lack the enzyme required to synthesise taurine from L-cysteine and methionine, including the closely related Japanese yellowtail, it is highly likely that taurine will be also important to YTK. The taurine content of rotifers enrichment on UM Plus and analysed by the ChemCentre was 2.53%DW, higher than that of most wild zooplankton. As previously described, we followed the method of Salze et al. (2011) for supplementing the UM Plus rotifers with taurine (4 g taurine /L). These authors did not report the resulting concentration of taurine in their live

foods, however, in a subsequent study (Salze et al., 2012), it was reported that this enrichment protocol resulted in a rotifer taurine level of approximately 0.08% on a wet weight basis. Assuming a rotifer water content of 85% (http://rotifersolutions.com/?p=29), this equates to a dry weight content of approximately 0.52%. Likewise, Rotman et al. (2012) enriched rotifers using a similar protocol to the current study (4 g/L for 12 hours) and reported the resulting taurine content on a WW basis. Again, assuming an 85% water content of rotifers, the reported values convert to approximately 0.73%DW, higher than that of Salze et al. (2012), but still significantly less than the 2.53%DW achieved in the current trial. In a study by Matsunari et al. (2005), rotifers were enriched with lower taurine concentrations of 0.4 to 1.2 g/L, which resulted in rotifer taurine concentrations ranging from ca. 0.35 to 0.60%DW. Figure 9-6 demonstrates the large differences between studies, and also shows that our results are more closely aligned with those of Matsunari et al. (2005) (assuming a continuing linear relationship) than Salze et al. (2012) and Rotman et al. (2012). The reasons for such variable results between studies are unclear. There is evidence that taurine uptake by rotifers is time dependent (Chen et al., 2005; Hawkyard et al., 2014b) and it is therefore possible that the differences in rotifer taurine content between studies are at least partially attributable to differences in enrichment time. Rotman et al. (2012), for example, enriched for 12 hours and Matsunari et al. (2005) for 22 hours compared with 18 hours in the current study. The enrichment time of Salze et al. (2012) was reported only as ‘per day’. To further elucidate the effect of enrichment dose rate on rotifer taurine content, we conducted further studies detailed in Section 9.3.7.

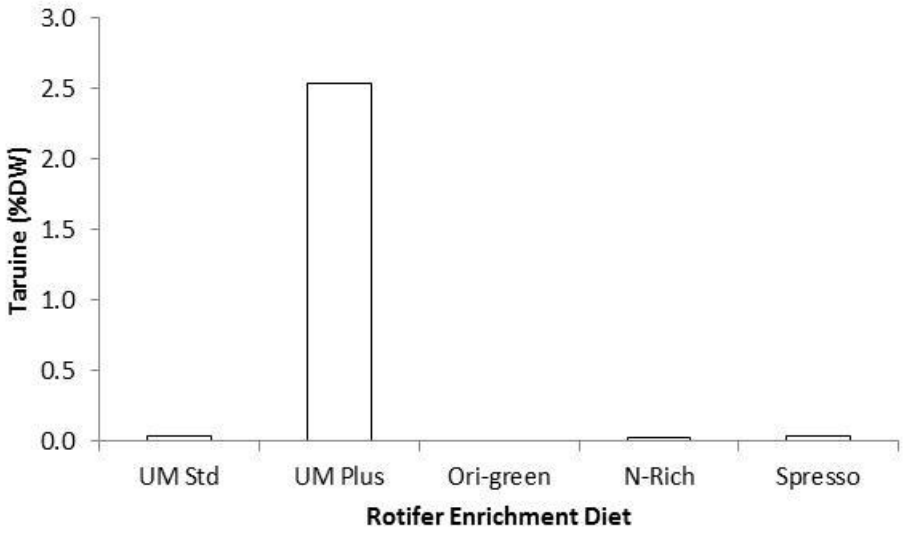

Figure 9-5: Taurine content of rotifers enriched on various products. Ori-Green samples analysed by DAFWA and all other samples by the ChemCentre.

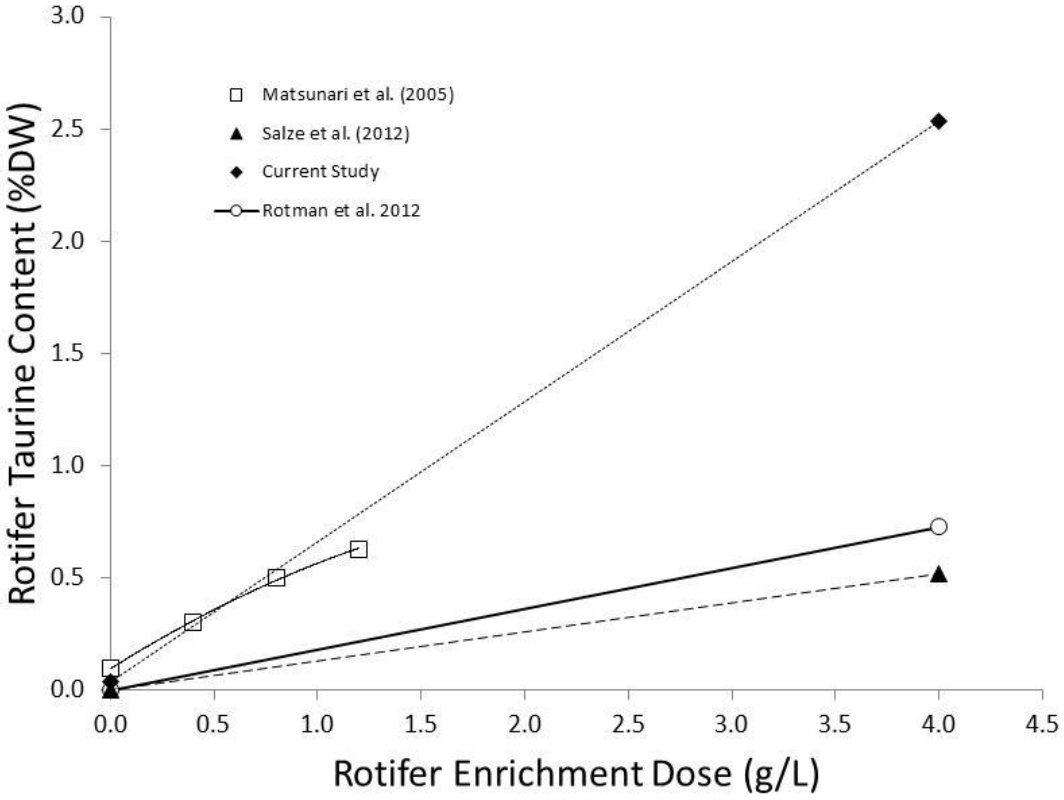

Figure 9-6: A comparison of the taurine content of rotifers from different studies.

Rotifer Ash and Carotenoids

Enriched rotifers had an ash content ranging from 11% (Spresso) to 19% (N-Rich PL plus). Given that ash is indigestible and has no nutritional value, N-Rich PL plus appears to be the least favourable enrichment diet from this point of view.

There were significant differences in the total carotenoids in rotifers enriched on the different diets and analysed by the CSIRO (P = 0.05)(Figure 9-7). Multiple t-tests revealed that N-Rich PL Plus enriched rotifers had higher carotenoids (227 ± 56 mg/kg) than all other treatments except Ori-Green (182 ± 48 mg/kg). The majority of the total carotenoids (>50%) in all enriched rotifers were canthaxanthin and its isomers and the concentrations and levels of significant differences followed those of the total carotenoids.

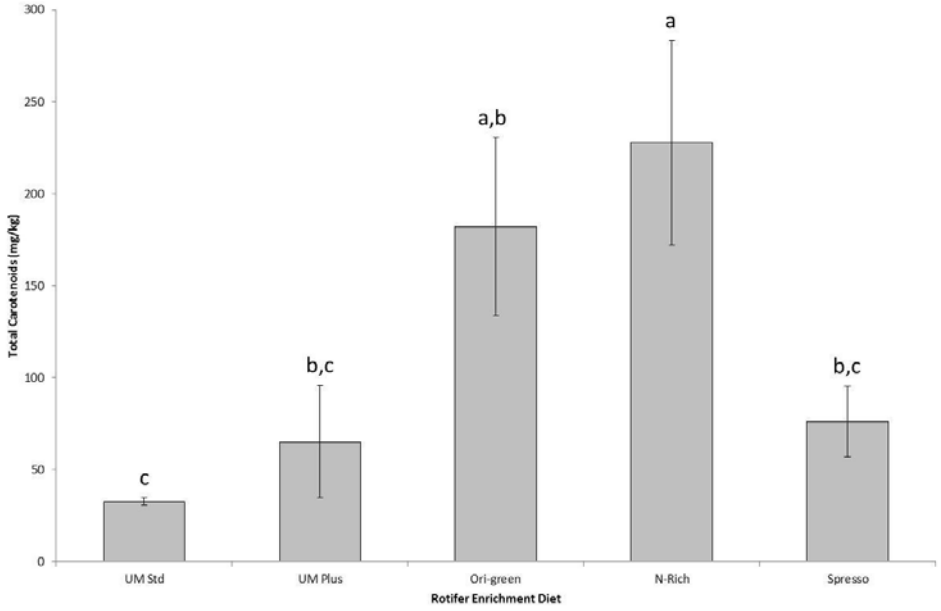

Figure 9-7: Total carotenoids (mg/kg) of rotifers enriched on various products. Columns sharing the same letter are not significantly different (P>0.05).

There was no effect of treatment diet on the level of astaxanthin (P = 0.4) (pooled average 20 ± 3 mg/kg) in enriched rotifers analysed by CSIRO and the preliminary samples analysed by the ChemCentre showed a similar pattern (Figure 9-8). These levels are significantly lower than those found naturally in copepods (413 to 1422 mg/kg)(van der Meeren et al., 2008). Despite both UM diets containing AstaRose, a natural source of astaxanthin

derived from a unique strain of yeast and containing 10,000 mg/kg of astaxanthin (http://www.aquafauna.com/Profiles-AstaRose.htm), rotifers enriched on these two diets had the lowest levels of carotenoids (33 ± 2 and 65 ± 30 mg/kg for UM Standard and UM Plus, respectively) and levels of astaxanthin no higher than the other diets, suggesting that the AstaRose is not well assimilated by the rotifers during the enrichment process. The slightly higher level of carotenoids and astaxanthin in the UM Plus diet may have been the result of the added lecithin to this diet, however any such improvement was only marginal and insufficient to reach the levels of astaxanthin seen in copepods.

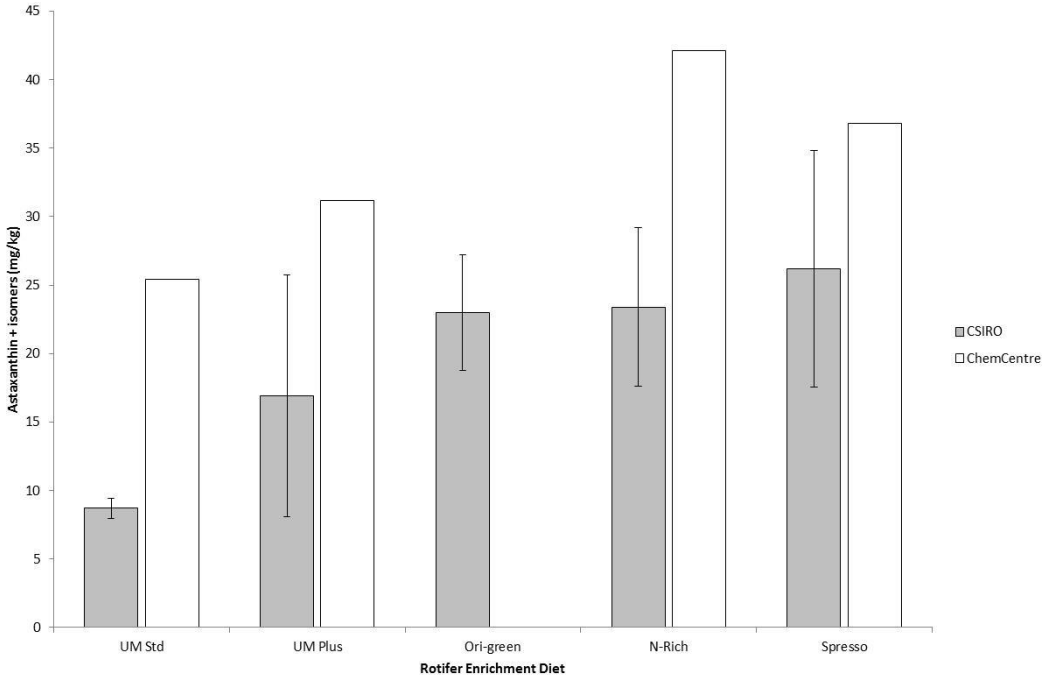

Figure 9-8: Asataxanthin content (mg/kg) of rotifers enriched on various products

Rotifer Selenium

The selenium content of rotifers in the preliminary samples analysed by the ChemCentre ranged from 0.1 to 0.4 mg/kg, significantly lower than copepods which typically contain approximately 3 mg/kg. The UM Plus enrichment diet (to which organic selenium had been added) produced rotifers containing 6.9 mg Se/kg; higher than our target value of 3 mg/kg. The selenium dose for UM

Plus enriched rotifers was subsequently reduced from 2.4 to 1.7 mg/million and a new duplicate set of rotifers enriched on all diets (including Ori-Green) was prepared and analysed by Murdoch University (Figure 9-9). The selenium content of rotifers enriched on Ori-Green was below the detectable level of 0.1 mg/kg and was subsequently excluded from the analysis of variance. The selenium content of rotifers enriched on Spresso (0.45 ± 0.05 mg/kg) was significantly higher than those enriched on N-Rich (0.15 ± 0.05 mg/kg) and UM Standard (0.10 ± 0.01 mg/kg). The reduction in the dose rate of organic selenium in the UM Plus diet was effective and the target dose of 3 mg/kg was achieved in the UM Plus enriched rotifers (3.05 ± 0.05 mg/kg).

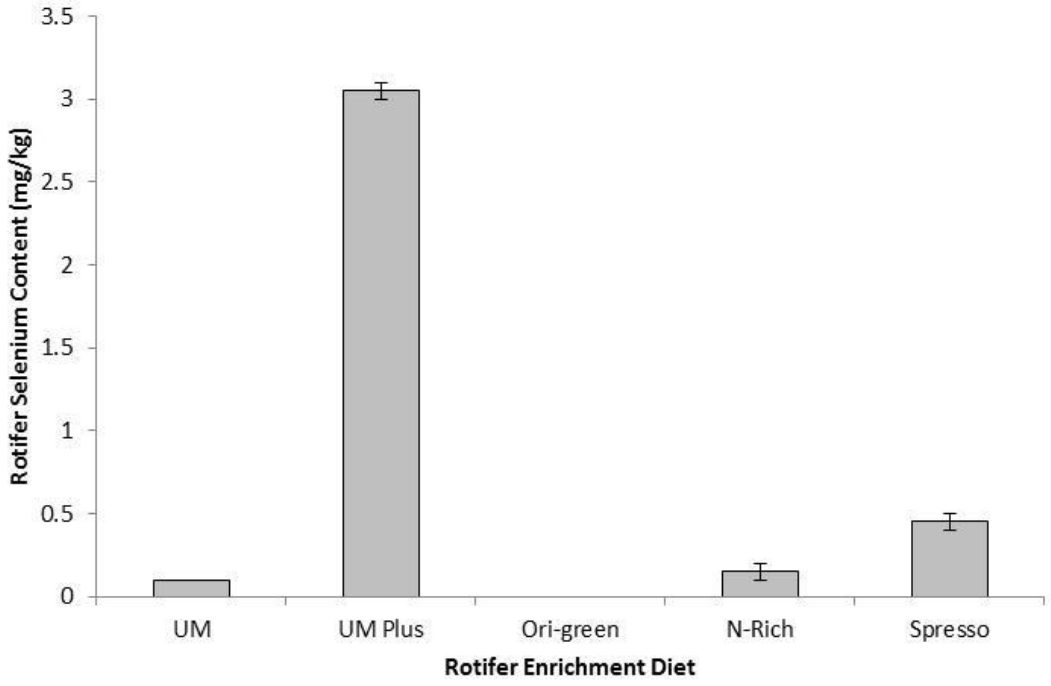

Figure 9-9: Selenium content of rotifers enriched on various products and analysed by Murdoch University.

Rotifer Lipids

Rotifer Total Lipid and Lipid Classes

The preliminary lipid class and fatty acid data provided by the ChemCentre was erroneous and could not be utilised. For example, it suggested that the rotifers enriched on marine microalgae and emulsions of marine lipids contained no DHA or EPA. All of the lipid data presented below are from the

analyses performed by CSIRO. All data are summarised in Table 9-3 and are described in further detail below.

Total lipid ranged from 10.5 ± 0.8% in Ori-Green enriched rotifers to 15.4 ± 0.8% in Spresso enriched rotifers (Figure 9-10). The total lipid content was not significantly different between treatments (P = 0.07) due to the high variation around the two UM Plus replicates. Exclusion of this treatment from the analysis resulted in a significant difference between treatments (P = 0.01), with the lipid content of Spresso being significantly higher than all other treatments, which did not differ from each other. The addition of lecithin to the UM Plus diet was necessary to emulsify the fat soluble vitamin E, but it did not appear to result in any further uptake of lipid by the rotifers. The level of total lipid in the Spresso enriched rotifers is very similar to that reported by Ma and Qin (2012)(15.77 ± 1.61%). These authors reported a higher lipid content for rotifers enriched on AlgaMac (18.5%) compared with those enriched on UM diets in the current study (ca. 11%), probably due to the fact that the UM diets contain only 42.5% AlgaMac.

Lipids were broadly categorised into two classes, polar lipids (phospholipids + glycolipids) and neutral lipids (triacylglycerols + diacylglycerols + free fatty acids + wax esters + hydrocarbons + sterols + sterol esters) and expressed as a percentage of total lipid (Figure 9-11).

Spresso enriched rotifers had the highest percentage of neutral lipids (54 ± 9%) and the lowest percentage of polar lipids (46 ± 9%) of all diets, whilst the two UM diets had the highest percentage of polar lipids (63%) (Figure 9-11). There were, however, no differences in lipid class composition between treatments (P = 0.33). The range of % polar lipid contents reported here are similar to the %phospholipid values reported by (Fernandez-Reiriz et al., 1993) of between 36% and 51% for rotifers enriched for different times on different INVE enrichment products. The slightly higher values we obtained may be due to the fact that our % polar lipids included glycolipids in addition to phospholipids.

	UM Std	UM Plus	Ori-green	N-Rich	Spresso	*p**
%Total Lipid	11.3 ± 0.2	11.8 ± 1.7	10.5 ± 0.8	11.4 ± 0.4	15.4 ± 0.8	0.07/0.01
% Polar Lipid	63 ± 5	63 ± 8	62 ± 1	56 ± 2	46 ± 9	0.33
Total Fatty Acids (mg/100g)						
DHA	1687 ± 90	1736 ± 458	803 ± 73	1666 ± 227	2080 ± 223	0.1/0.02
EPA	436 ± 26	422 ± 40	461 ± 37	469 ± 35	551 ± 11	0.16
ARA	243 ± 10^{a}	249 ± 42^{a}	33 ± 4^{b}	12 ± 1^{b}	213 ± 15^{a}	0.0009
DHA:EPA	3.9 ± 0.0^{a}	4.0 ± 0.7^{a}	1.7 ± 0.0^{b}	3.5 ± 0.3^{a,b}	3.8 ± 0.3^{a,b}	0.02
Fatty acids in Phospholipid fraction (mg/100g)						
DHA	273 ± 29^{a}	250 ± 20^{a,b}	101 ± 16^{c}	149 ± 15^{b,c}	206 ± 21^{a,b,c}	0.009
EPA	136 ± 11	119 ± 4	133 ± 20	105 ± 4	122 ± 19	0.54
ARA	80 ± 19^{a}	76 ± 2^{a}	9 ± 2^{b}	4 ± 0^{b}	49 ± 3^{a,b}	0.004
DHA:EPA	2.0 ± 0.1^{a}	2.1 ± 0.2^{a}	0.8 ± 0.0^{b}	1.4 ± 0.1^{a,b}	1.7 ± 0.1^{a}	0.003
%DHA in Phospholipid	16 ± 3%	15 ± 3%	13 ± 1%	9 ± 0%	10 ± 2%	0.17
%EPA in Phospholipid	31 ± 4%	28 ± 4%	29 ± 2%	22 ± 1%	22 ± 4%	0.29
%ARA in Phospholipid	33 ± 6%	31 ± 4%	28 ± 2%	36 ± 1%	23 ± 3%	0.3

Table 9-3: Key total lipid and phospholipid data for rotifers enriched on various diets.

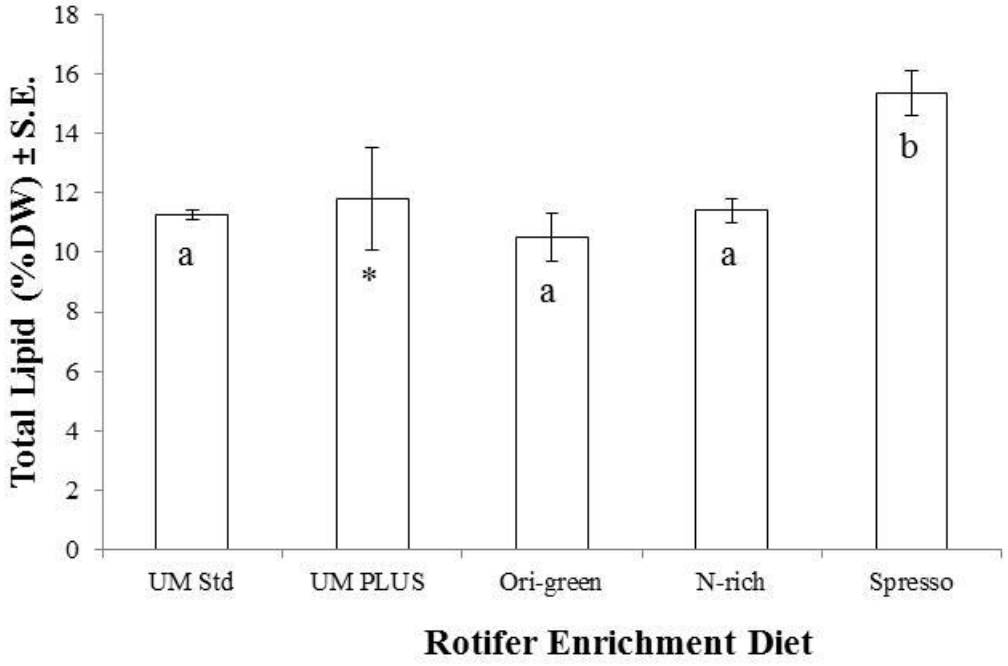

Figure 9-10: Total lipid content of rotifers enriched on various diets. Columns sharing the same letter are not significant different (P > 0.05). * excluded from ANOVA.

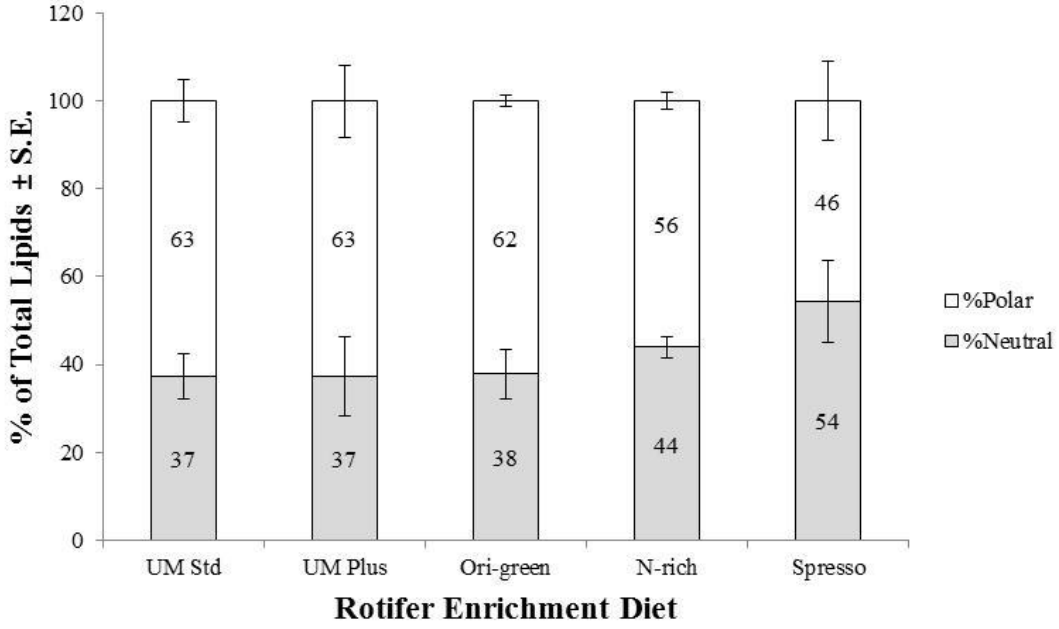

Figure 9-11: The ratio of polar and neutral lipids in rotifers enriched on various products.

Rotifer Fatty Acid Profiles in Total Lipid and within the Phospholipid Class

The absolute and relative amounts of DHA, EPA and ARA in the total lipids and phospholipid fractions of the rotifers enriched on the various diets are shown in Table 9-3, along with various important EFA ratios.

Largely due to Spresso enriched rotifers having the highest lipid content, they also had the highest absolute amount of DHA in total lipid (2080 ± 223 mg/100 g). Ori-Green enriched rotifers had the lowest amount of DHA in total lipid at 803 ± 73 mg/100 g. Despite this large range there was no difference in the total DHA content between rotifers enriched on the various diets; again due to the high variation between the two UM Plus replicates. Excluding UM Plus from the analysis shows the absolute amount of DHA in the total lipid to be significantly higher in Spresso enriched rotifers compared with Ori-Green enriched rotifers ($P = 0.02$) and with no other differences between treatments. Within the phospholipid fraction, those rotifers enriched on Ori-Green had significantly less DHA (101 ± 16 mg/100g) than those enriched on UM Standard (273 ± 29 mg/100g) and UM Plus (250 ± 20 mg/100g) (Figure 9-12). The proportion of the total DHA that was found in the phospholipid fraction ranged from 9 to 15% (Table 9-3), with no significant difference between treatments. This relatively small percentage is believed to be one of the limitations in the use of enriched rotifers for marine fish larvae. In copepods, a much higher percentage of the total DHA is found with the phospholipid fraction (ca. 30% (Coutteau, 1997)).

There was no difference in the total EPA concentration in enriched rotifers between treatments (422 to 551 mg/100 g) ($P = 0.16$), nor in the amount of EPA within the phospholipid fraction (105 to 136 mg/100g)($P= 0.54$)(Figure 9-13). The proportion of the total EPA found in the phospholipid fraction was therefore also similar between treatments (22 to 31%) and unlike DHA, this value is similar to copepods (ca. 30%)(Coutteau, 1997).

Both the total amount of ARA and the amount of ARA in the phospholipid fraction of enriched rotifers was significantly affected by treatment ($P = 0.0009$

and 0.004, respectively)(Figure 9-14). Those rotifers enriched on Ori-Green and N-Rich had significantly lower total ARA (33 ± 4 and 12 ± 1 mg/100g, respectively) and phospholipid ARA (9 ± 2 and 4 ± 2 mg/100 g, respectively) than all other treatments. The percentage of the total ARA found in the phospholipid fraction was similar between treatments (23 to 36%; P = 0.3). The amount of total ARA expressed as a % of total lipid for Spresso and UM diets were1.7% and 2.9%, respectively. This compares very favourably with the results of Ma and Qin (2012) who found 1.66 ± 0.04% ARA in Spresso enriched rotifers and 2.35 ± 0.14% in AlgaMac enriched rotifers. The slightly higher percentage found in the current study may indicate that the ARA powder added to the UM diets is effective in slightly increasing the content of ARA in UM enriched rotifers. Hamre et al. (2007) attributes poor pigmentation and eye migration in flatfish larvae to a high ARA concentration and points out that whilst most enriched *Artemia* contain > 2% of their fatty acids as ARA copepods contain <1%. If high concentrations of ARA are also detrimental to round fish larvae such as YTK then the UM diets and Spresso are the least favourable rotifer enrichment diets in this regard.

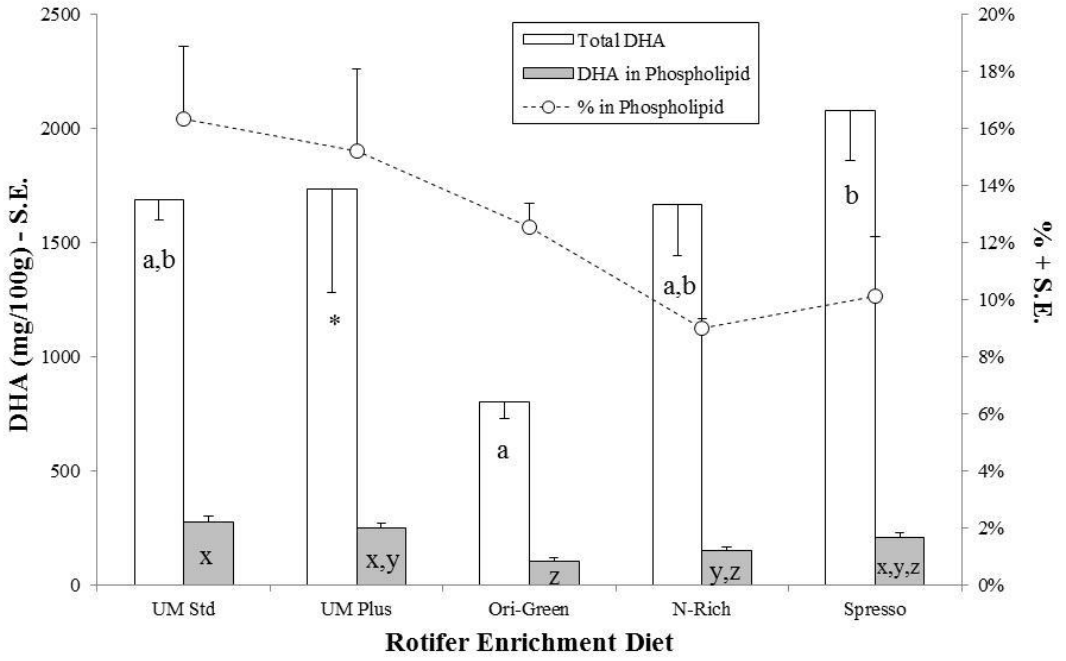

Figure 9-12: Quantity of DHA in total lipid and phospholipid fractions and the % of DHA found within the phospholipid fraction of enriched rotifers. Columns sharing the same letter are not significantly different.

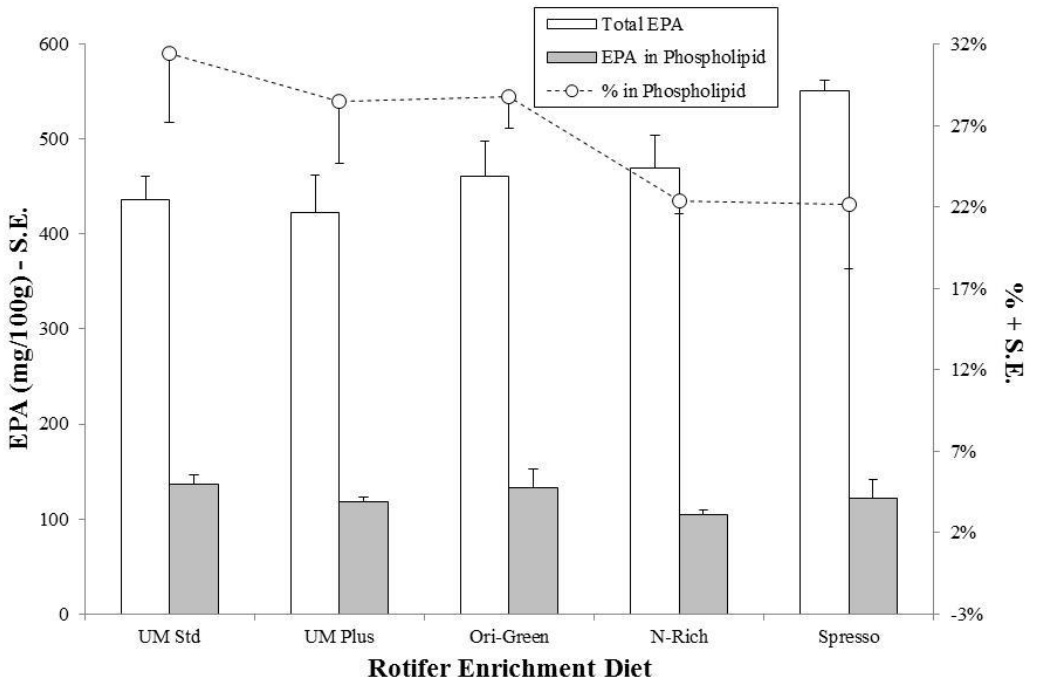

Figure 9-13: Quantity of EPA in total lipid and phospholipid fractions and the % of EPA found within the phospholipid fraction of enriched rotifers. Columns sharing the same letter are not significantly different.

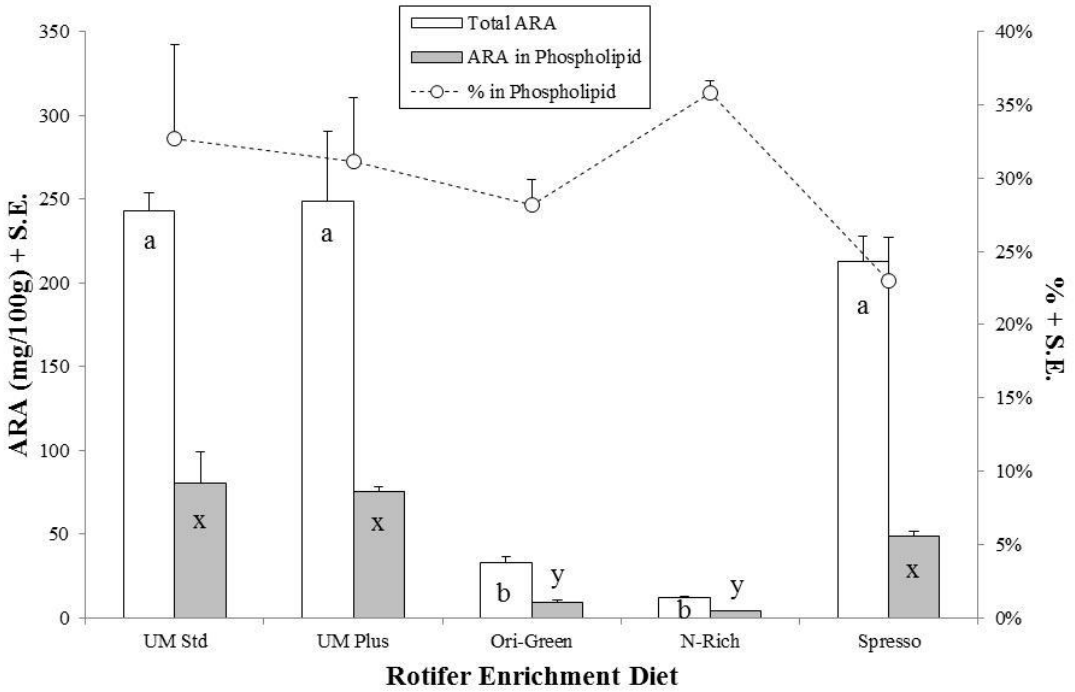

Figure 9-14: Quantity of ARA in total lipid and phospholipid fractions and the % of ARA found within the phospholipid fraction of enriched rotifers. Columns sharing the same letter are not significantly different.

In both total lipid and the phospholipid fraction, those rotifers enriched on Ori-Green had a significantly lower DHA:EPA ratio (1.7 ± 0.0 and 0.8 ± 0.0) than all other treatments, which did not differ from each other (pooled average 3.8 and 1.8, respectively)(Figure 9-15). The DHA:EPA ratio we achieved in total lipid for Spresso enriched rotifers (3.8 ± 0.3) is similar to that reported by Ma and Qin (2012)(4.8 ± 0.12). The ratio found in AlgaMac enriched rotifers by Ma and Qin (2012) was, however, 10.8, much higher than the ratio of 4.0 found in UM enriched rotifers in the current trial. We believe this is due to the longer enrichment time used in the current study, which enables further retroconversion of DHA into EPA and this is discussed in further detail below (Section 9.3.4.3). Given that a high DHA:EPA ratio is considered ideal for marine fish larvae, the UM diets and Spresso appear superior in this regard.

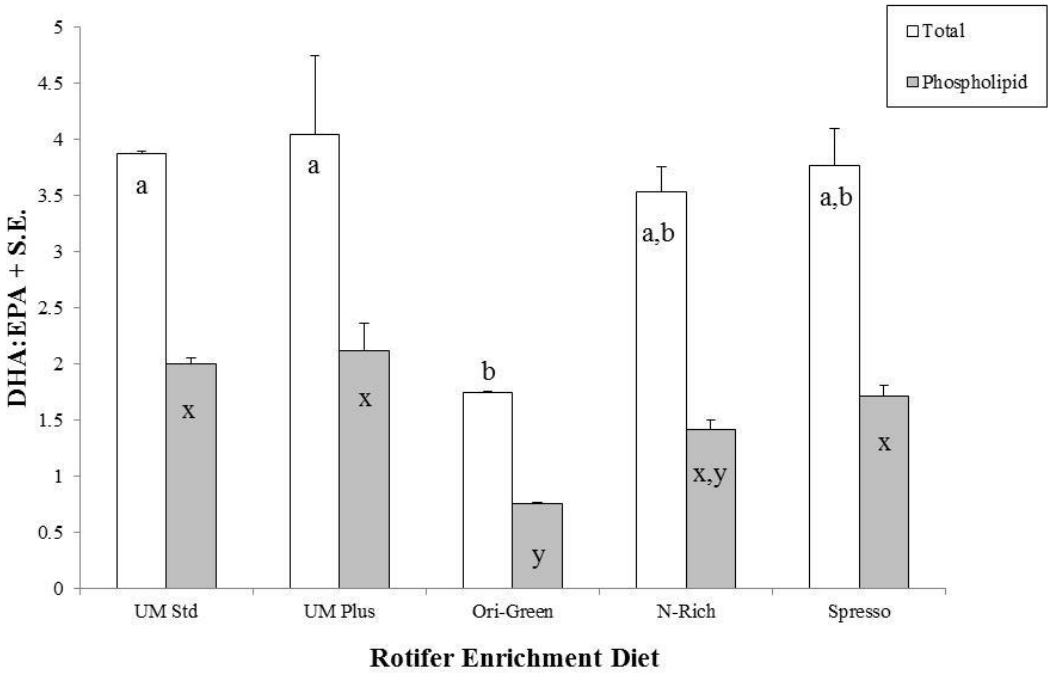

Figure 9-15: DHA:EPA ratios in total lipid and phospholipid fractions of enriched rotifers. Columns sharing the same letter are not significantly different.

The EPA:ARA ratio in total lipid was significantly higher in rotifers enriched on N-Rich (39.4 ± 1.0) than all other treatments (Figure 9-16). The value in Ori-Green (14.1 ± 0.4) was significantly higher than the Spresso (2.60 ± 0.13) and UM treatments (1.76 ± 0.09), which did not differ from each other. The values found in N-Rich and Ori-Green compare more favourably to the values found

in copepods (23-27)(van der Meeren et al. 2008) than all other treatments. The value we achieved in Spresso (2.60 ± 0.13) is very similar to that reported by Ma and Qin (2012)(2.46 ± 0.02), whilst the values we achieved from the UM diets (1.76 ± 0.09) is slightly higher than that reported by Ma and Qin (2012) for AlgaMac enriched rotifers (1.38 ± 0.24). Interestingly, Ma and Qin (2012) attributed the poor performance of larval YTK fed AlgaMac enriched rotifers to the low EPA:ARA of this diet and this is discussed in further detail below (see Section 9.3.2.3). Whilst a high EPA:ARA ratio (from a low ARA content) has been implicated in successful pigmentation of metamorphosing flatfish (Hamre et al., 2007)), its importance to round fish larvae has not been well researched.

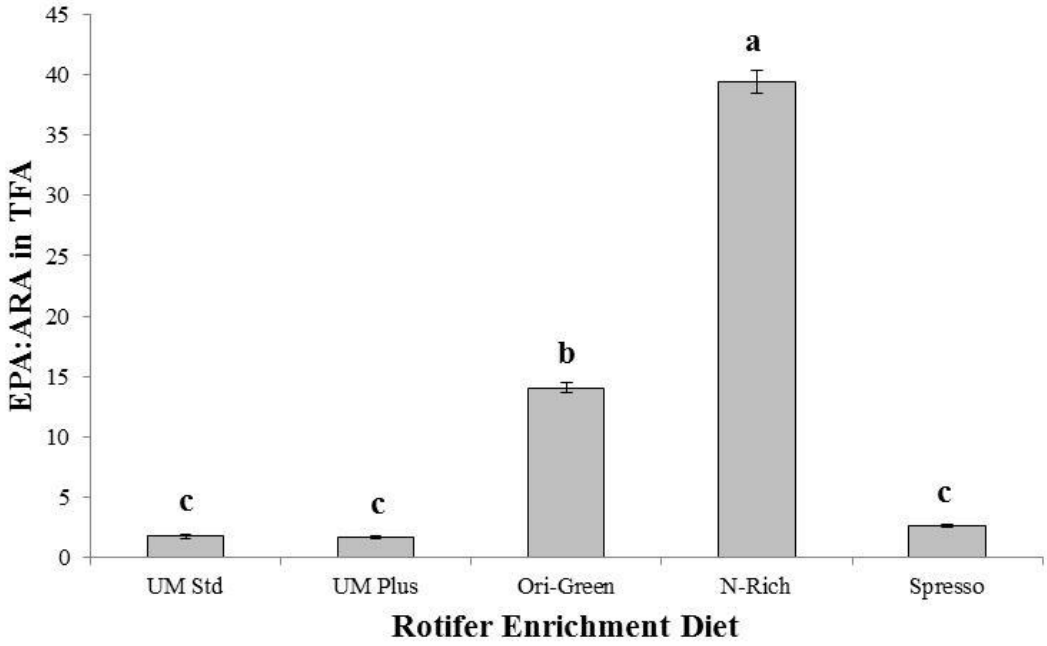

Figure 9-16: EPA:ARA ratios in total lipid and phospholipid fractions of enriched rotifers. Columns sharing the same letter are not significantly different.

Rotifer Vitamins

Rotifer vitamin C content was significantly affected by enrichment diet (P = 0.01)(Figure 9-17). Those rotifers enriched on UM Standard had a significantly lower content of vitamin C (324 ± 49 µg/g DW) than all other treatments except N-Rich PL Plus (638 ± 91 µg/g DW). Supplementing the

UM Plus diet with vitamin C in the form of ascorbyl palmitate was effective in increasing the level to that found in Spresso (797 ± 54 µg/g DW) and Ori-green (791 ± 46 µg/g DW) enriched rotifers.

Rotifer vitamin E (free α tocopherol) content was also affected by enrichment diet (P = 0.03)(Figure 9-17). Again, those rotifers enriched on the UM Standard diet had the lowest vitamin E content (103 ± 11 µg/g DW), significantly lower than only Spresso (343 ± 78 µg/g DW). Supplementing the UM Plus diet with alpha tocopherol doubled the vitamin E content of the rotifers to 231 ± 11 µg/g DW, relative to those enriched on UM Standard, but this difference was not significant.

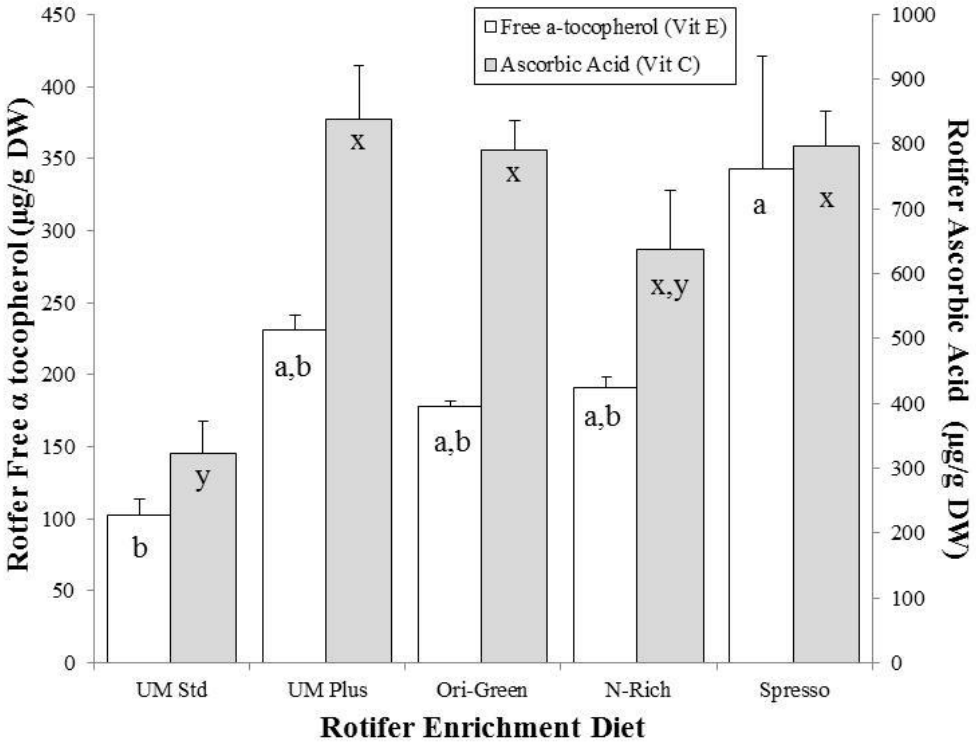

Figure 9-17: α-tocopherol (vitamin E) and ascorbic acid (vitamin C) contents of rotifers enriched on different diets. Columns sharing the same letter are not significantly different.

9.3.2.3. Conclusions

These analyses demonstrated some significant differences in the nutritional composition in rotifers enriched on different enrichment products that could potentially result in significant differences to the growth, survival and malformation rates of YTK larvae.

As expected, all diets were deficient in taurine and selenium relative to wild zooplankton and the level of both were successfully elevated in the UM Plus diet.

Within the three commercial diets of Spresso, N-rich and Ori-Green there were differences in the levels of ash, lipid and essential fatty acids both within the total lipid and phospholipid fractions. Spresso enriched rotifer generally had better levels and ratios of essential fatty acids than N-Rich and Ori-Green rotifers in both the total and phospholipid fractions. Whilst N-Rich and Ori-Green had higher levels of total carotenoids, all diets had similar levels of astaxanthin. Spresso, Ori-Green and N-Rich enriched rotifers had similar levels of vitamins C and E, whilst UM Standard was lacking in both. The addition of lecithin, ascorbyl palmitate and was effective in increasing the levels of vitamins C and E in the UM Plus enriched rotifers.

Ori-Green was deficient in a number of components relative to the other diets. This may have been the result of the very short enrichment period that the manufacturers recommend for this product, which may result in only ‘gut loading’ rather than changes in the biochemical composition of the rotifers themselves. Trials investigating the effects of longer enrichment periods would be required to test this hypothesis.

The major component of the UM diets is AlgaMac 3050. In a study by Ma and Qin (2012), YTK larvae exhibited significantly lower survival when fed rotifers enriched on AlgaMac 3050, compared with those enriched on live microalgae or Spresso. The former rotifers had a very high percentage of DHA in total

lipid (34.5 ± 2.1%), considerably higher than those enriched on UM diets in the current study (19.8 ± 1.1%). Furthermore, those rotifers enriched on AlgaMac 3050 by Ma and Qin (2012), had considerably lower %EPA in total lipids (3.2 ± 0.5%) than those enriched on UM diets in the current study (5.0 ± 0.3%). This difference resulted in Ma and Qin's rotifers having a DHA:EPA ratio of 10.8 ± 1.0, compared with only 3.8 ± 0.3 in the current study. It has been previously demonstrated that AlgaMac 3050 itself is very high in DHA and contains virtually no EPA. Rotifers, however, have the capability to retroconvert DHA into EPA (Barclay and Zeller, 1996). The differences in DHA and EPA contents of the rotifers enriched by Ma and Qin and the current study is likely due to the shorter enrichment time employed by the former authors (12 hours) compared to the 18 hours used in the current study, reducing the time for such retroconversion to occur. With a longer enrichment period, retroconversion both decreases the amount of DHA and increases the amount of EPA and thereby improves the DHA:EPA ratio. Given that AlgaMac 3050 constituted 42.5% of the 300 mg/L enrichment dose for the UM diets, this resulted in rotifers receiving 127 mg/L of AlgaMac 3050; lower than that used by Ma and Qin (2012). This may have contributed to the lower DHA content of the rotifers in the current study, but the lower dose alone cannot account for the differences in EPA or DHA:EPA ratio between the two studies. In a study by Barclay & Zeller (1996) rotifers enriched on AlgaMac for an even longer period of 24 hours achieved a DHA content of 7.3% and an EPA content of 5.3% (DHA:EPA = 1.4) supporting our hypothesis that the DHA:EPA ratio decreases with time as more DHA is retroconverted to EPA.

9.3.3. Part 2: A comparison of the performance of YTK larvae fed rotifers enriched on three enrichment products

9.3.3.1. Introduction

Following the aforementioned biochemical analyses, a larval rearing trial was conducted in which the performance of YTK larvae was compared when fed on rotifers enriched with either Spresso, UM Standard or UM Plus. Only three treatments could be included in the trial due to the number of available larval rearing tanks. Spresso was selected as the current industry standard, whilst UM and UM Plus were included to determine if improvements would be achieved through the increased levels of selenium, taurine and vitamins C and E.

9.3.3.2. Materials and Methods

Each treatment was tested with 4 replicates using the ACAAR larval rearing system described in detail in Section 9.2.2. Larvae were stocked at a density of 53 /L and fed on rotifers enriched following same protocols described above (Section 9.3.2.1) from 3 to 12 dph using the hybrid rotifer feeding regime described in Section 9.2.2. Larval standard length and dry weight was measured on days 4, 6 8, 10 and 12 and rotifer mastaxes counted in larval guts on days 5, 7, 9 and 11 as described in Section 9.2.2. The trial was concluded on 13 dph when all remaining larvae in each tank were hand-counted to determine survival. A subsample was collected, washed in freshwater and frozen at -80°C, then freeze dried. Samples were then ground and analysed for selenium and taurine. Malformation rates were not assessed at the conclusion of this trial as it was discovered from the previous trial that larvae at the end of the rotifer stage are not large enough to detect malformations.

Subsamples of the enriched rotifers fed to the larvae during the rearing trial were sampled on three separate occasions directly the from enrichment tanks, rinsed in freshwater and frozen at -80°C for biochemical analyses. Two of the three samples were analysed by the CSIRO for lipids, pigments and vitamins and the results were presented in the previous section. Taurine

content of the rotifers was analysed by DAFWA. Whilst it was our intention to have fully analysed and interpreted the enrichment data prior to a larval rearing trial, this was not achievable due to the delays and erroneous data received from the ChemCentre.

9.3.3.3. Results and Discussion

Survival of YTK larvae to 13 dph ranged from 16 ± 5% to 19 ± 8% and did not differ between treatments (P = 0.9; Figure 9-18). This level of survival falls within the normal range achieved in other studies on YTK during the rotifer period. Stuart and Drawbridge (Stuart and Drawbridge, 2011) for example achieved 9.2 ± 3.1% survival in their best performing treatments to 16 dph. In our previous experiment described in Section 9.2, we achieved survival rates between 17 and 28% to the same age (13 dph) and Ma and Qin (2012) achieved survival rates ranging from 10 to 41% to 12 dph.

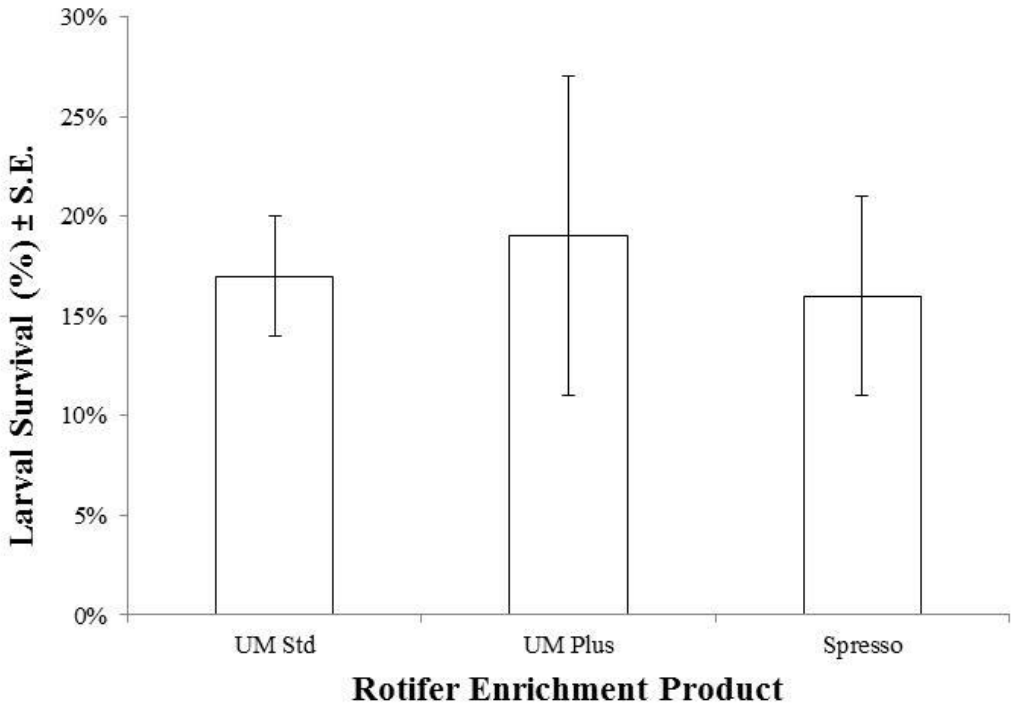

Figure 9-18: Survival of YTK larvae reared to 13 dph on rotifers enriched with different products.

Repeated measures analysis of variance demonstration that rotifer ingestion by larvae significantly increased with age (P<0.001) but was not affected by treatment (P = 0.49) (Figure 9-19). Despite previous suggestions in the

literature that taurine may assist in retinal development via improved utilisation of dietary DHA, our observation that rotifer ingestion was equal across treatments suggests that the taurine level in the UM Plus rotifers did not improve the vision of these larvae to the extent where prey capture was improved under the environmental conditions employed in this trial. As a result of equal rotifer ingestion rates, growth of larvae in terms of both length and dry weight was also equal across treatments (P = 0.58 and 0.71, respectively)(Figure 9-20). On 12 dph larvae had an average standard length of 6.30 ± 0.07 mm and an average dry weight of 0.30 ± 0.05 mg/larvae (pooled across treatments). This rate of growth is much greater than that achieved by Ma and Qin (2012) who achieved a standard length of 5.59 ± 0.06 in their fastest growing treatments by 14 dph and by Ma and Qin (2012) who appeared to achieve a standard length of only 4.84 mm in the best performing treatments by 12 dph. These data demonstrate that conditions and general performance of the larvae was acceptable.

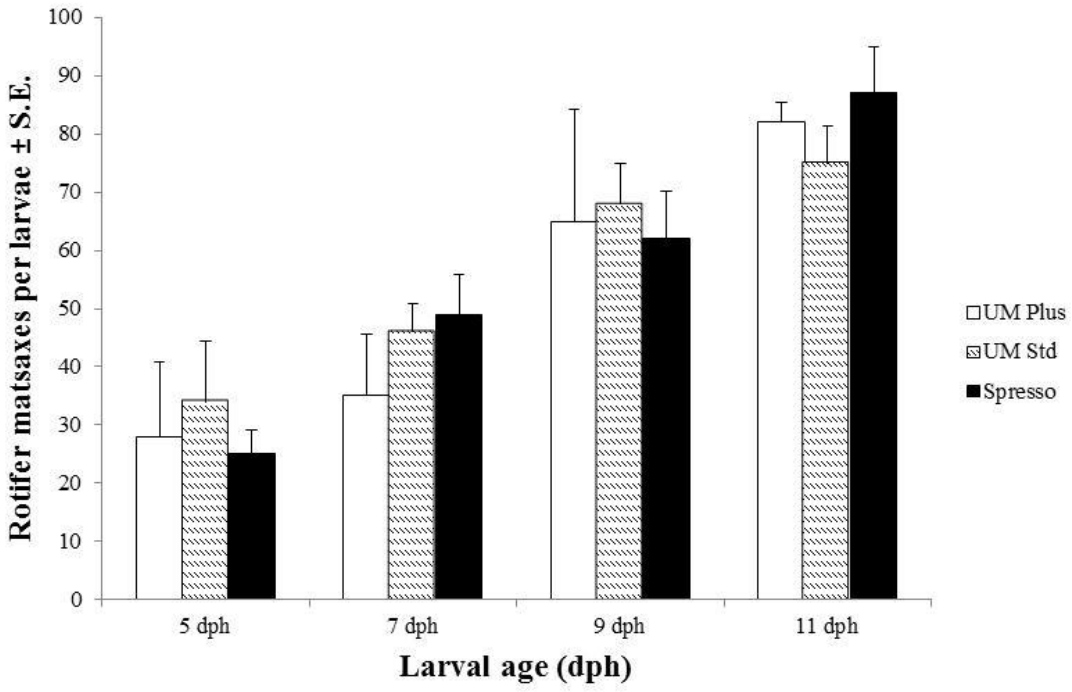

Figure 9-19: Ingestion rates by YTK larvae of different ages on rotifers enriched with different products.

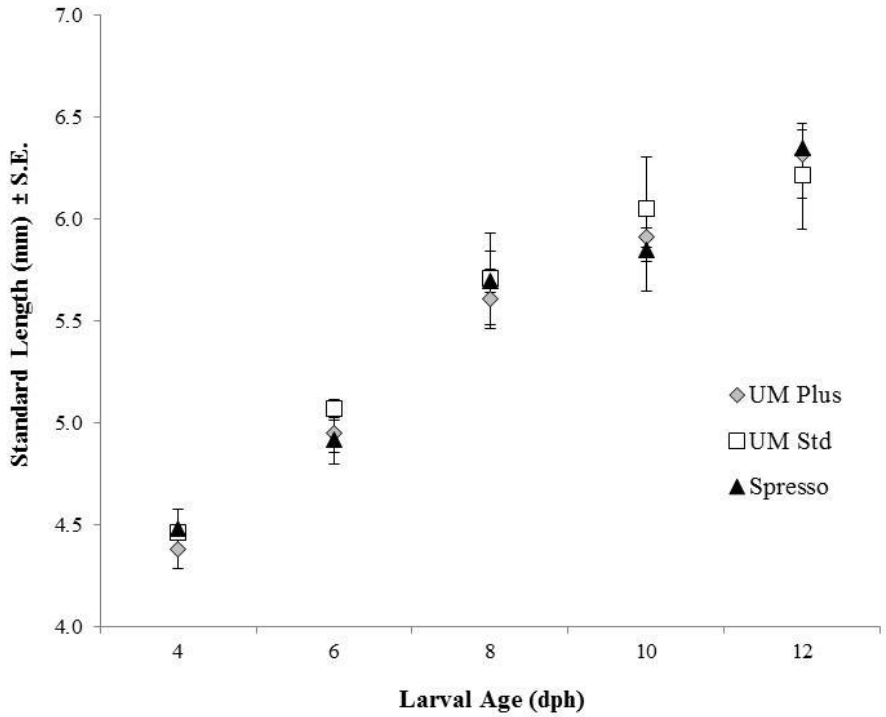

Figure 9-20: Growth (standard length) of YTK larvae fed rotifers enriched with different products.

The whole-body selenium content of larvae at the conclusion of the trial was significantly affected by the rotifers on which they were fed ($P < 0.0001$, Figure 9-21). Those larvae fed on rotifers enriched with UM Plus had a significantly higher whole-body selenium content (4.5 ± 0.6 mg/kg) than those fed on UM Standard (0.7 ± 0.0 mg/kg) and Spresso (1.1 ± 0.1 mg/kg), which did not differ from each other. These data demonstrate that the selenium incorporated into the UM Plus enriched rotifers was effectively transferred to the larvae. The levels of selenium in the Se-enriched and unenriched YTK larvae were very similar to those found in cod *Gadus morhua* larvae receiving enriched (3.99 ± 0.15 mg Se/kg) and unenriched rotifers (0.88 ± 0.02 mg Se/kg)(Penglase et al., 2010). In the aforementioned study by Penglase et al. (2010), no difference in growth or malformation rates were found between cod larvae fed rotifers enriched with or without selenium, however cod larvae fed selenium enriched rotifers had a higher expression and activity of Se-dependent glutathione peroxidases. Whilst we hypothesised that the warm-water and faster growing YTK larvae may benefit more than cod-larvae from the antioxidant properties of selenium, we also found no significant benefit in terms of growth in YTK larvae.

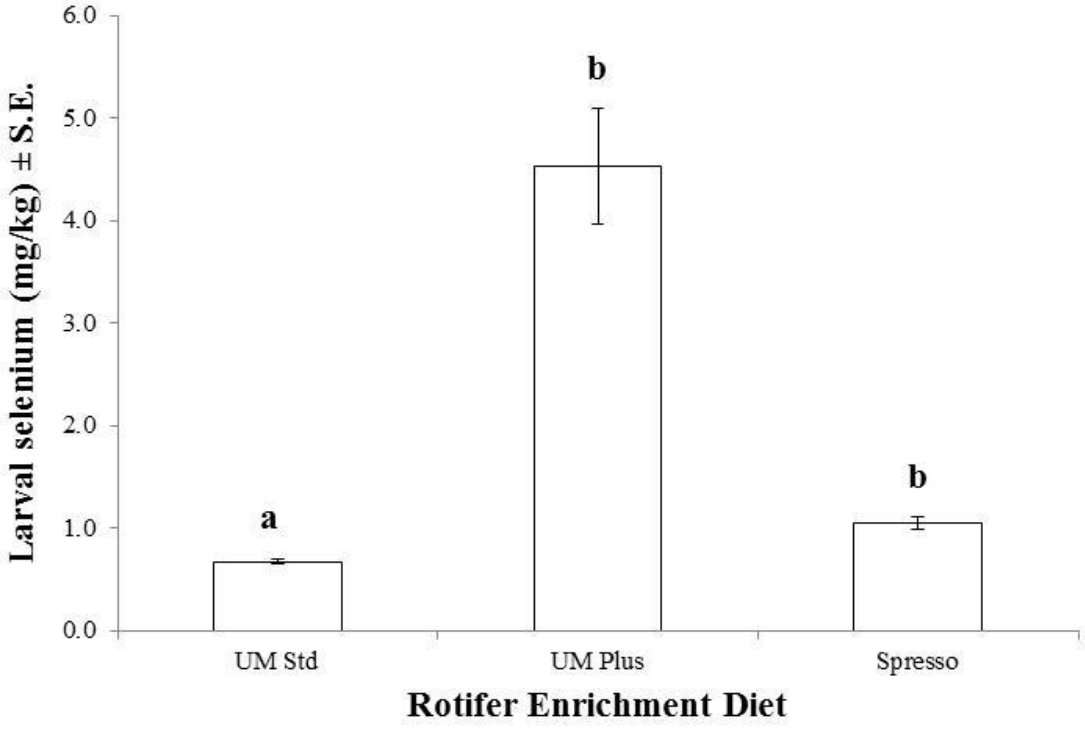

Figure 9-21: Whole body selenium contents of 13 dph YTK larvae fed rotifers enriched on different products.

The rotifers fed to the larvae in the current trial and analysed by DAFWA contained 3.47 ± 0.42%DW of taurine, even higher than the value reported by the ChemCentre, which was higher than other studies which have used similar enrichment protocols (see Section 9.3.2.2). We are unsure whether this difference was due to differences in analytical methods between the ChemCentre and DAFWA or another factor.

Rotman et al. (2012) enriched rotifers using a similar protocol to the current study (4 g/L for 12 hours) and fed them to YTK larvae for 10 days. As previously described (see Section 9.3.2.2), the taurine content of these rotifers was reported on a WW basis, which we have calculated equates to a dry weight content of approximately 0.73%DW; significantly lower than the values we achieved. Unlike the current study, survival to 10 dph was found by to be significantly better in those larvae fed taurine enriched rotifers. Larvae were bigger in the taurine-supplemented treatment (5.43 mm vs 5.13 mm),

however these differences weren't significant. Interestingly, both the survival and growth rates achieved in the taurine supplemented treatment (20% and 5.43 mm TL) in Rotman's study were similar that achieved in the control treatments in the current study (average survival 17% and TL at 10 dph = 6.00 mm). These results suggest that the taurine content of the rotifers in the current study may have been too high. Section 9.3.7 describes a further study in which we investigated the effect of taurine dose rate on the taurine content of rotifers.

Larvae fed rotifers enriched on UM Plus had significantly higher whole-body taurine levels (1.9 ± 0.1%DW) than those fed rotifers enriched on Spresso (0.2 ± 0.1%DW) and UM Standard (0.3 ± 0.0%DW)(Figure 9-22). Japanese yellowtail reared on non-taurine enriched diets had a similar whole-body taurine content on 15 dph to those in the current trial (0.2%DW), whilst wild caught juvenile Japanese yellowtail of ca. 30 mm in length, have been shown to contain 2.3%DW taurine, a value similar to those fed taurine enriched rotifers in the current trial (1.9%) (Matsunari, 2003; Yamamoto et al., 2008). Likewise, in the aforementioned study by Rotman et al. (2012), those larvae fed taurine-enriched rotifers to 10 dph contained approximately 1.6%DW taurine (calculated from a wet content of 0.24% and a larval water content of 85%). Furthermore, cod larvae reared for 25 days on non-taurine enriched rotifers had a whole-body taurine content of 0.14%DW whilst those fed on rotifers enriched with 1.2 g/L of taurine had a whole-body content of 1.1%DW (Matsunari et al., 2005). Therefore, despite the very large difference in taurine content between wild zooplankton (range 0.6 to 1.2%DW) and the enriched rotifers used in the current trial (3.47%DW), the final whole-body content of wild Japanese yellowtail juveniles and larvae from the current trial appear to be similar. Combined, these data suggest that the larval whole-body taurine content in Seriola probably has a maximum 'saturation' value close to 2% and that increases in the taurine content of the live prey past the values naturally seen in wild zooplankton (maximum value ca. 1.2%) are probably not required. The effects of different taurine enrichment dose rates on rotifer taurine content and the subsequent performance and whole-body taurine content of YTK larvae are described in Section 9.3.7.

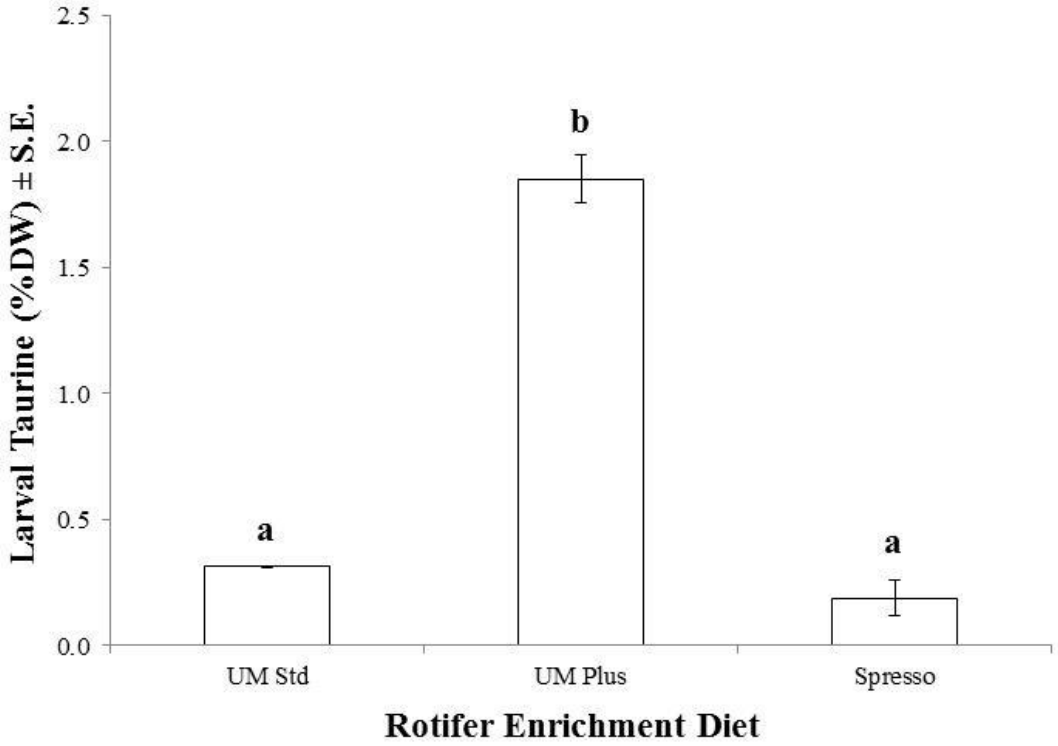

Figure 9-22: Whole body taurine contents of 13 dph YTK larvae fed rotifers enriched on different products.

In terms of taurine supplementation, the results of this study are consistent with those in larval sole and seabream, which demonstrated no benefit of enriching rotifers with taurine (Pinto et al., 2010; Pinto et al., 2013b). However, in these species, feeding taurine-enriched rotifers did not result in an increased level of taurine in the whole body of the larvae, which is in contrast to the current study. Cod larvae, on the other hand, increased their whole body content of taurine and grew significantly faster (14% faster to 25 dph) when fed rotifers enriched with taurine (Matsunari et al., 2005). There was no effect of taurine supplementation on cod larval survival, similar to seen in the current study (Matsunari et al., 2005). Chen et al. (2004) also found no significant improvement in survival in red sea bream by 20 dph fed rotifers with and without taurine enrichment. In a subsequent paper, Chen et al. (2005) reported similar survival results for Japanese flounder at 16 dph fed rotifers with and without taurine enrichment. Northern rock sole (*Lepidopsetta polyxystra*) fed rotifers enriched to contain 0.28%DW of taurine grew significantly faster than those fed on control rotifers containing 0.04% taurine

and the former had a higher whole body taurine content (0.43%) than the latter (0.08%DW).

Salze et al. (2011) achieved both significantly higher growth and survival in cobia larvae fed taurine-enriched rotifers and *Artemia* to 27 dph and the degree of improvement in both factors was far greater than seen in other species for which improvements due to taurine enrichment have been reported. It was not determined whether the benefits seen in this study were accrued during the rotifer stage, *Artemia* stage or a combination of both. In a subsequent study (Salze et al., 2012) it was determined that taurine supplementation significantly enhances trypsin and lipase activity during early larval development and pepsin-like enzymatic activity from 16 dph. That we achieved no improvement in growth during the rotifer feeding phase suggests that either taurine does not have the same mode of action on trypsin and amalyse activity in YTK, or that any increases in these activities as a result of taurine supplementation do not lead to significant improvements in prey digestibility and growth. That taurine induced a very significant increase in pepsin activity in cobia from 16 dph and given that YTK develop gastric glands and acid-digestion from approximately the same age (Chen et al., 2006a; Chen et al., 2006b) suggests that taurine supplementation may be of greater benefit during the *Artemia* feeding phase.

Although the level of taurine in the UM Plus enriched rotifers was considerably higher than all other reported studies on wild plankton and enriched rotifers, the resulting taurine content of the larvae receiving this diet was still slightly less than that seen in wild juvenile Japanese yellowtail. This suggests that the very high taurine content of the enriched rotifers should not have been problematic, however given the only other study on rotifer taurine supplementation with YTK larvae found a significant improvement in larval survival with a much lower rotifer taurine content suggests that the high rotifer taurine content in the current trial may have been problematic.

9.3.4. Part 3: The effect of various enrichment products on the biochemical composition of enriched *Artemia*

9.3.4.1. Materials and Methods

In a similar manner to that described in Part 1 above, the effect of different enrichment products on the biochemical composition of *Artemia* were determined. The enrichment diets tested were Spresso (current industry standard)(lipid emulsion; INVE), Ori-Green (artificial diet; Skretting) and 'UM', an enrichment diet comprising a blend of commercially available products used in the commercial hatchery production of cobia at the University of Miami. In addition, a diet of UM Plus was also prepared in which taurine, and vitamins C and E were added.

Artemia enriched on Spresso were enriched according to the manufacturer's directions at a density of 100 *Artemia*/mL. A background feed of 1000 ppm was initially dosed into the enrichment tank at 11 am followed by a second dose of 1000 ppm at 3 pm. *Artemia* were harvested the following morning at 9 am, giving a 22 hour enrichment.

Those *Artemia* enriched on Ori-Green were enriched following the manufacturer's directions by feeding 600 grams/m^3 at a density of 1000 Artemia/mL for 12 hours.

The UM Standard enrichment diet for *Artemia* is a blend of various commercially available products described by Benetti et al. (2008) that differs from that described above for rotifers. Its constituents are summarised in Table 9-4. It was included in this study as juvenile cobia produced on this diet exhibit a low level of malformations. These *Artemia* were enriched at 100/mL and fed at the rate of 0.3 g/million for 18 hours.

UM Plus was prepared by the addition of taurine, soy lecithin, ascorbyl palmitate (vitamin C) and Alpha tocopherol (vitamin E) to the standard UM

diet according to Table 9-4. Unlike the UM Plus diet fed to rotifers, organic selenium (Sel-Plex®, Alltech) was not added to the *Artemia* enrichment as preliminary analyses and information from the literature (Solbakken et al., 2002) demonstrated that the naturally occurring level of selenium in *Artemia* is similar to that found in copepods (ca. 3 mg/kg). Taurine was added following the methods of Salze et al. (2011) at 4 g/L for a period of 18 hours. Vitamins C and E were added following preliminary analyses demonstrating both to be lacking in UM Standard diet relative to other commercial enrichment diets. The UM Plus and UM Standard *Artemia* analysed here were the same *Artemia* actually fed to the larvae in the subsequent trial.

Ingredient	UM Std	UM Plus
Algamac 3050	85.0%	68.0%
Sel-Plex (organic selenium)	0.0%	0.0%
AstaRose (astaxanthin)	5.0%	5.0%
Ascorbyl palmitate	0.0%	10.0%
Soy lecithin	0.0%	5.0%
Alpha tocopherol	0.0%	2.4%
ARA	10.0%	10.0%
TOTAL	100%	100%

Table 9-4: Composition of the two UM diets used to enrich *Artemia*

Duplicate samples of enriched *Artemia* were submitted to CSIRO for analysis of lipids (including lipid class profiles and fatty acid composition within class), pigments and vitamins. Subsamples of the same duplicate subsamples were sent to DAFWA for analysis of amino acids, including taurine.

9.3.4.2. Results and Discussion

Artemia Amino Acid Profile and Taurine

An essential amino acid index was calculated for *Artemia* enriched on the various enrichment diets, using the essential amino acid content of fertilised Japanese yellowtail as the reference protein (Matsunari et al. 2003)(Figure 9-23). Whilst this figure demonstrates that leucine and isoleucine are potentially limiting in enriched *Artemia* for YTK larvae, it also demonstrates that the deficiency is equal across all enrichment diets and that no enrichment diet is superior in terms of its essential amino acid profile. The deficiency of leucine in *Artemia* was also reported by Conceicao et al. (1997).

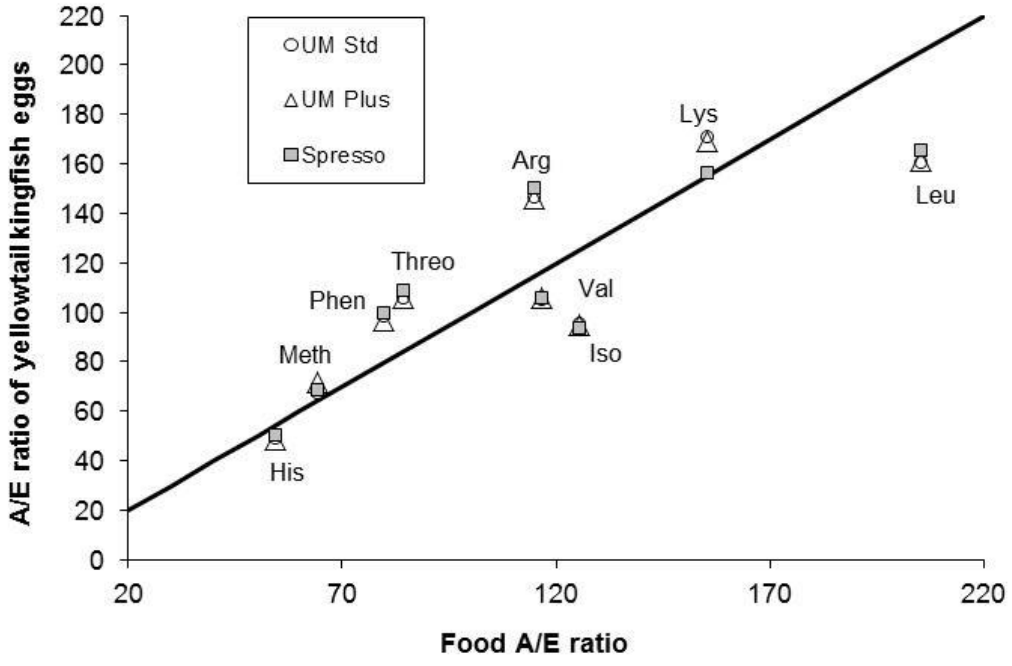

Figure 9-23: A comparison of the A/E ratio of fertilised Japanese yellowtail (*Seriola quinqueradiata*) eggs (from Matsunari et al. 2003) and *Artemia* enriched on various products. Amino acids below the line are potentially limiting.

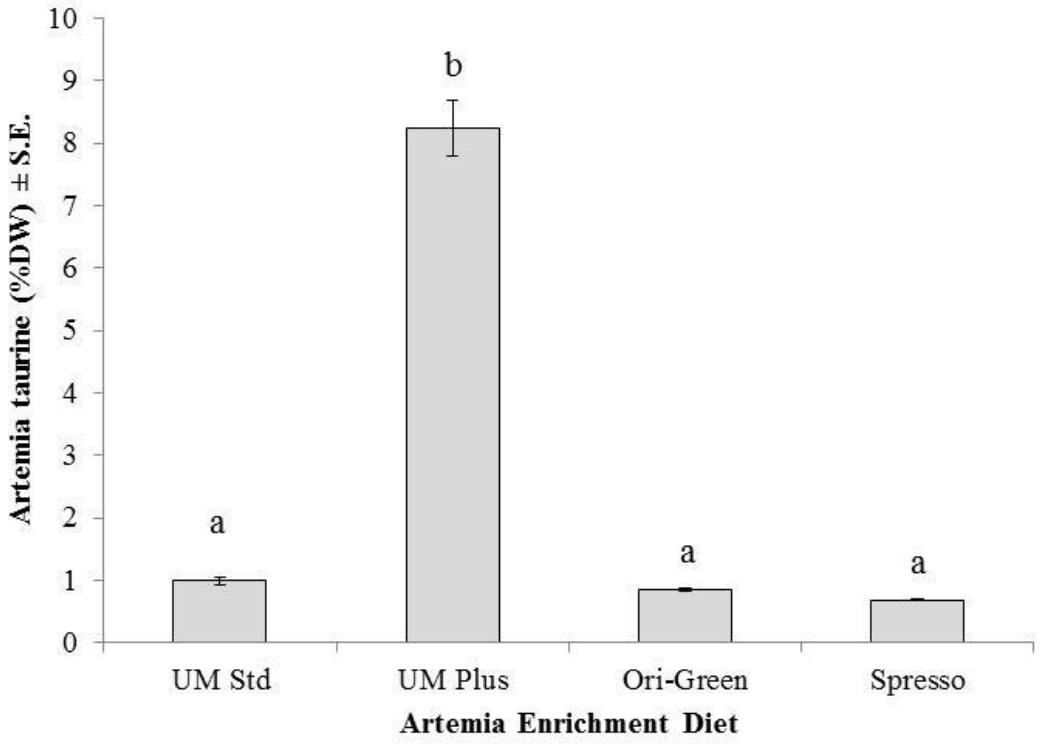

Figure 9-24: Taurine content of *Artemia* enriched on various products.

The effect of enrichment diet on the taurine content of enriched *Artemia* is shown in Figure 9-24. Consistent with other studies, the level of taurine in non-taurine supplemented *Artemia* ranged from 0.68 to 0.99 %DW (Yamamoto et al., 2008), with no difference between these unsupplemented treatments. Similar to the rotifer protocol described above, we followed the Salze et al. (2011) protocol of enriching *Artemia* with 4 g/L. Using the same protocol, Salze et al. (2012) reported a wet weight taurine content of ca. 0.23% in taurine-enriched *Artemia* which equates to approximately 2.3%DW (based on an *Artemia* water content of 90% that was confirmed during this study). Similar to the UM Plus enriched rotifers described previously (see Sections 9.3.2.2 and 9.3.3.3), the taurine content of *Artemia* enriched with UM Plus (8.24 ± 0.45 %DW) was much higher than that calculated from the wet weight values presented by Salze et al. (2012) and higher than any reported values for live prey. In the aforementioned study by Rotman et al. (2012), the taurine content of *Artemia* enriched at 1 g/L for 12 hours was 0.15%WW, equivalent to only ca. 1%DW. To investigate potential causes for such large differences seen between studies, we conducted a further study outlined in

Section 9.3.6 investigating the effect of different taurine dose rates on the taurine content of *Artemia*.

Artemia Carotenoids

There were no significant differences in the total carotenoids in *Artemia* enriched on the different diets, which ranged from 721 to 807 µg/g DW (P = 0.59)(Figure 9-25). The vast majority of the total carotenoids (>98%) in all enriched *Artemia* were canthaxanthin and its isomers.

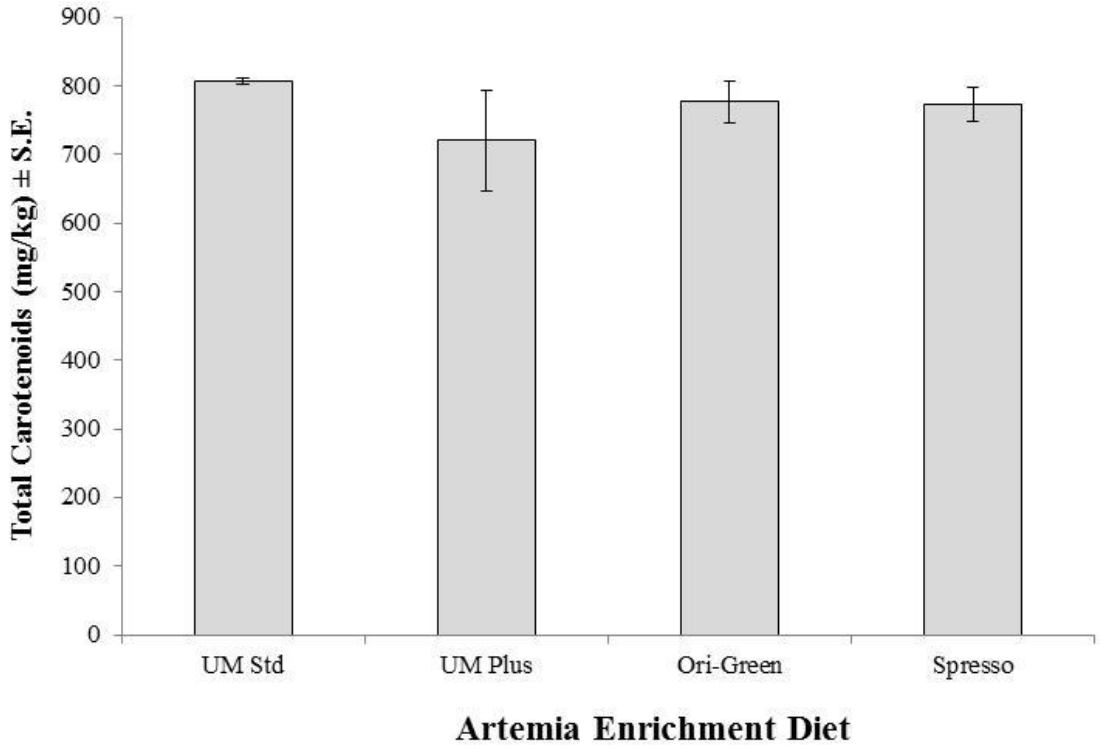

Figure 9-25: Total carotenoids in *Artemia* enriched on various products.

The concentration of astaxanthin in all *Artemia* was subsequently low but significantly affected by treatment (P = 0.003)(Figure 9-26). *Artemia* enriched on Ori-Green contained no astaxanthin, whilst those enriched on Spresso contained significantly more astaxanthin (14.6 ± 1.8 mg/kg) than all other treatments. These levels are significantly lower than those found naturally in copepods (413 to 1422 mg/kg)(van der Meeren et al., 2008). Despite both UM diets containing AstaRose, a natural source of astaxanthin derived from a unique strain of yeast and containing 10,000 mg/kg of astaxanthin (http://www.aquafauna.com/Profiles-AstaRose.htm), *Artemia* enriched on

these two diets still had significantly lower levels of astaxanthin than those enriched on Spresso, suggesting that the AstaRose is not well assimilated by the *Artemia* during the enrichment process.

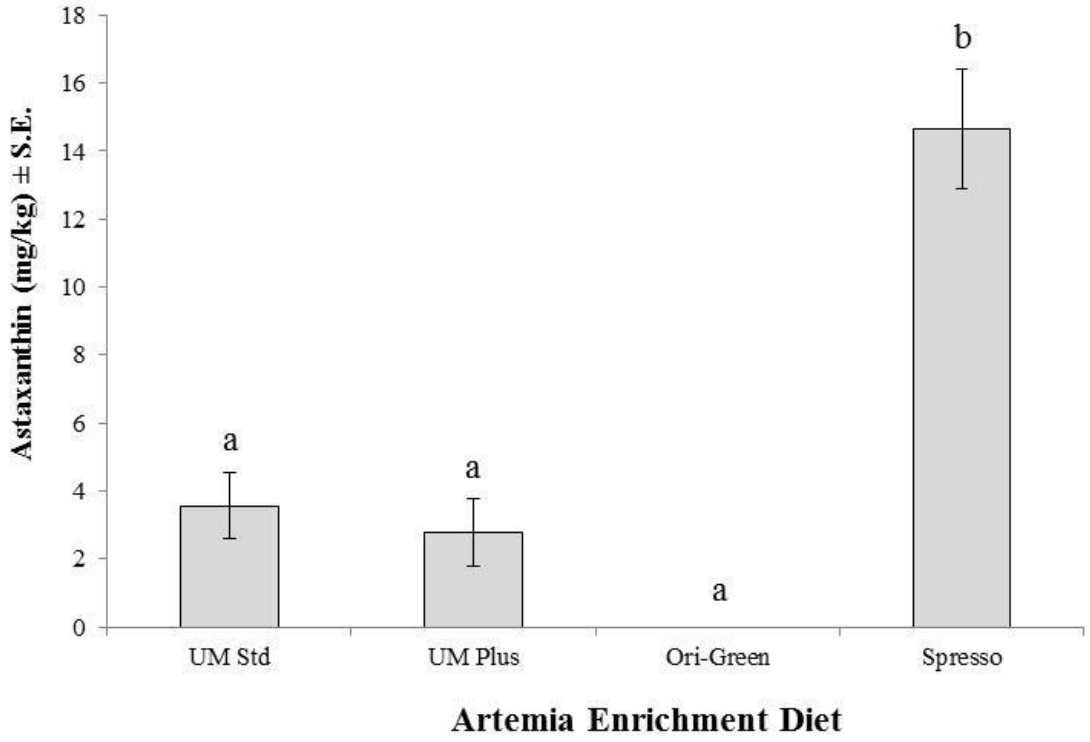

Figure 9-26: Astaxanthin in *Artemia* enriched on various products. Columns sharing the same letter are not significantly different (P>0.05).

Artemia Lipids

Artemia Total Lipid and Lipid Classes

There were significant differences in the total lipid content of *Artemia* enriched on the different products (P = 0.002)(Figure 9-27). Those enriched on Spresso contained significantly more lipid (26.9 ± 0.8%) than all other treatments. Those *Artemia* enriched on Ori-Green had a significantly higher lipid content (19.0 ± 0.3%) than those enriched on UM Standard (12.5 ± 0.4%). The addition of lecithin to the UM Plus diet was necessary to emulsify the fat soluble vitamin E. Whilst this addition appeared to also elevate the lipid content of UM Plus enriched *Artemia* (15.7 ± 1.8%) compared with the UM Standard enriched *Artemia* (12.5 ± 0.4%), this difference was not significant.

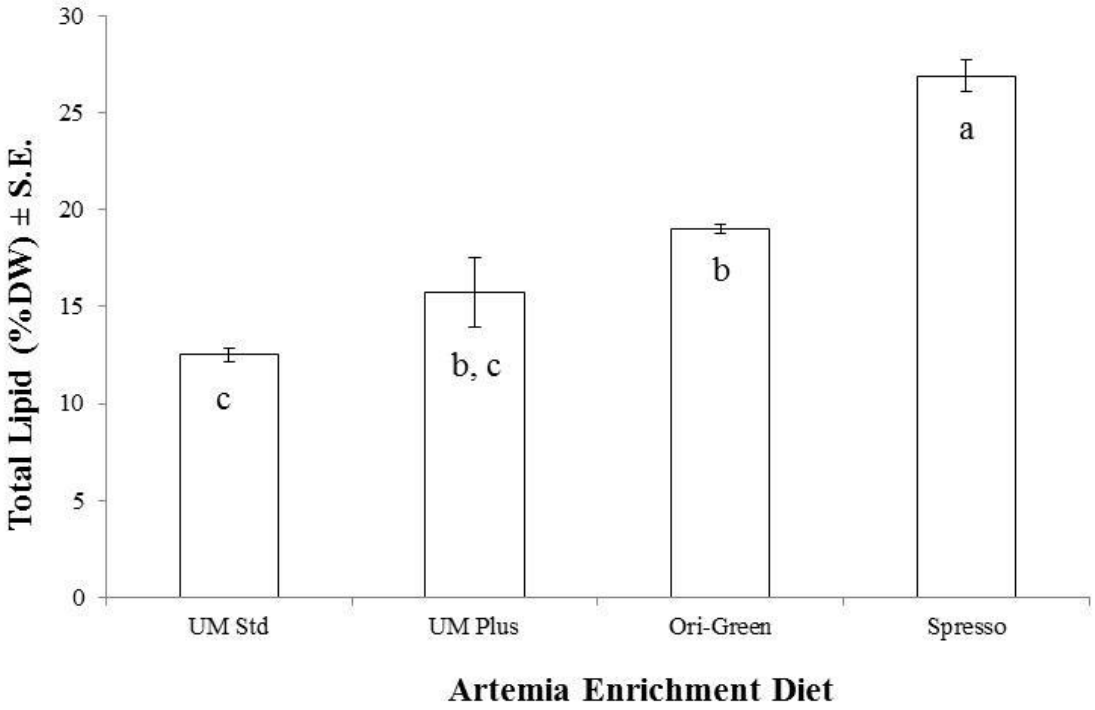

Figure 9-27: Total lipid content (%DW) of *Artemia* enriched on various products. Columns sharing the same letter are not significantly different (P>0.05).

The lipid class composition analyses revealed a high level of lipid degradation in one of each of the two duplicate samples for both of the UM treatments, as evidenced by a high percentage of free fatty acids in each sample. Whilst all other samples had between 3% and 15% of their total lipid as free fatty acids, these two samples had free fatty acid contents of 44% and 82%. The degradation of the sample with 82% of free fatty acids was evident upon freeze-drying where it became a sticky mass rather than a dry powder, however this was not the case with the former sample. The reason for this degradation is unclear given that all samples were treated and stored in the same manner. These degraded samples are not included in the graphs or analyses which follow. This degradation did not affect the total lipid content of the samples and the fatty acids themselves were not degraded based on the fatty acid profile within the total lipid.

Artemia lipids were broadly classed into neutral and polar lipids following the same criteria as previously described for rotifers and are presented in Figure 9-28. Ori-Green enriched *Artemia* had a significantly higher percentage of polar lipids (42 ± 1%)(P = 0.02) than those enriched on Spresso (26 ± 9%).

Whilst the UM treatments had to be excluded from the ANOVA, it appears that the UM Plus enriched *Artemia* had the highest proportion of polar lipids and this is worthy of further investigation.

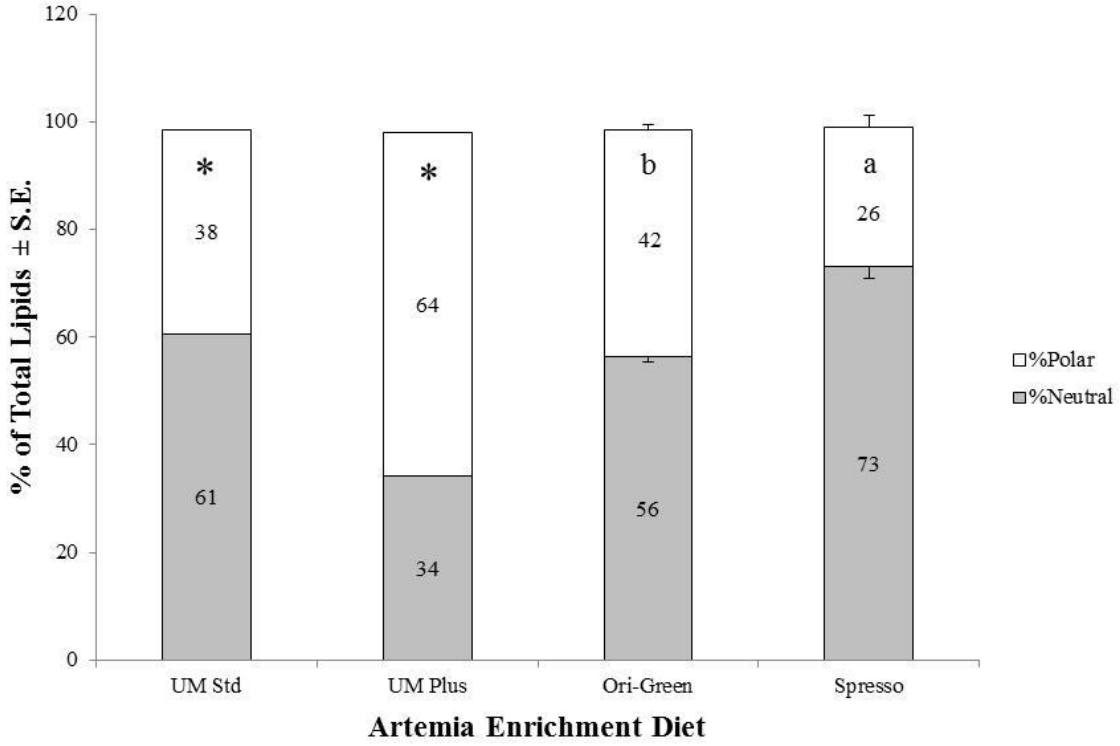

Figure 9-28: Ratio of polar and neutral lipids in *Artemia* enriched on different products. Columns sharing the same letter are not significantly different. * indicates only 1 replicate value for that sample.

Artemia Fatty Acid Profiles in Total Lipid and within the Phospholipid Class

The absolute and relative amounts of DHA, EPA and ARA in the total lipids and phospholipid fractions of the *Artemia* enriched on the various diets are shown in Table 9-5, along with various important EFA ratios.

	UM Std	UM Plus	Ori-Green	Spresso	*p*
%Total Lipid	12.5 ± 0.4	15.7 ± 1.8	19.0 ± 0.3	26.9 ± 0.8	0.002
% Polar Lipid	38*	64*	42 ± 1[b]	26 ± 2[a]	0.02
Total Fatty Acids (mg/100g)					
DHA	648*	126*	429 ± 103[b]	2382 ± 18[a]	0.001
EPA	734*	404*	722 ± 38[b]	1150 ± 24[a]	0.005
ARA	382*	215*	207 ± 6[b]	437 ± 8[a]	0.001
DHA:EPA	0.88*	0.31*	0.59 ± 0.11[b]	2.07 ± 0.03[a]	0.003
Fatty acids in Phospholipid fraction (mg/100g)					
DHA	62*	28*	42 ± 0	83 ± 17	0.13
EPA	231*	171*	292 ± 9	257 ± 53	0.57
ARA	130*	108*	93 ± 3	118 ± 24	0.42
DHA:EPA	0.27*	0.16*	0.14 ± 0.00[b]	0.32 ± 0.00[a]	0.0005
%DHA in Phospholipid	10*	22*	10 ± 2	3 ± 1	0.07
%EPA in Phospholipid	31*	42*	41 ± 3	22 ± 4	0.06
%ARA in Phospholipid	34*	50*	45 ± 3	27 ± 5	0.09

Table 9-5: Key total lipid and phospholipid data for *Artemia* enriched on various diets.

The DHA data for the various enriched *Artemia* are shown in Figure 9-29. Largely due to Spresso enriched *Artemia* having the highest lipid content, they also had the highest absolute amount of total DHA (2382 ± 18 mg/100 g), significantly higher than Ori-Green (429 ± 103 mg/100g) (P = 0.0001), which had a similar concentration to the two UM treatments (648 and 126 mg/100g).

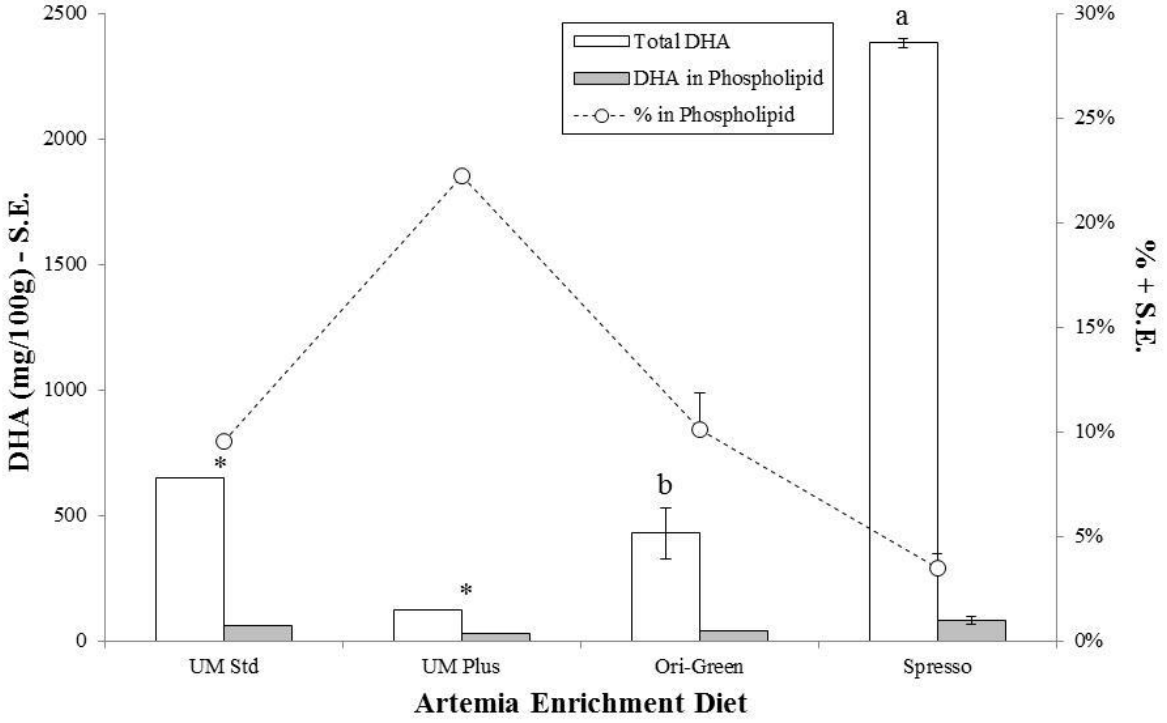

Figure 9-29: Quantity of DHA in total lipid and phospholipid fractions and the % of DHA found within the phospholipid fraction of *Artemia* enriched on different products. Columns sharing the same letter are not significantly different. * indicates only 1 replicate value for that sample.

There was, however, no significant difference in the DHA concentration within the phospholipid fraction of *Artemia* enriched on Spresso (83 ± 17 mg/100 g) and Ori-Green (42 ± 0 mg/100 g)(P = 0.13). This is despite the fact that the percentage of polar lipids in the Ori-Green enriched *Artemia* was significantly higher than the Spresso enriched *Artemia* and is the net result of Spresso having a significantly higher total DHA, despite its lower percentage of lipids in the polar lipid fraction. The amount of DHA in the phospholipid fraction of the two UM treatments (62 and 28 mg/100 g) was similar to that of the former two treatments.

The proportion of the total DHA that was found in the phospholipid fraction ranged from 3 to 16% (Figure 9-29,Table 9-5), with no significant difference between Ori-Green and Spresso enriched *Artemia* (P = 0.07). This small percentage is believed to be one of the limitations in the use of enriched *Artemia* for marine fish larvae as in copepods, a much higher percentage of the total DHA is found with the phospholipid fraction (ca. 30%).

There was a significant difference in the total EPA concentration between *Artemia* enriched on Spresso (1150 ± 24 mg/100g) and Ori-Green (722 ± 38 mg/100g)(P = 0.005)(Figure 9-30), however the differences in phospholipid EPA were not different (257 ± 53 and 292 ± 9 mg/100g, respectively). The amount of phospholipid EPA was also similar in the *Artemia* enriched on the two UM diets (231 and 171 mg/100g for UM Standard and UM Plus, respectively).

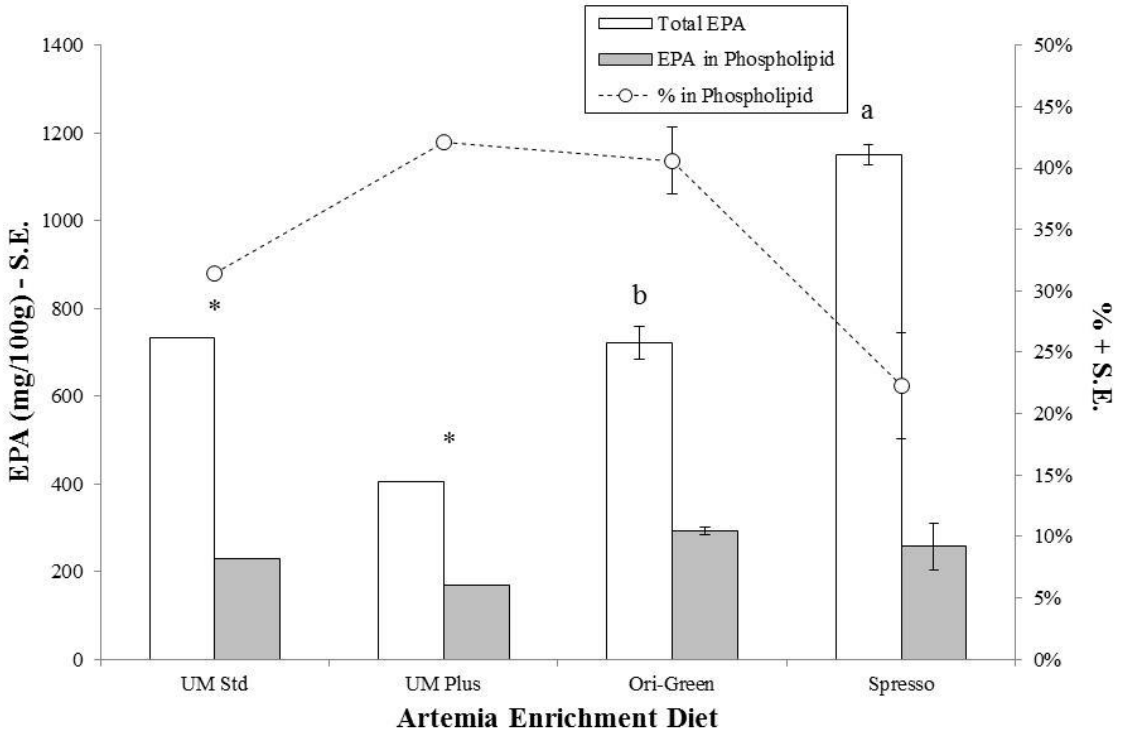

Figure 9-30: Quantity of EPA in total lipid and phospholipid fractions and the % of EPA found within the phospholipid fraction of *Artemia* enriched on different products. Columns sharing the same letter are not significantly different. * indicates only 1 replicate value for that sample.

The total amount of ARA in enriched *Artemia* was significantly affected by treatment (P = 0.001)(Figure 9-31). Those enriched on Ori-Green had

significantly lower total ARA (207 ± 6 mg/100g) than those enriched on Spresso (437 ± 8 mg/100 g). The amount of total ARA in the UM Standard and UM Plus treatments was similar at 382 and 215 mg/100 g, respectively. The amount of ARA in the phospholipid fraction was not effected by treatment (P = 0.42). The percentage of the total ARA found in the phospholipid fraction was also not effected by treatments and ranged from (27 to 50%; P = 0.09). Expressed as a % of TFA, ARA ranged from 1.4% in Ori-Green enriched *Artemia* to 2.5% in UM Standard enriched *Artemia*.

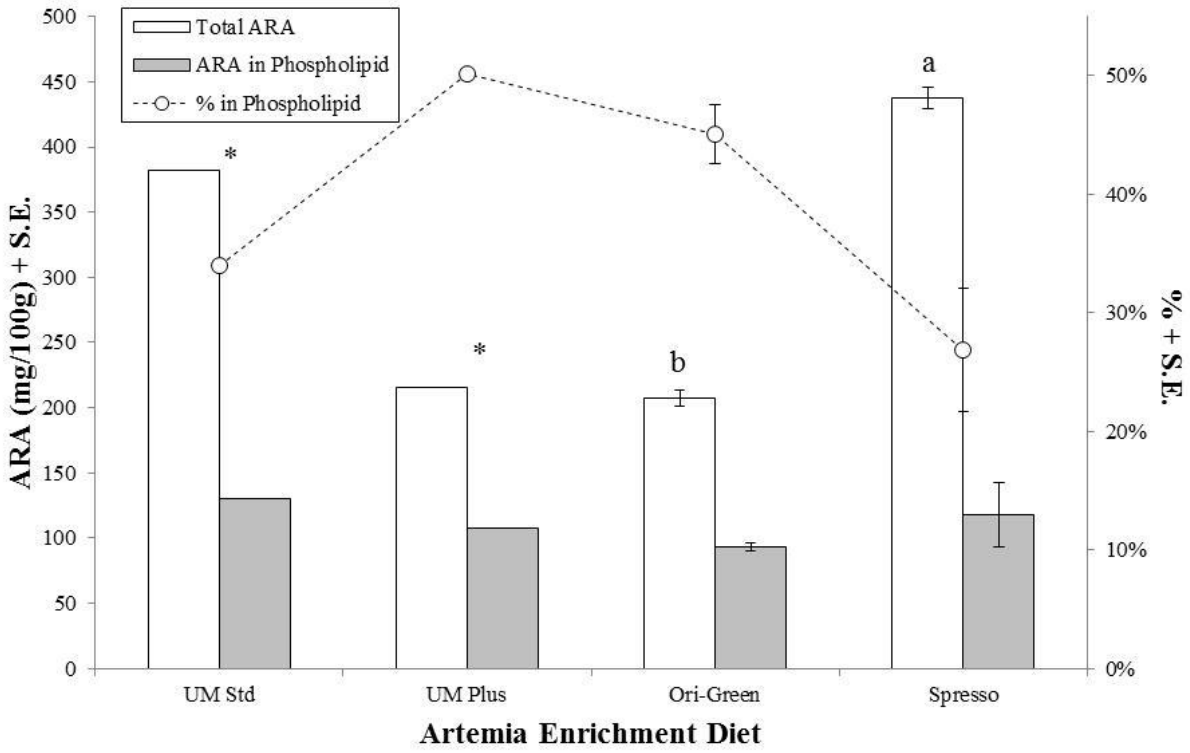

Figure 9-31: Quantity of ARA in total lipid and phospholipid fractions and the % of ARA found within the phospholipid fraction of *Artemia* enriched on different products. Columns sharing the same letter are not significantly different. * indicates only 1 replicate value for that sample.

In the total lipid fraction, those *Artemia* enriched on Ori-Green had a significantly lower DHA:EPA ratio (0.59 ± 0.11)(P = 0.003) than those enriched on Spresso, which had a ratio similar to that considered optimum for marine fish larvae (2.07 ± 0.03). There was no difference in the DHA:EPA ratio within the phospholipid fraction, which was low in all treatments (range 0.14 to 0.32) (Figure 9-32).

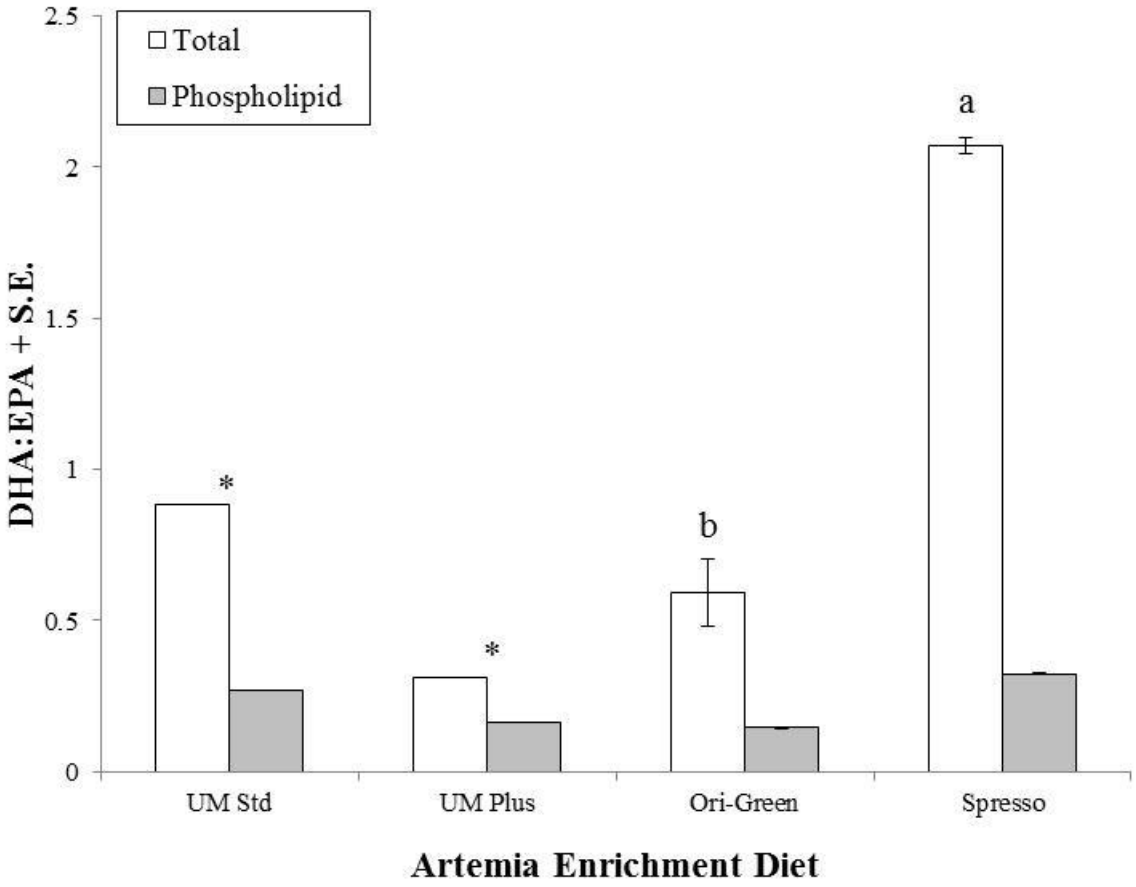

Figure 9-32: Ratio of DHA to EPA in total lipid and phospholipid fractions of *Artemia* enriched on different products. Columns sharing the same letter are not significantly different. * indicates only 1 replicate value for that sample.

Artemia Vitamins

The contents of free α-tocopherol (vitamin E) and ascorbic acid (vitamin C) in the enriched *Artemia* are shown in (Figure 9-33). The variation between the two replicates for both α-tocopherol and ascorbic acid in the UM Standard enriched *Artemia* was very high. As previously described, one of these two samples exhibited heavy lipid oxidation into free fatty acids. This sample contained only 18 µg/g DW of free α-tocopherol and 76 µg/g DW of ascorbic acid compared with 608 and µg/g DW and 396 µg/g DW, respectively in the second replicate that did not exhibit lipid oxidation. As no additional vitamin E or vitamin C was added to the UM Standard diet, this suggests that the free α-tocopherol and ascorbic acid were oxidised prior to the oxidation of the lipids.

Unlike the rotifers, the addition of vitamin E and vitamin C to the UM Plus diet did not result in higher concentrations of these vitamins in the enriched *Artemia*. Excluding the UM Standard treatment from the analysis of variance, there was no effect of treatment on the free α-tocopherol content (P = 0.74) or ascorbic acid content (P = 0.57) of enriched *Artemia*.

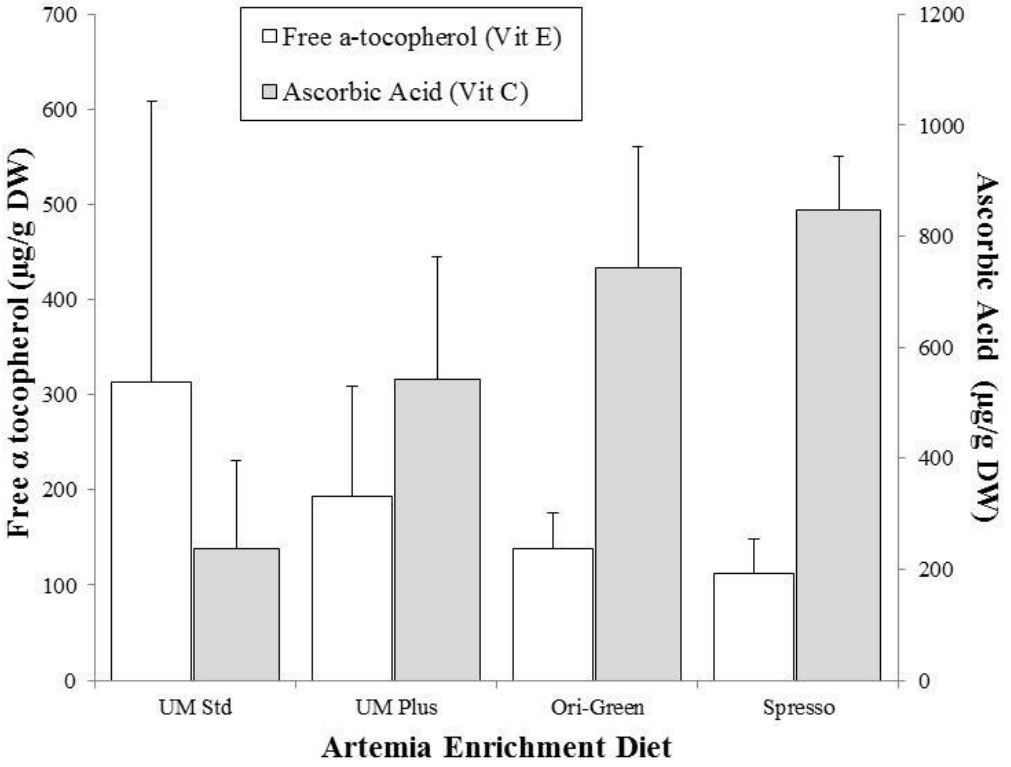

Figure 9-33: Free α-tocopherol (vitamin E) and ascorbic acid (vitamin C) concentrations of *Artemia* enriched on different products.

9.3.4.3. Conclusions

The differences between enriched *Artemia* were not as great as those found in rotifers. Interpretation of the data from UM enriched *Artemia* was hampered by inconsistent replicates and as previously discussed in the rotifer section, this variability may be a function of the complex mixture of the UM diets. Based on the results of the biochemical analyses many of the additional components of the UM diets may not be warranted. AstaRose, for example, did not increase the carotenoid content of UM diets, vitamin contents were similar between UM and UM Plus diets and the ARA content of UM enriched

Artemia was similar to that described by Barclay and Zeller (1996), suggesting that the addition of ARA powder to the UM diets had little effect in increasing the ARA content of the *Artemia*. A comparison of the performance of YTK fed *Artemia* enriched on UM versus those enriched only on AlgaMac would be required to confirm this hypothesis.

Spresso-enriched *Artemia* appeared superior to Ori-Green enriched *Artemia* in many respects. They had significantly more astaxanthin, the highest total lipid, the highest absolute amount of DHA in the phospholipid fraction. Spresso enriched *Artemia* had a better DHA:EPA ratio in both the total and phospholipid fractions than all other treatments.

Of potential importance is a comparison between enriched rotifers and *Artemia*, particularly a comparison of the data in Table 9-3 and Table 9-5, which demonstrate that enriched rotifers have considerably higher levels of phospholipid DHA and better DHA:EPA ratios within this fraction. Spresso enriched rotifers, for example, had 206 mg/100 g of DHA in the phospholipid fraction, more than double that of Spresso-enriched *Artemia*, with a similar pattern across all enrichment diets. Most enriched rotifers had a DHA:EPA ratio in the phospholipid fraction close the optimum value of 2, whilst *Artemia* had ratios in this fraction of 0.3 at best. These data suggest that extending the rotifer feeding period and minimising the amount of *Artemia* fed to YTK larvae may be beneficial in terms of their nutrition and development, in addition to those benefits previously described by Woolley et al. (2012).

9.3.5. Part 4: A comparison of the performance of YTK larvae fed rotifers and *Artemia* enriched on two enrichment products

9.3.5.1. Introduction

This study compared the performance of YTK larvae reared on UM Standard and UM Plus enriched rotifers and *Artemia*. Only two treatments were tested to provide 6 replicates per treatment to increase the likelihood of detecting significant differences between treatments.

9.3.5.2. Materials and Methods

Larvae were reared within the same larval rearing system described in Sections 9.2.2 and 9.3.3.2 above. During the rotifer feeding phase (3 to 15 dph), rotifers were enriched on UM Standard and UM Plus following the protocols described in Sections 9.3.2.1 and 9.3.4.1. Based on the results of the previous larval rearing trial (see Section 9.3.3.3), we made the assumption that survival in the two treatments would have been equal at the start of the *Artemia* feeding phase. During the rotifer feeding phase, larvae were fed with the 'hybrid' feeding method described in Section 9.2. *Artemia* were introduced on 12 dph and larvae were fed according to the 'adaptive' feeding method described by Woolley et al. (2012). The trial was concluded on 21 dph. The *Artemia* fed during this trial were the same as those sampled for biochemical analyses reported in the previous section 9.3.4.2.

9.3.5.3. Results and Discussion

There was a significant difference in survival at 21 dph between treatments. Larvae fed *Artemia* enriched on UM Standard had significantly higher survival (14.9 ± 0.5%) compared to those fed UM Plus enriched *Artemia* (9.9 ± 1.5%, $P = 0.01$)(Figure 9-34).

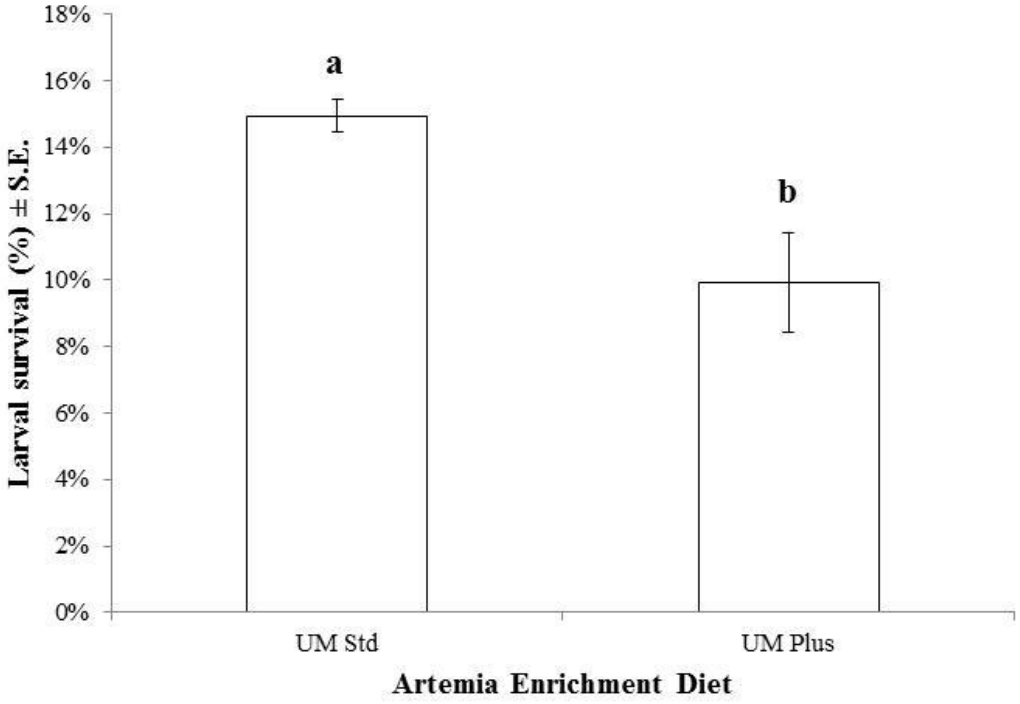

Figure 9-34: Survival of YTK larvae reared to 21 dph on rotifers and *Artemia* enriched with different products.

Growth data, measured as dry weight and standard length, are shown in Figure 9-35 and Figure 9-36. These data confirm the findings from the previous rotifer enrichment trial that there were no differences in growth between larvae fed UM Standard and UM Plus enriched rotifers (i.e. no significant difference in size on 12 dph P = 0.27; pooled average = 6.1 mm). Growth during the *Artemia* feeding phase, however, was significantly affected by enrichment product (P = 0.027 and P = 0.021 for dry weight and length, respectively). Larvae fed UM Plus enriched *Artemia* were significantly longer and heavier by 20 dph (8.89 ± 0.87 mm, 3.15 ± 1.15 mg) compared to those fed UM enriched *Artemia* (8.07 ± 0.22 mm, 2.54 ± 2.02 mg).

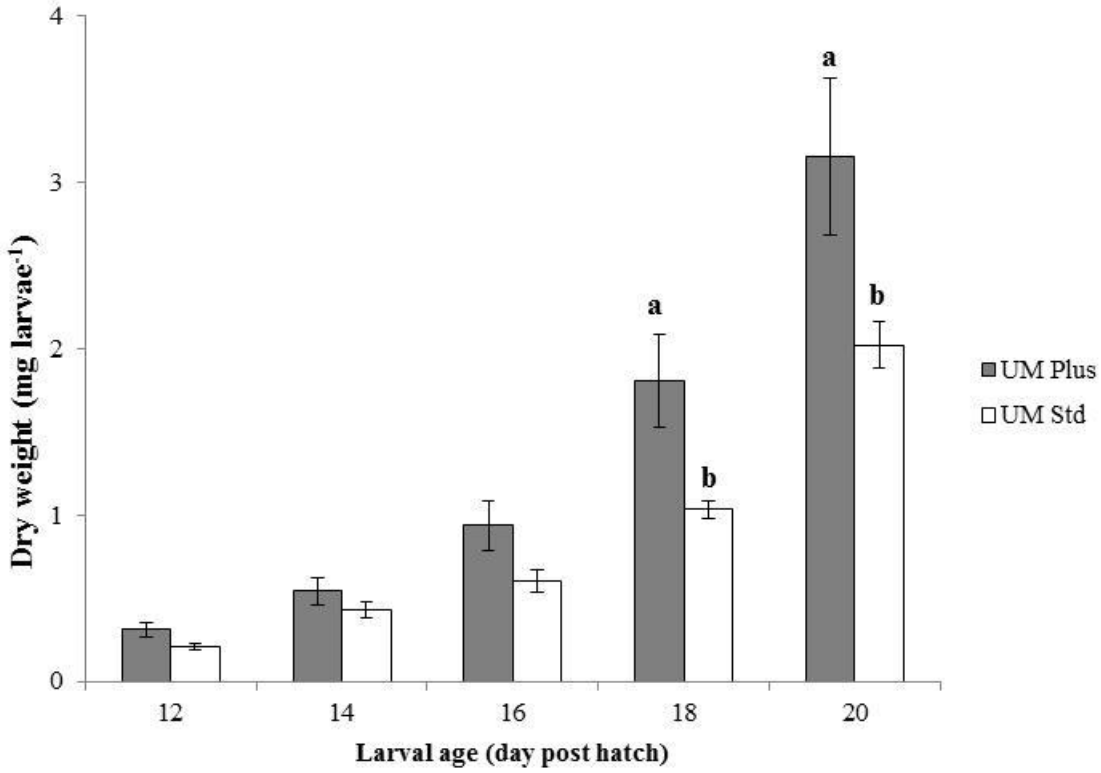

Figure 9-35: Growth (dry weight) of YTK larvae reared to 21 dph on rotifers and *Artemia* enriched with different products.

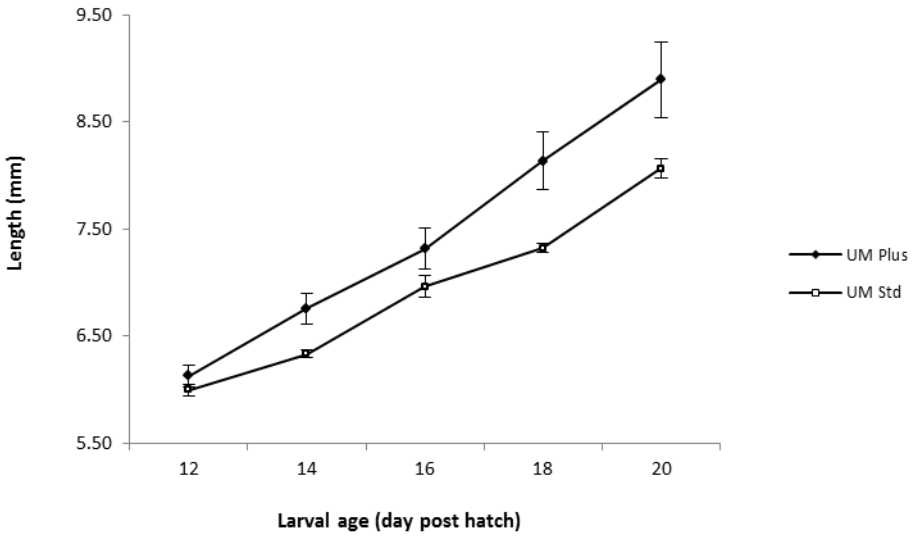

Figure 9-36: Growth (standard length) of YTK larvae reared to 21 dph on rotifers and *Artemia* enriched with different products.

Given the significant differences in survival, we cannot attribute the improved growth of larvae in the UM Plus treatment only to the enrichment diet and the larger size of the larvae in this treatment may have been attributable to them receiving more food (on a 'per larvae' basis) or due to differences in final

larval density. Indeed, there was a positive correlation between fish size and survival within each treatment (Figure 9-37). Whilst the correlation between size and survival is strong, it does not indicate why survival in the UM Plus treatment was lower than in the UM treatment and we believe it is unlikely that food limitation was the cause of the difference in the survival. For example, based on the survival rates achieved in each treatment and with the feeding regime used, we have calculated that larvae in the UM Plus and UM Standard treatments were offered 214 and 126 *Artemia* per larvae per feed, respectively, at the end of the trial. This is similar to those in Woolley et al. (2012) which received approximately 140 *Artemia* per larvae/feed at the same age. That there was no correlation between survival and larval size in this aforementioned trial, even with a lower feed rate, suggests that larvae in the current trial should not have been food limited. As we describe in further detail below (Section 9.3.6.3), differences in final larval stocking density (as a result of differing survival rates) are likely to have impacted on growth, as such effects have been reported both in YTK larvae within the current CRC (see CST Fingerling RD Report_12 Nov 2012) and in other species such as tuna.

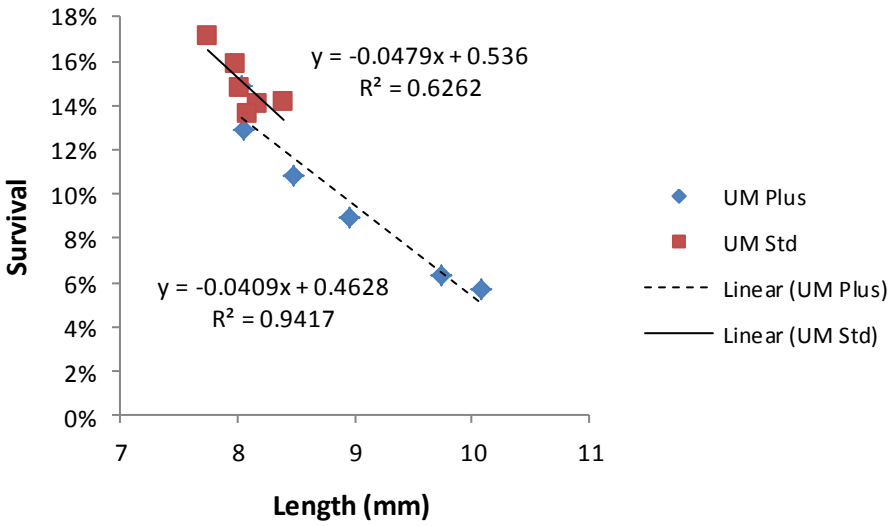

Figure 9-37: Relationship between final length and survival of YTK larvae reared to 21 dph on rotifers and *Artemia* enriched with different products.

Artemia ingestion over time is shown in Figure 9-38. Larvae fed UM Plus enriched *Artemia* had significantly higher numbers of *Artemia* (measured as

individuals consumed per larva, one hour after feeding) in their gut compared to those fed UM enriched *Artemia* (*P* = 0.004). Whilst it could be argued that the higher ingestion of *Artemia* in the UM Plus treatment is evidence supporting food limitation in the UM Standard treatment, it is equally likely that the increased ingestion by larvae in the UM Plus treatment was simply the result of their larger size, and indeed differences in *Artemia* ingestion between treatments increased with time, in line with the increasing difference in larval size.

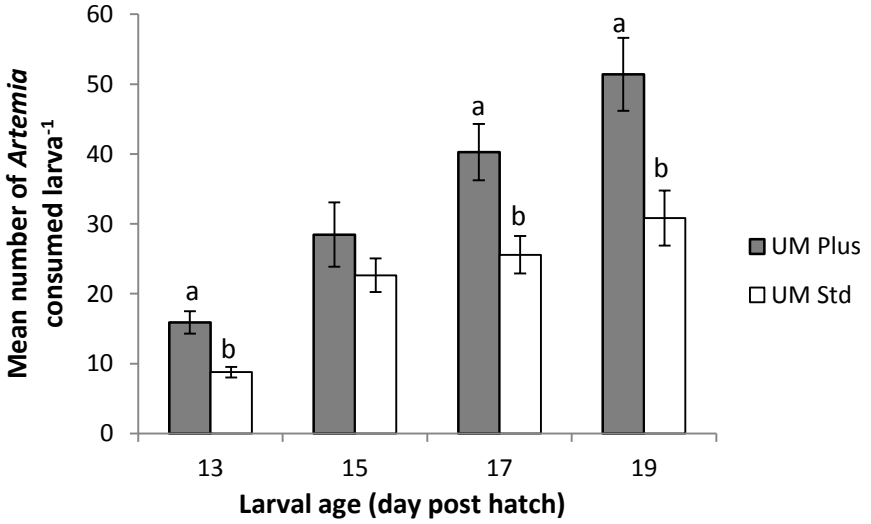

Figure 9-38: Ingestion rates by YTK larvae of different ages on *Artemia* enriched with different products.

Larvae fed taurine-enriched *Artemia* (UM Plus) also had significantly fewer malformations than those fed on non-taurine enriched *Artemia* (UM Standard)(P = 0.002)(Figure 9-39). Due to the significant differences previously reported between treatments, we cannot attribute these differences in malformation rate only to treatment and indeed there was a strong correlation between survival and malformation rate in both treatments (Figure 9-40), pointing to the possibility that those larvae which died were malformed.

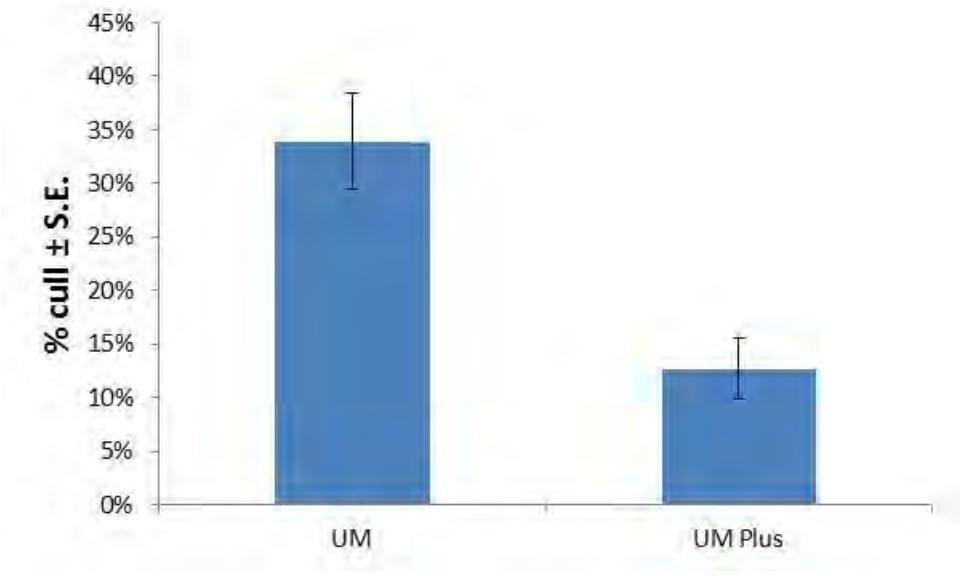

Figure 9-39: Percentage of YTK larvae with a jaw malformation score ≥2.

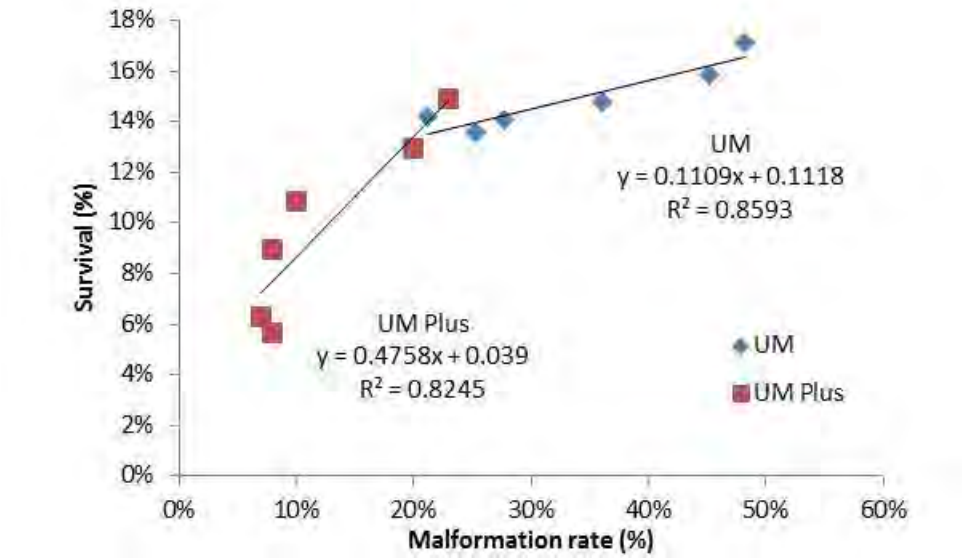

Figure 9-40: Relationship between survival and malformation rate (% of larvae with a jaw malformation score ≥2) between treatments.

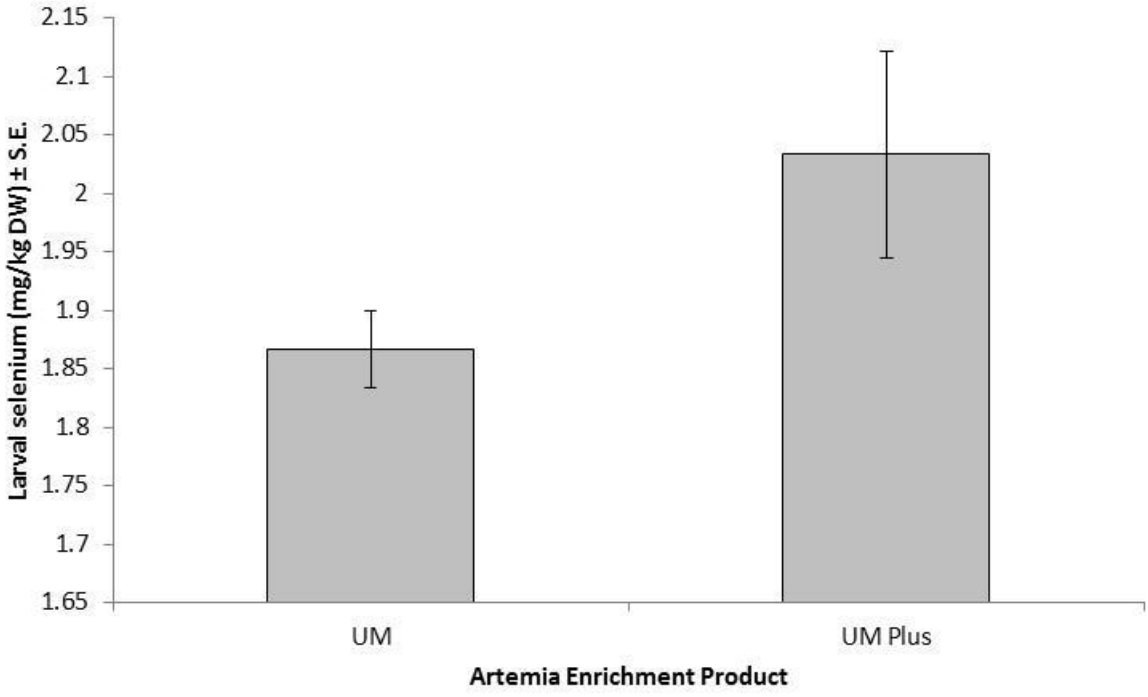

Figure 9-41: Whole body selenium contents of 21 dph YTK larvae fed rotifers and *Artemia* enriched on different products.

The whole body content of selenium and taurine of larvae at 21 dph is shown in Figure 9-41 and Figure 9-42. Despite having highly significant differences in whole body selenium content at the end of the rotifer feeding phase (see Figure 9-21 in Section 9.3.3.3), whole body selenium at the end of the *Artemia* feeding phase was no longer significantly different (P = 0.15)(Figure 9-41). No *Artemia* were supplemented with additional selenium due to their relatively high natural content of 2.0 mg/kg (vs 0.48 and 3 mg/kg in unsupplemented and Se-supplemented rotifers). By the end of the *Artemia* feeding phase those larvae which had not received selenium supplementation during the rotifer phase had increased their whole body selenium content from 0.67 ± 0.03 to 1.87 ± 0.03 mg/kg, whilst those which did receive selenium supplementation during the rotifer phase decreased their whole body selenium from 4.52 ± 0.56 to 2.03 ± 0.09 mg/kg. The increase in the former larvae demonstrates that the naturally occurring selenium in *Artemia* is bioavailable, however the large drop in whole body selenium in the latter larvae suggests that it may not be as bioavailable as the organic selenium used to supplement the rotifers or

the ca. 1 mg/kg higher concentration of selenium in the enriched rotifers results in the higher whole body selenium content at the end of the rotifer phase. Given that the optimum whole body content of selenium is unknown, it remains unclear whether further supplementation of selenium to *Artemia* is necessary and this requires further investigation.

The whole body taurine content of larvae fed UM Plus enriched *Artemia* (2.65 ± 0.06%DW) was significantly (P = 0.001) higher than those fed on UM enriched *Artemia* (1.78 ± 0.09%DW) and higher than those larvae receiving UM Plus enriched rotifers (1.9 ± 0.1%DW)(see Section 9.3.3.3). Based on the results from the rotifer feeding trial, those larvae offered UM Standard enriched rotifers during the first phase of this current trial would have had a whole body taurine content of ca. 0.3%DW at the end of the rotifer feeding phase (see Figure 9-22). The increase to 1.78%DW by the 21 dph can therefore only be attributable to the naturally occurring taurine in the UM Standard enriched *Artemia* (0.91%DW). Given that this level is only slightly lower than that found in wild juvenile Japanese yellowtail (ca. 2.3 %DW) implies that there may be only a small additional requirement during the *Artemia* feeding phase to increase the whole body content to that found in wild caught juveniles of a similar species. Salze et al. (2011) reported that the whole body content of cobia larvae fed unsupplemented and supplemented live feeds was 18,360 and 27,521 nmol/g WW, which we have calculated to be ca. 1.53 and 2.3%DW, respectively (assuming an 85% water content in cobia larvae). These values are similar to those reported here, despite the great differences in the calculated taurine contents for the live feeds themselves. This calculated value for whole body taurine of cobia on a dry weight basis is equal to that seen in wild caught juvenile Japanese yellowtail.

Given that taurine-enriched *Artemia* fed to larvae in the current trial contained nearly 7 times the maximum amount of taurine found in wild-zooplankton but the whole body content increased only from ca. 2.3 to 2.65 %DW supports our previous hypothesis that those larvae receiving taurine-enriched rotifers were close to ‘taurine-saturation’ (see Section 9.3.3.3) and furthermore may suggest that the larvae eating taurine-enriched *Artemia* may be

‘supersaturated’ and that this level of dietary taurine may have contributed to the poor survival of larvae in this treatment.

These two studies (Part 1 - 9.3.2 and Part 3 - 9.3.3) highlight that further detailed dose-response data are required to determine the exact requirements for larval YTK for taurine and the potential deleterious effects of excessive taurine. This was subsequently investigated in the following trials.

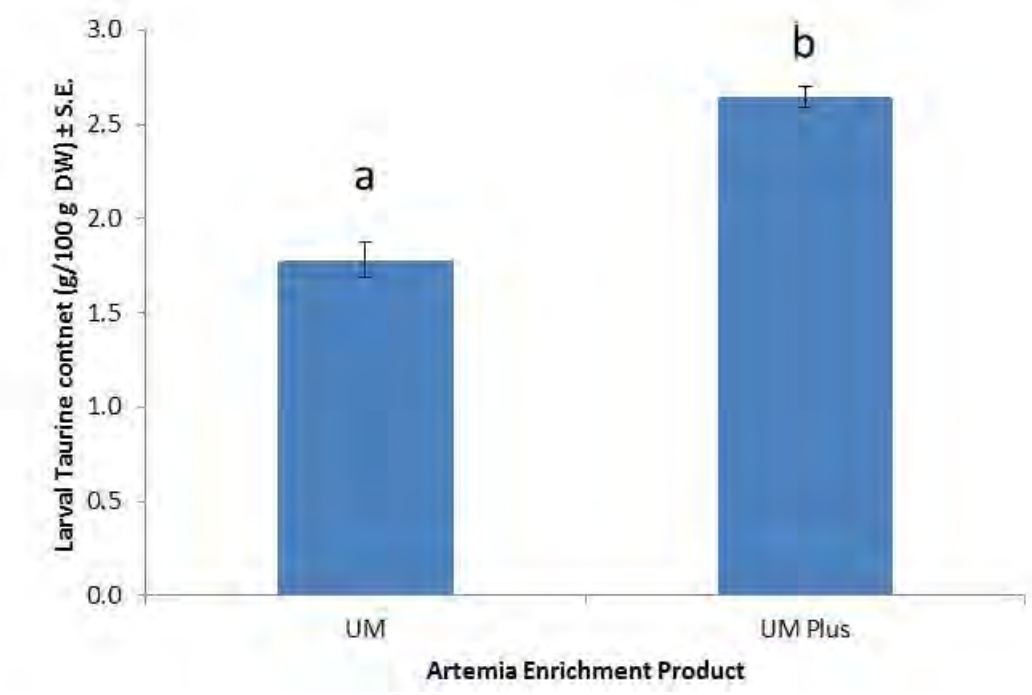

Figure 9-42: Whole body taurine contents of 21 dph YTK larvae fed rotifers and *Artemia* enriched on different products.

9.3.6. The effect of taurine dose rate on the taurine content of *Artemia* and the effect of these *Artemia* on the performance of YTK larvae

9.3.6.1. Introduction

Data from all of the aforementioned previous trials combined with results of other published studies suggest that large differences in the taurine concentration of enriched live foods can occur, even when following similar enrichment protocols. Furthermore, the previous trial also suggested that high concentrations of dietary taurine may be detrimental to larval YTK.

This study therefore sought to determine the relationship between taurine dose rate during enrichment and *Artemia* taurine concentration and the subsequent effect these enriched *Artemia* had on the larval performance of YTK. In this trial *Artemia* were co-enriched with the Australian industry standard diet Spresso, a similar INVE product to that used by Salze et al., (2011).

9.3.6.2. Materials and Methods

Larvae were reared in the same experimental system as previously described. Between 3 and 12 dph larvae were fed rotifers enriched with Spresso® (INVE Aquaculture, Belgium) and without taurine enrichment under the previously described hybrid feeding protocol. From 12 dph, feeding on taurine-enriched *Artemia* metanauplii began. *Artemia* were enriched with Spresso® (INVE Aquaculture, Belgium) according to manufacturer's directions. *Artemia* were co-enriched with taurine (Henan Aowei International, China) during the 18 hour enrichment period at one of six doses 0, 0.8, 1.6, 2.4, 3.2 and 4 g TAU/L following the method of Salze et al. (2011). Treatments were randomly allocated across the 12 experimental tanks, with duplicate larval rearing tanks per treatment. *Artemia* were fed to larvae according to the 'adaptive' feeding method described by Woolley et al. (2012) .

Sampling Protocol

Sub-samples (ca. 100 g) of *Artemia* from each enrichment tank were taken on two different days, rinsed in freshwater, frozen and then freeze-dried. Total taurine was analysed on each sample via HPLC following homogenisation then hydrolysis in 6 M HCl with 0.5% phenol for 24 hours at 110°C. Purity of the taurine used throughout this report was also determined via this method by comparing against a pure taurine standard (Sigma Alrich, T-0625). Heavy metal analysis of the taurine was conducted by preparing an acidified 1% solution in deionised water following analysis on a Agilent 730 Axial Simultaneous CCD ICP-OES.

Larval standard length and dry weight were assessed on 12, 14, 16, 18, 20 and 23 dph on 20 randomly selected larvae per tank. Individual *Artemia* were quantified in the larval guts (5 per tank) by counting undigested *Artemia* eyes under a stereomicroscope one hour after the first feed on 13, 15, 17 and 19 dph in squash-mounted fresh larvae. The trial was terminated on 23 dph and larvae from each tank were hand counted to determine the survival. One hundred larvae from each tank were anesthetised without recovery, and fixed in 10% formaldehyde then transferred to 70% ethanol for jaw deformity assessment according to methods described by Cobcroft et al. (2004). Jaw malformations classified as an intermediate or malformed jaw by the industry were expressed as a percentage of the total sample assessed. The remaining larvae from each tank were rinsed to remove seawater and frozen for analyses of total amino acids.

Statistics

A repeated measures ANOVA was used to determine the effect of larval age and *Artemia* enrichment treatment on the growth of larvae (*i.e*. length and dry weight) and number of *Artemia* consumed (*i.e. Artemia* eye per larval gut). For all repeated measures ANOVA tests, larval age (dph) was selected as the within-subject factor and feeding treatment as the between-subject factor and

each tank was used as the replication unit. If Mauchly's test of sphericity was violated, the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity. Pairwise comparison with Bonferroni tests were used if a significant difference between or within subjects was found. One-way ANOVA was used to determine differences between treatments and parameters of jaw deformity rates and taurine uptake. Regression analyses was used to determine the effect of dosage concentration on the taurine uptake in *Artemia* and between taurine content in *Artemia* and larval taurine uptake. Data was arcsine transformed where necessary to ensure homogeneity of variance. Significance was set at $P < 0.05$ and values are presented as mean ± SE. All statistical analyses were performed using IBM Statistics 20 (Release 20.0, Chicago, IL, USA).

9.3.6.3. Results and Discussion

Artemia taurine content was significantly affected by enrichment dose ($P < 0.0001$) and was described by Equation 9-1, with an R^2 value of 0.99 (Figure 9-43).

$$Artemia\ taurine\ content\ (\%DW) = 0.76 + 4.35 \times (1 - \exp^{(-0.34 \times taurinedoserate)})$$

Equation 9-1: Relationship between taurine enrichment dose rate and *Artemia* taurine content.

Those *Artemia* not receiving taurine supplementation had a total taurine content of 0.76 ± 0.04%DW, similar to the value of 0.68% reported above for *Artemia* enriched on Spresso (see Section 9.3.4.2). This value was significantly lower than all treatments receiving supplementation. Total *Artemia* taurine content increased from 1.79 ± 0.27%DW at the lowest dose of 0.8 g/L to 3.77%DW at 3.2 g/L. *Artemia* taurine plateaued at an enrichment dose of 3.2 g/L, with no significant difference in *Artemia* taurine content between a dose of 3.2 and 4.0 g/L (3.77 ± 0.13%DW and 3.95 ± 0.16%DW, respectively). The taurine content of the *Artemia* enriched at 4 g/L was only half that we obtained in the previous trial where the *Artemia* were enriched at

the same dose and duration but with a UM Plus enrichment diet (8.24 ± 0.45 %DW), suggesting that a strong interaction exists between enrichment diet and taurine uptake. In the study by Salze et al. (2012), it was reported that *Artemia* enriched at this same dose rate of 4 g/L contained ca. 0.23% taurine, on a wet weight basis, equating to 2.30% DW (using an *Artemia* water content of 90%). This is approximately half again of the content achieved in this current study (3.95%DW) and may again be due to an interaction between enrichment diet type and taurine uptake. Given that we obtained similar taurine levels for unsupplemented *Artemia* (0.76%DW) to that of Salze et al. (2012) (0.08% WW ≈ 0.8% DW) suggests that the differences in enriched values are not due to differences in analytical method. Alternatively, the differences between the current study and that of Salze may be due to differences in enrichment time, as it has been demonstrated that taurine uptake by live feeds is time-dependent (Chen et al., 2005; Hawkyard et al., 2014b). Enrichment in the current study was for 18 hours, whilst that of Salze et al. (2012) was described only as 'per day'.

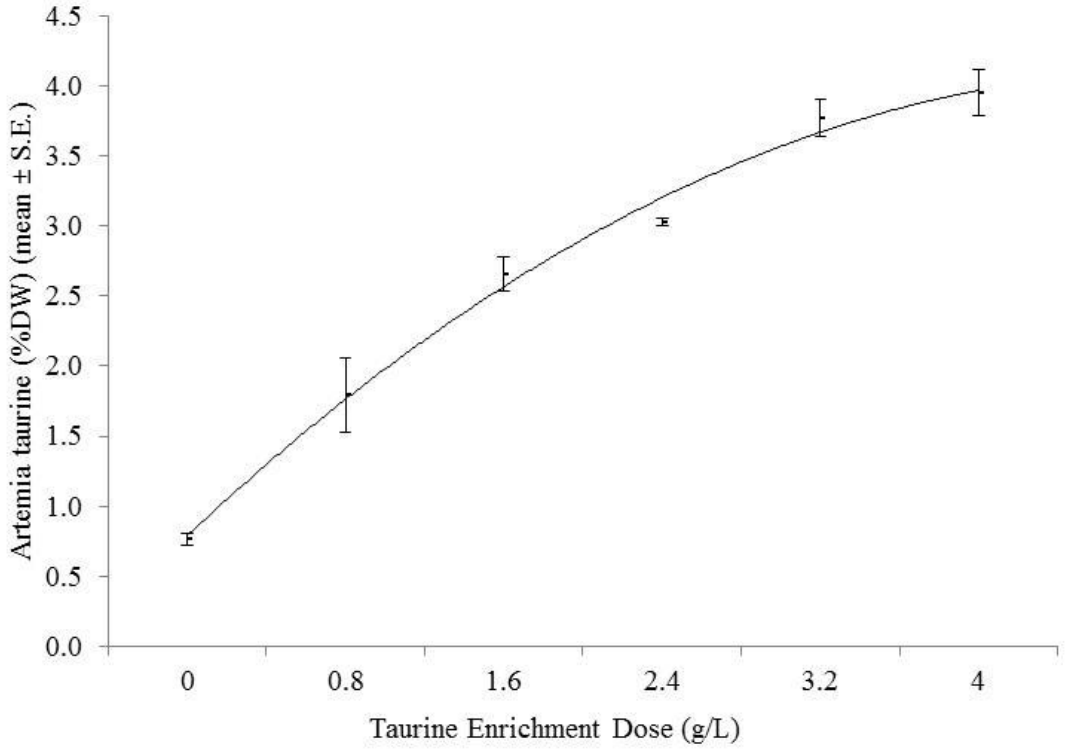

Figure 9-43: Relationship between taurine dose rate (g/L) and *Artemia* taurine content (%DW) following an 18 hour enrichment period in Spresso.

Similarly to the previous trial, survival of larvae in all treatments receiving taurine enriched *Artemia* was significantly and inversely related to *Artemia* taurine dose rate (Figure 9-44). Survival in the control (0 g TAU/L) treatment (10.4 ± 1.1%), was significantly higher than all taurine enriched treatments, which did not differ from each other (pooled average 4.7 ± 1%)(*P* = 0.001). The treatment receiving the lowest enrichment dose of 0.8 g/L yielded a taurine content of 1.8 %DW in the enriched *Artemia*, lower than extrapolated from Salze et al. (2012)(2.3%DW) and which resulted in significantly improved survival in cobia larvae.

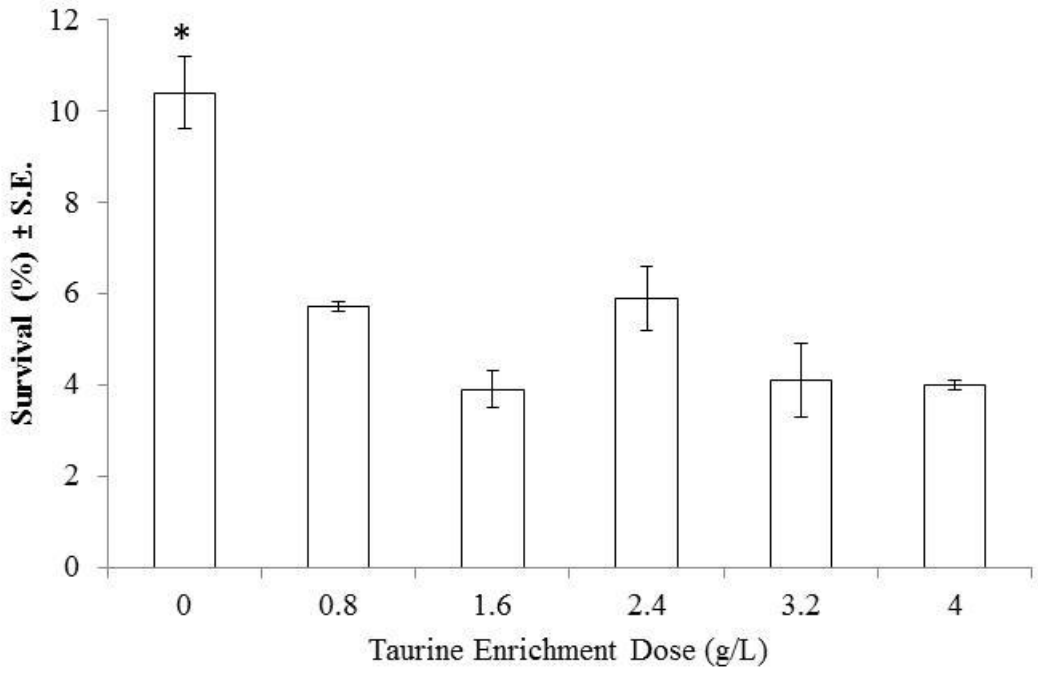

Figure 9-44: The effect of *Artemia* taurine dose rate on the survival of YTK (* indicates a significant difference at P < 0.05).

This appears to be the first study in which taurine has caused a negative impact on marine fish larvae. Most published studies have dealt with taurine enrichment during the rotifer feeding phase, due to the fact that the deficiency of taurine in rotifers is greater than in *Artemia*. Of those studies, either a positive benefit or no benefits have been reported, but never a negative effect. Co-enrichment of taurine improved survival and growth of cobia larvae,

Rachycentron canadum (Salze et al., 2012). Conceicao et al. (1997) suggested that the poorer performance of larval turbot *Scophthalmus maximus* fed enriched *Artemia* compared with zooplankton was at least partially attributable to the lower taurine content of the former diet. Pinto et al. (2013a) fed live prey containing 1.5%DW taurine to gilthead seabream larvae for 31 days and found no improvements (or detrimental effects).

We could not attribute the poor performance of larvae on supplemented *Artemia* to a low purity of the taurine, or contamination with heavy metals as the taurine purity was measured at 99.55% and all heavy metals were below their respective detectable limits.

Final larval dry and wet weights were significantly affected by enrichment dose rate (P = 0.01 and P = 0.04, respectively). Those larvae fed *Artemia* without taurine enrichment were significantly smaller (31.1 ± 2.4 mg WW and 3.8 ± 0.3 mg DW) than those receiving *Artemia* at all enrichment doses rates, except those enriched at 2.4 g/L. The average final wet weight of the larvae was strongly and negatively correlated with their final stocking density (R^2 = 0.85).

Whilst we achieved significantly greater growth rates in larvae in all taurine supplemented treatments, we cannot attribute this difference to the taurine alone as such differences could be attributable to differences in food availability and/or larval stocking density. As we have described above, we believe that larvae should not have been food limited. Based on the survival rates achieved in each treatment and with the feeding regime employed, we have calculated that larvae in the taurine enriched treatments would have had access to 416 ± 30 *Artemia*/larvae/feed at the end of the trial, whilst those in the control treatment had access to 182 ± 13. Whilst this difference is significant, those larvae in the control treatment had access to a similar number of *Artemia* than those in Woolley et al. (2012), which received approximately 140 *Artemia/* larvae/feed at the same age. That there was no correlation between survival and larval size in this aforementioned trial, even with a lower feed rate, suggests that larvae in the current trial should not have

been food limited to the extent it influenced survival. We therefore consider that differences in larval density could have played a more important role in the differences in fish size. The significant differences in survival between treatments subsequently resulted in a significant difference in final larval density (P = 0.001). Larval stocking density at the end of the trial in the control treatment (5.5 ± 0.4 larvae/L) was more than double that in all taurine enriched treatments, which did not differ from each other (pooled average 2.5 ± 0.18 larvae/L) and the average final wet weight of the larvae was strongly correlated to their final stocking density (R^2 = 0.72). Density dependent growth has been described in other marine fish larvae. Increasing the density of yellowfin tuna larvae from 2 to 18/L, for example, resulted in growth reductions of up to 35 % (Margulies et al., 2007). Studies by Clean Seas Tuna within this CRC have also found a significant effect of stocking density on growth and survival of YTK larvae (see CST Fingerling RD Report_12 Nov 2012).

Artemia ingestion by larvae increased significantly with age (*P* > 0.001) but was equal across all treatments (*P* = 0.05). This further supports our hypothesis that larvae were not food limited, as such limitation would result in a reduction in *Artemia* ingestion rates in those treatments which were food limited.

Larval whole body taurine content was significantly affected by *Artemia* taurine content (P < 0.001). Those larvae receiving unsupplemented *Artemia* had a significantly lower whole body taurine content (1.85 ± 0.03%DW) than all taurine supplemented treatments, which did not differ from each (pooled average 2.48 ± 0.03%DW). The relationship between the taurine content of the *Artemia* and the whole body taurine content of the larvae was described by Equation 9-2 with an R^2 of 0.98 (Figure 9-45).

$$Larval\,taurine\,content\ (\%DW) = 0.79 + 1.75 \times (1 - \exp^{(-1.22 \times Artemia\,taurine)})$$

Equation 9-2: Relationship between *Artemia* taurine content and larval whole body taurine content.

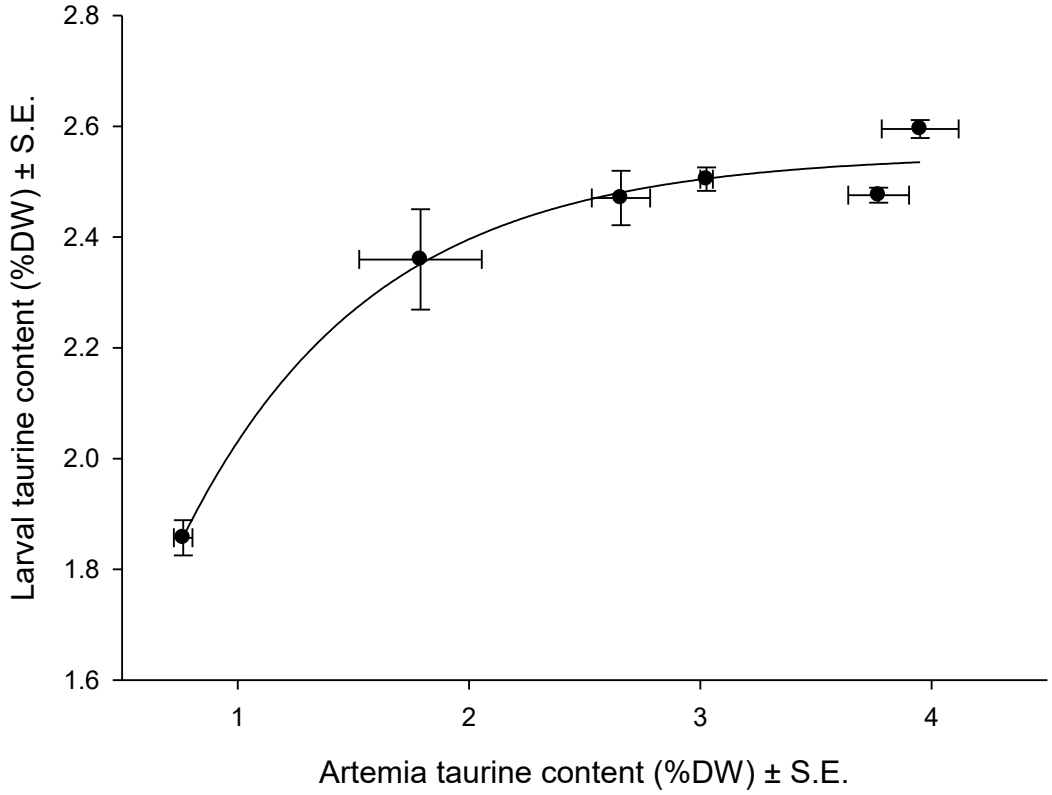

Figure 9-45: The relationship between *Artemia* taurine content and larval taurine content.

Whilst the *Artemia* receiving the higher doses of taurine had much higher levels of taurine than wild zooplankton and despite significant differences in the taurine content of the *Artemia* at most supplementation rates, there were no significant differences in the whole body taurine contents of larvae receiving taurine-supplemented *Artemia*. This suggests that the larvae of this age either possess a functional mechanism for the excretion of excess taurine, or that passive loss is occurring at levels beyond saturation. Excess taurine is excreted by a net tubular secretive process within the kidneys and passes out in the urine (King and Beyenbach, 1982). If the plateauing of whole body taurine was due to a functional regulatory mechanism, this would suggest that any taurine beyond the larvae's requirements could be excreted and not cause detrimental effects to the larvae. Whilst the first structures of the kidneys in most larval marine fish are observed at around 3 dph, with the haemopoietic tissues appearing shortly afterward (Castro Cunha et al., 2003),

the level of development and differentiation within the kidney at these early stages may not be adequate for excreting excess taurine. T

The whole body taurine content of larvae fed supplemented *Artemia* (2.48%DW) in this trial is lower than the previous trial, where larvae that received *Artemia* containing taurine at 8.24%DW had a whole body taurine content of 2.65%DW. Both of these values are higher than the taurine content reported for 30 mm wild Japanese yellowtail (2.3%DW) (Matsunari et al. 2005). Juvenile Japanese flounder (50 mm) fed on mysids contained a similar amount of taurine (2.4%DW). Whilst the absolute differences between our two studies and that of Matsunari et al. (2005) are not great, they may represent saturated or supersaturated (and subsequently detrimental) levels. The relationship described above at
Equation 9-2, suggests that the *Artemia* taurine content required to obtain a whole body larval taurine content equal to that of wild Japanese yellowtail (2.3%DW) is 1.6%DW. Using Equation 9-1 subsequently suggests that the taurine enrichment dose required to achieve this *Artemia* taurine concentration is 0.6 g/L, lower than the lowest dose rate used in this study. Furthermore, we also suggest below (Section 9.3.7.3) that pre-metamorphic YTK larvae may have a lower tolerance to taurine than post-metamorphic larvae and if this is the case, then aiming to achieve the same whole body taurine content of juvenile (post-metamorphic) Japanese yellowtail may still result in detrimental effects. Further studies are therefore required to determine if an enrichment dose rate of 0.6 g/L would lead to significant improvements in YTK larval performance relative to the higher doses used in these studies and which were found to be detrimental to larval survival.

There was no effect of taurine supplementation dosage on jaw deformity levels (P= 0.75). The percentage of larvae with a malformation score ≥ 2 was 17.3 ± 0.9% (pooled mean ± S.E.). This finding is in contrast to the previous trial where we found that larvae fed *Artemia* enriched with taurine had significantly lower malformations than those without, however the malformation rate in this trial was lower than the previous trial.

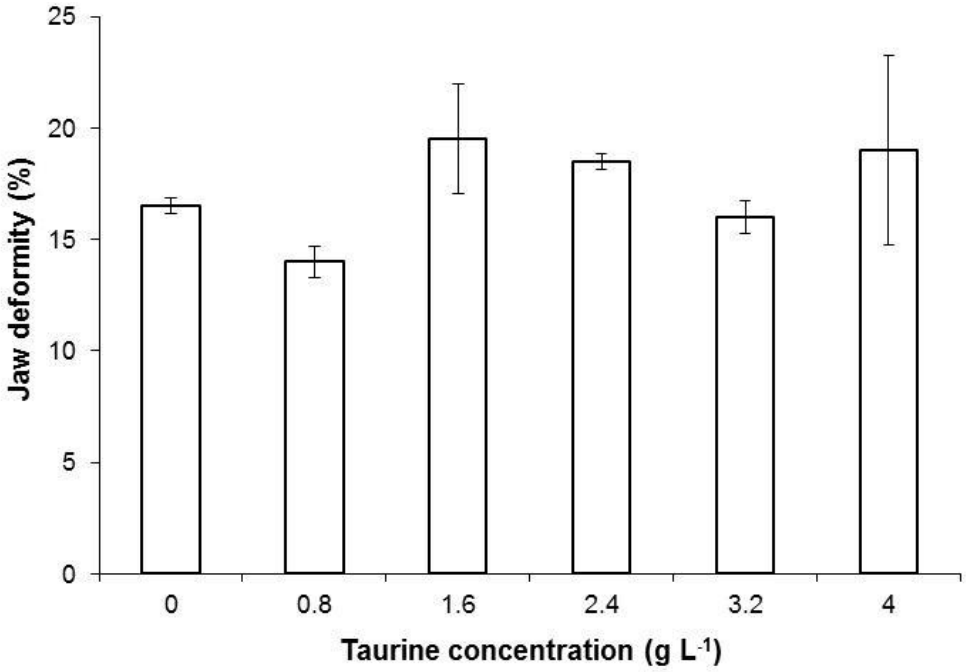

Figure 9-46: Percentage of YTK larvae with a jaw malformation score ≥2.

9.3.7. The effect of taurine dose rate on the taurine content of rotifers and the effect of these rotifers on performance of YTK larvae

9.3.7.1. Introduction

As previously described, data from all of the aforementioned previous trials combined with results of other published studies suggests that large differences in the taurine concentration of enriched live foods can occur, even when following similar enrichment protocols. Following similar methods described above for *Artemia* (see Section 9.3.6) , this trial aimed to determine the relationship between taurine dose rate during rotifers enrichment and rotifer taurine concentration and the subsequent effect these enriched rotifers had on the larval performance of YTK..

9.3.7.2. Materials and Methods

To determine the relationship between taurine dose rate and rotifer taurine content, rotifers were enriched for 18 hours on Spresso following the manufacturer's directions and taurine at dose rates of 0, 0.8, 1.6, 2.4, 3.2 and 4.0 g/L. After 18 hours rotifers were harvested, thoroughly rinsed in freshwater before dewatering and freezing. Prior to analysis samples were freeze-fried then ground. Total taurine content was determined via acid hydrolysis as previously described (see Section 9.3.6.2.).

A larval rearing trial was then undertaken in which YTK larvae were fed Spresso-enriched rotifers co-enriched with taurine at doses of 0, 0.4, 0.8, 1.6, 2.4 and 3.2 g/L. Rotifers were fed these diets in duplicate in the aforementioned larval rearing system from 3 to 12 dph using the hybrid feeding method described in Section 9.2. At the completion of the trial, larvae were hand-counted to determine survival, growth was assessed by measuring larval standard length and a subsample of larvae from each tank was taken for analysis of total taurine using previously described methods (see Section 9.3.6.2).

9.3.7.3. Results and Discussion

The relationship between taurine dose rate (g/L) and rotifer taurine content (%DW) is shown in Figure 9-47 and was described by Equation 9-3 with an R^2 of 0.99.

$$Rotifer\ \ taurine\ content\ (\%DW) = -0.147 \times (dose)^2 + 1.27 \times (dose)$$

Equation 9-3 Relationship between taurine enrichment dose (g/L) and taurine content in rotifers enriched on Spresso for 18 hours.

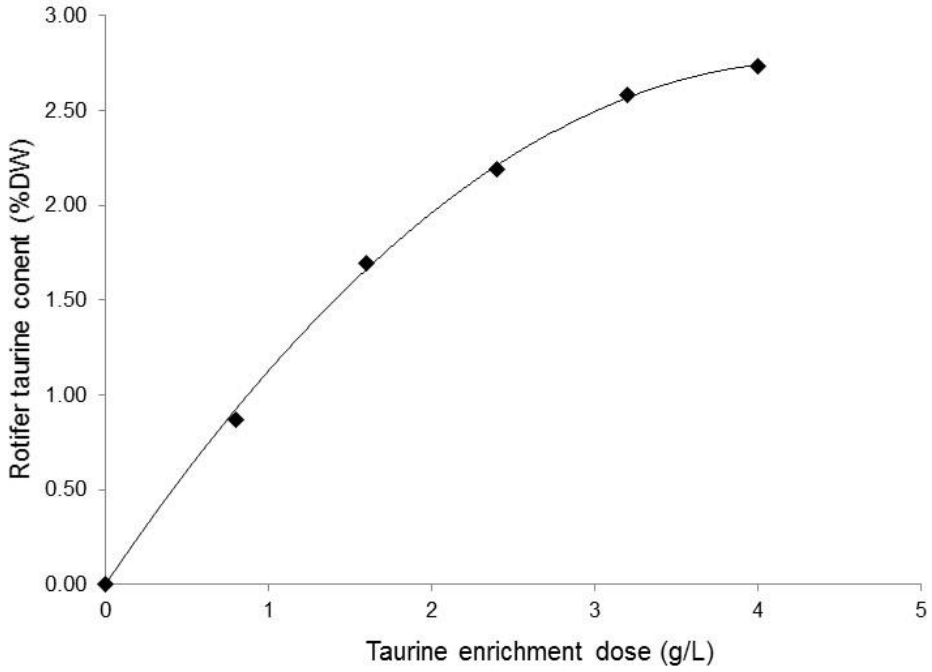

Figure 9-47: Relationship between taurine dose rate (g/L) and rotifer taurine content (%DW) following an 18 hour enrichment period in Spresso.

The total rotifer taurine content increased from close to zero without supplementation to 2.73% DW at 4 g/L. Based on this relationship and on the results from our previous trial on *Artemia*, the highest dose rate of taurine (4.0 g/L) was not tested in the larval rearing trial, but was replaced with a lower dose rate of 0.4 g/L. Based on this relationship, this dose rate would have resulted in a rotifer taurine content of 0.5% DW.

Survival of YTK larvae to 12 dph on the various treatment diets is shown in Figure 9-48. Due to the high variation around the 0.8 g/L treatment, ANOVA showed there to be no significant difference in survival between treatments (P = 0.09), despite survival rates ranging from 6 to 22%. Excluding this treatment from the analysis revealed that the survival in the treatment without taurine supplementation was significantly higher than all other treatments, with the exception of the 3.2 g/L treatment (P = 0.01). These finding are similar to those reported above for taurine supplemented *Artemia* and were unexpected. However, there does appear to be an emerging body of very recent evidence that taurine can be detrimental to pelagic marine fish larvae. In a recent conference abstract, Hawkyard et al (2014a) reported a 40% reduction in survival of YTK larvae fed rotifers containing 1.5% DW taurine, relative the control of unsupplemented rotifers. Koven et al. (2014) also recently reported in a conference presentation that rotifers enriched to contain 0.64%DW taurine resulted in a significant reduction in survival when fed to northern bluefin tuna larvae compared with those enriched to contain 0.44%DW taurine. Based on our standard curve above, rotifers enriched on 0.4 g/L would have contained 0.5%DW of taurine. YTK larvae therefore appear similar to northern bluefin tuna larvae and may therefore benefit from a lower rotifer taurine content than the lowest investigated in this study.

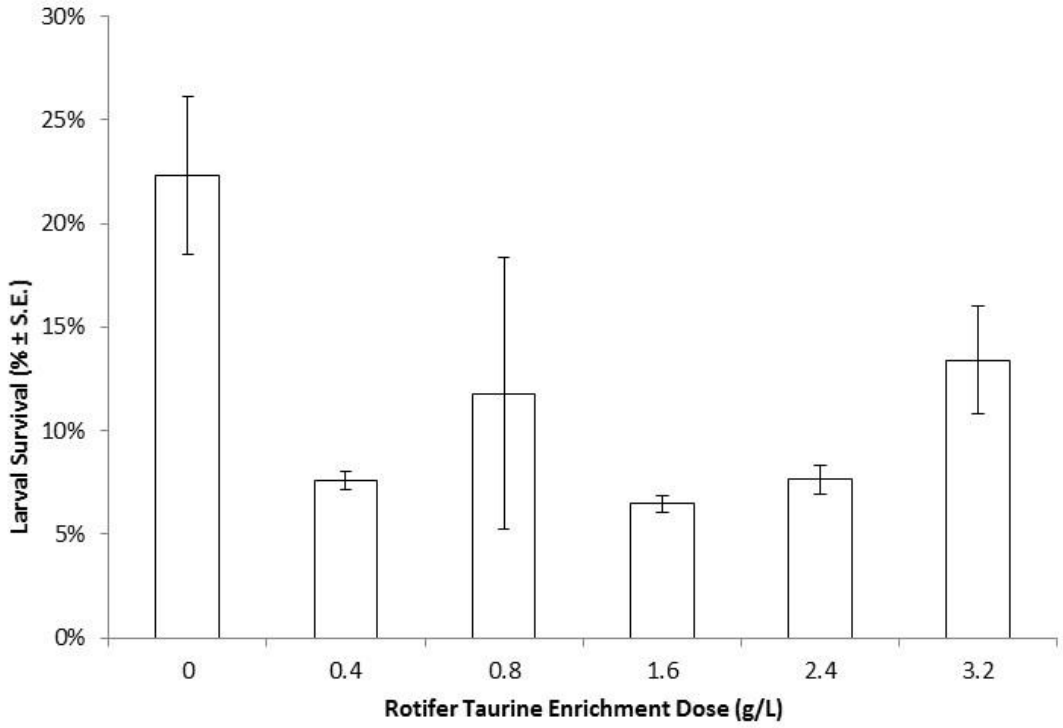

Figure 9-48: Effect of feeding rotifers enriched on different dose rates of taurine (g/L) on the survival of YTK larvae to 12 dph.

The growth of larvae fed rotifers enriched with different doses of taurine is shown in Figure 9-49. Again, due to a high variation between the duplicates of the 2.4 g/L treatment, ANOVA suggested there to be no significant differences between treatments (P = 0.10). Exclusion of this treatment from the analysis, however, suggested a significant effect of rotifer taurine enrichment dose on final standard length (P = 0.02), with those larvae fed rotifers enriched with 0.4 g/L of taurine being significantly larger (6.19 ± 0.01 mm) than those not receiving supplementation (5.50 ± 0.06 mm) and those fed rotifers enriched with 3.2 g/L (5.52 ± 0.02 mm). Koven et al (2014) also reported improved growth in northern bluefin tuna larvae when fed rotifers containing 0.44%DW taurine relative to a control of unsupplemented rotifers, but then a reduction in growth in those larvae fed rotifers containing a higher taurine content of 0.64%DW.

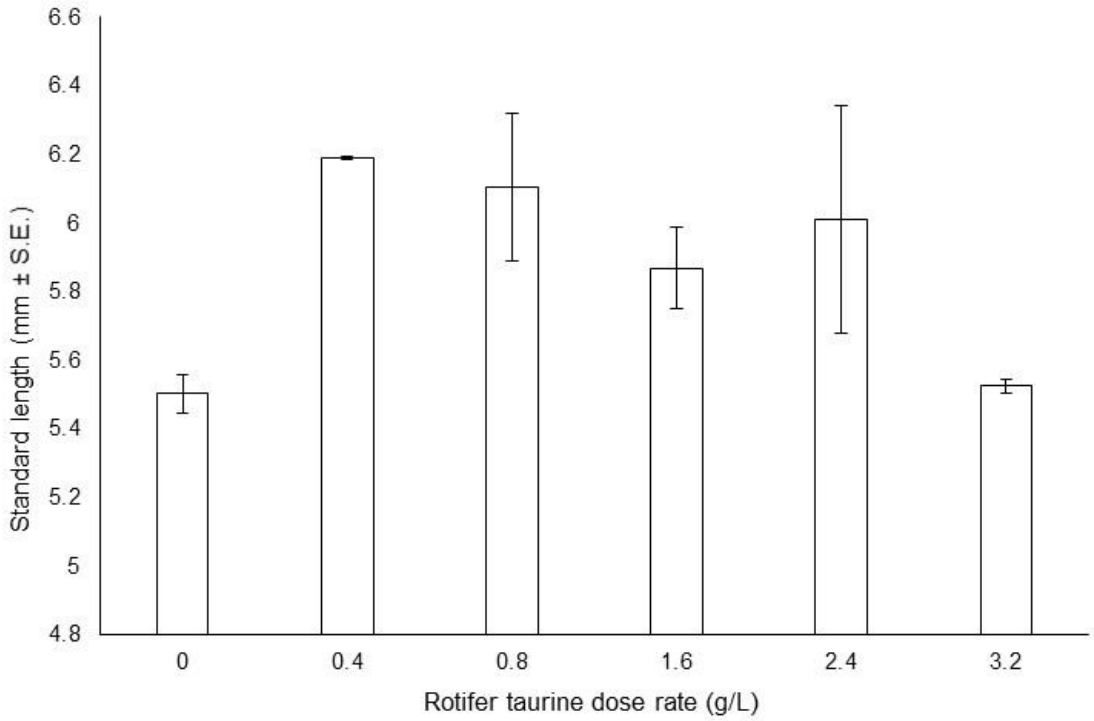

Figure 9-49: The effect of rotifer taurine dose rate of the standard length of yellowtail kingfish larvae on 12 dph.

The relationship between rotifer taurine content (%DW) and larval taurine content (%DW) in 12 dph YTK larvae is shown in Figure 9-50. The relationship between these two factors was described by the same exponential function described above for *Artemia* with an R^2 of 0.99 (Equation 9-4).

$$Larval\,taurine\,content\ (\%DW) = 0.29 + 1.56 \times (1 - \exp^{(-2.56 \times \text{rotifer taurine})})$$

Equation 9-4: The relationship between *Artemia* taurine content and larval taurine content.

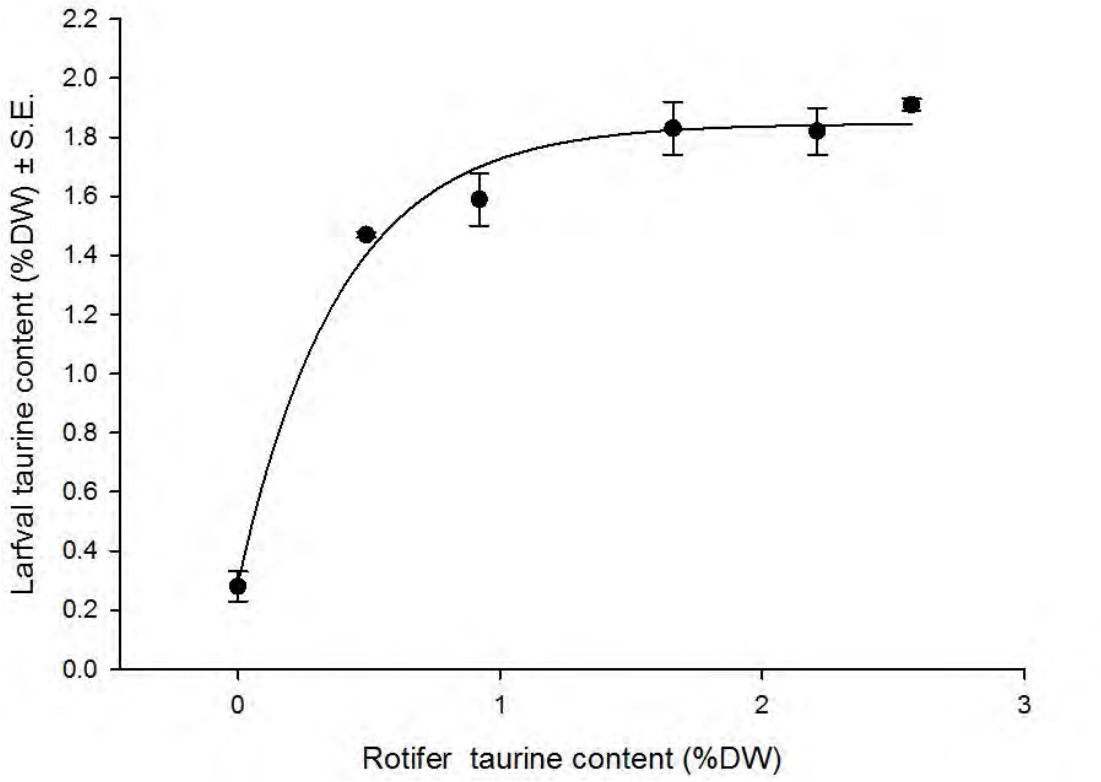

Figure 9-50: The relationship between *Artemia* taurine content and larval taurine content.

These functions demonstrate that rotifer-fed YTK larvae have a lower whole body taurine asymptotic (saturation) level (ca. 1.9% DW) than *Artemia*-fed YTK larvae (ca. 2.5 to 2.6%DW) and this is likely the result of differing body sizes or taurine excretion abilities with age. These data also suggest that the taurine requirement and toxicity levels to be different between rotifer-fed and *Artemia*-fed larvae. For example we saw a significant reduction in survival in YTK larvae fed rotifers containing only 0.5% TAU, whereas we hypothesised that YTK larvae fed Artemia may benefit from having their taurine content increased to 1.65% in order to achieve the same whole body content of wild juvenile Japanese yellowtail (2.3% DW). It is clear from this trial that targeting a whole body taurine content of 2.3%DW in younger larvae is unachievable and that larval mortality occurs at a much lower corresponding taurine content of enriched rotifers.

Koven et al. (2014) achieved a whole body larval taurine content of 1.04%DW in northern bluefin tuna larvae offered rotifers with a taurine content of

0.44%DW and 1.59%DW in those larvae fed rotifers containing 0.64%DW taurine. Using Equation 9-4, YTK larvae would contain 1.34%DW if fed rotifers containing 0.44%DW and 1.55% if fed rotifer containing 0.64%DW taurine, demonstrating a very similar response between northern bluefin and YTK.

The mechanisms for the apparent toxicity of taurine seen in these studies is unclear. Given that other studies with juvenile fish have demonstrated no detrimental effects of dietary taurine contents far exceeding those tested in these trials (Salze et al., 2014), suggests that the observed toxicity is specific to larval fish. This is therefore likely due to one of the many physiological mechanisms that taurine is involved with being overwhelmed in pre-metamorphic YTK and suggests that the ability to effectively deal with high dietary taurine contents only becomes functional after metamorphosis. Given this, our previous hypothesis that *Artemia*-fed YTK (i.e. pre-metamorphic larvae) may benefit from increasing the taurine content of *Artemia* to 1.65% DW to match the whole body content of juvenile (i.e. post-metamorphic) Japanese yellowtail may not be valid. The requirement for taurine in pre-metamorphic YTK therefore appears to be less than juvenile fish and more research is required to quantity the effects of lower taurine dose rates in both the rotifer and *Artemia* feeding stage of YTK.

9.3.8. References

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9.4. Objective 3 - Bacterial Management

9.4.1. Introduction

Microbial dynamics within aquaculture settings is a growing concern during the culture of various marine larval species and may inhibit larval performance. “Green-water culture” is a commonly used strategy where various species of live or inert microalgae is introduced to the culture environment. Some benefits of this strategy are prey selection and even distribution of larvae caused by the increased turbidity of the culture water. By utilising inert algae paste a potential issue of creating environments for higher bacterial loads may exist.

A recent strategy of substituting “green-water culture” for non-organic alternatives is being used for various challenging marine species. For example, within the past 20 years discoveries in the freshwater culture of walleye *Stizostedion vitreum* have led researchers and aquaculturists to investigate the artificial introduction of clay. Published research utilising a suspended clay slurry to increase water turbidity has been shown to significantly increase walleye larvae survival, growth, and increased swim bladder inflation over traditional “clear-water” culture (Bristow and Summerfelt 1994; Bristow et al. 1996; Rieger and Summerfelt 1997; Clayton and Summerfelt 2010). Many of these publications’ discussions accredit the high survival and growth to an increased food acceptance, likely caused by uniform larval dispersion and a high contrast of food particles within the turbid water.

Limited research exists regarding the use of this strategy in marine larval finfish rearing. Most research on the effects of increased turbidity levels in marine settings is centered on anthropogenic activities such as dredging in coastal areas and the interactions on various marine species during egg and larval stages (Isono et al. 1998; Partridge and Michael 2010). Many coastal regions naturally possess low turbidity levels (Auld and Schubel 1978; Isono et al. 1998) which gives an ‘unorthodox’ designation of exposing marine larval

finish to artificially introduced inorganic turbidity in an aquaculture setting. Anecdotal evidence exists that implementing this strategy in the culture of highfin amberjack S*eriola rivoliana* is showing promising survival results (Benetti pers. comm. 2010).

In 2012 ACAAR Production Manager Mr. Robert Michael was awarded a CRC travel bursary (CRC report number 2012/70) to travel to the University of Miami and learn the techniques used to deliver clay to cobia larval rearing tanks. During this travel bursary, Mr. Michael worked collaboratively with UM Master's student Mr. Zack Daugherty to conduct the experiment detailed in Part 1 below. In short, the use clay particles in cobia larval rearing resulted in significant reductions in bacterial loads and significant savings relative to the use of microalgal pastes, with no negative impact on growth or survival.

On Mr. Michael's return to ACAAR, a simple and unreplicated trial was conducted with barramundi larvae to refine and adapt the techniques to ACAARs conditions and, like cobia, barramundi larvae were found to perform equally well between clay and micoalgal based tanks.

Following this preliminary trial, a fully replicated trial was then conducted with YTK larvae in which larval performance was compared between larvae grown in three different sources of turbidity. The results of this trial are outlined in Part 2 below.

9.4.2. Part 1 - Effects of an Inorganic Clay as an Algae Paste Substitute on the Larval Rearing Performance and Vibrio Community in the Culture of Cobia (*Rachycentron canadum*)

9.4.2.1. Introduction

This section forms part of the thesis of Mr. Zack Daugherty, a Masters student of the University of Miami who was co-supervised by Dr. Gavin Partridge.

A non-selective approach for the reduction of *Vibrio* during the 'greenwater' phase (3 to 13 dph) of cobia *Rachycentron canadum* larval rearing was assessed by comparing microalgal paste with inorganic clay. Reed Rotigrow *Nannochloropsis* microalgae paste and Old Mine #4 Kentucky ball clay were tested at equivalent Secchi disk depths (55 to 65 cm) with six replicates per treatment. The effects of these two treatments on larval survival, 13 dph total length and changes in *Vibrio* concentrations were assessed.

9.4.2.2. Materials and Methods

Pre-trial standardisation of water additives

A measure of Secchi disc depth (SDD) is the most common method of measuring turbidity in the larval rearing of marine fish, including cobia, and a SDD range of 55 to 65 cm is typically used. As our experimental larval rearing tanks were shallower than this range, a relationship between SDD and Nephelometric turbidity units (NTU) was obtained for both the microalgal paste and clay to enable us to achieve a SDD equivalent to 55 to 65 cm by measuring and maintaining an equivalent NTU.

Standardised curves for microalgal paste and clay were obtained by measuring SDD (TTG 120 cm Transparency Tube, Water Monitoring Equipment & Supply, P.O. Box 344, Seal Harbor, ME, USA, 04575) and NTU

(Hach 2100 Q Portable Turbidimeter (Hach Company, P.O. Box 389, Loveland, Colorado, 80539) after cumulative additions of material to each of one 250 L tank were made and homogenised via a single central air source. Twenty dosages of Reed Rotigrow (*Nannochloropsis occulata* algae paste, Reed Mariculture Inc.) ranging from 1 – 70 mL were made. Old Mine #4 Kentucky ball clay (Paoli Clay Company) was prepared in a stock solution (SS) at a concentration of 20 g clay/L of seawater. The clay SS was continuously stirred and incrementally added to the second 250 L tank at 37 dosages ranging from 1 – 1000 mL. The number of dosages for each treatment was determined by the measurable bounds of the 120 cm transparency tube (0 – 120 cm depth). SDD readings were plotted against NTU readings and fit to a power model (Equation 9-5).

$$y = c \times x^{b}$$

Equation 9-5: Function used to describe the relationship between Secchi disk depth (SDD = y) and turbidity (NTU = x).

Experimental Setup

This study was conducted at the University of Miami Experimental Hatchery (UMEH). Trials were performed using a 12 x 400 L tank array nested in a 15 m^3 water bath to mitigate diurnal temperature fluctuations. The experimental system was located outdoors under 90% shade cloth, where day time light intensity ranged from 1000 – 1400 lux (approximately 19 – 26 µmol/s/m^2 using sunlight conversion from Thimijan and Heins, 1983).

Turbidity (NTU) was measured 4 times daily (0800, 1100, 1400 and 1700) using the aforementioned Hach 2100 Q Portable Turbidimeter (Hach Company, P.O. Box 389, Loveland, Colorado, 80539). Dissolved oxygen levels, pH, salinity and temperature were measured twice daily (0800 and 1700) using a YSI Professional Plus Meter (YSI, Inc., 1700/1725 Brannum Lane, Yellow Springs, Ohio, 45387).

Clay and paste stock solutions were prepared in 1 µm filtered and UV treated seawater then stored in aerated 15 L conical tanks Each stock solution was pumped into a mixing chamber using a peristaltic pump (Masterflex L/S, Cole-Parmer) before gravity flowing to each replicate tank to maintain an SDD in each tank equivalent to 55 to 65 cm.

Larval Rearing

Cobia eggs were obtained using methodology described in Benetti et al. (2008b) and incubated at 500 eggs/L. Larvae were counted and stocked into each tank on 2 dph at a density of 15 /L under 300% water exchange/day. All standpipes were fitted with 300 µm mesh and central air rings at the base to provide a laminar current.

At 3 dph, the two treatment additives were introduced into each tank. The trial was conducted until 13 dph, or post-flexion stage, which is commonly associated with a significant drop in survival as a consequence of metamorphosis (Benetti et al., 2008b). Prior to first daily feedings, initial dosages of treatments were added to each designated tank with clay concentrations of 0.013 g/L of culture water and algae paste of 0.03 mL/L of culture water. Each initial treatment was mixed into 1500 mL stocked solution using saltwater to facilitate the addition into each tank

Rotifers were enriched using the UM Standard diet previously described in Section 9.3.2 and fed at 0800 hrs at 5 /mL. Thirty minutes prior to subsequent feedings, residual rotifers were counted and the appropriate number of new rotifers were added to maintain 5 rotifers/mL. Remaining live feeds were cold-stored at 10°C and fed at 1100, 1400, and 1700 hrs. Live feed concentrations were increased to 7 /mL at 11 dph to account for increased demand of prey.

Bacteriology

Water samples were obtained in sterile 1.5 mL microcentrifuge tubes from the seawater reservoir tank supplying water to the system, and each larval rearing

tank on days 5, 7, 10, and 12 to determine changes in total cultivable *Vibrio* communities. Each sample was plated in triplicate on TCBS (thiosulfate citrate bile sucrose, BD Diagnostics) using the membrane filter technique and incubated at 40°C for 24 hrs following a protocol outlined by Hernández-López et al. (1995). Total *Vibrio* colony forming units (CFU/mL) were enumerated after 24 hours.

Larval Sampling

Larval growth was assessed by measuring total length (TL, mm) at the end of trial period using digital microscopy (OptixCam Summit OCS 10.0). Survival was measured by hand-counting all remaining larvae at the end of the trial and expressing this number as a percentage of the number stocked.

Data Analysis

Independent t-tests were employed to determine if significant differences occurred in growth, survival, and total Vibrio concentrations between treatments. All results are reported as means ± SE. All statistical analyses were achieved using SAS JMP®, Version 10 (SAS Institute Inc., Cary, NC, 1989-2013).

9.4.2.3. Results

The Secchi disk depth-turbidity relationships for algae paste and clay are shown in Figure 9-51 and Equation 9-6 a and b.

$$SDD_{paste}\,(cm) = 90.4 \times T_{NTU}{}^{-0.78} \quad R^2 = 0.99 \quad (a)$$
$$SDD_{clay}\,(cm) = 454.3 \times T_{NTU}{}^{-0.86} \quad R^2 = 0.99 \quad (b)$$

Equation 9-6: Relationship between Secchi disk depth and turbidity for algae paste (a) and clay (b)

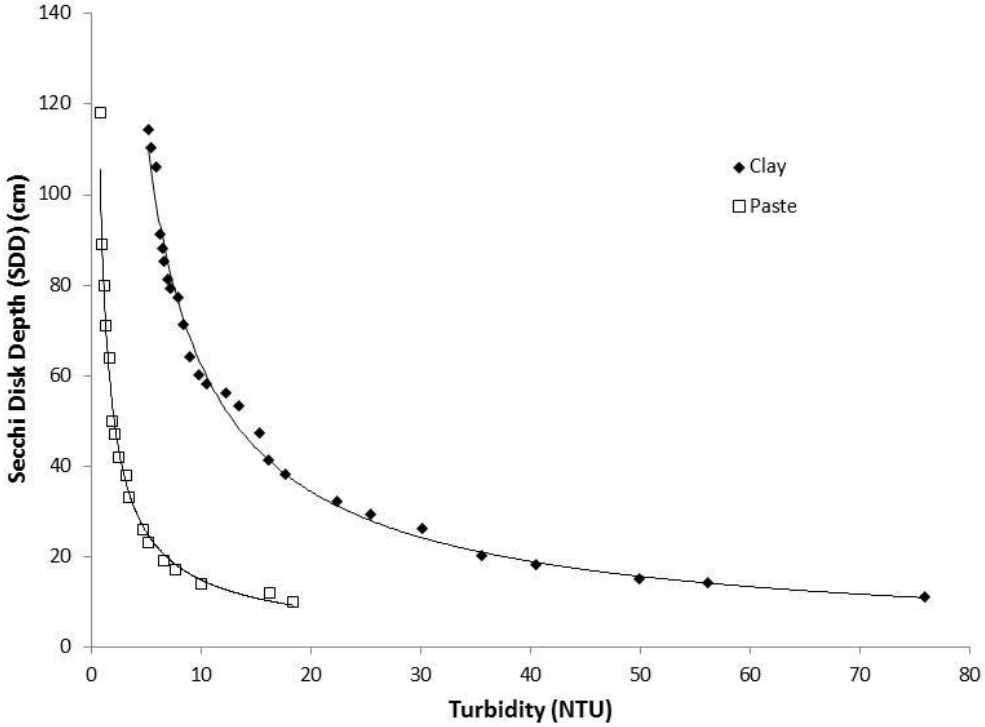

Figure 9-51: Relationship between Secchi disk depth and NTU for microalgal paste and clay.

The average turbidity in the two treatments during the rearing trial were 9.62 ± 0.06 NTU for clay and 1.96 ± 0.02 NTU for algae paste. Transformed mean SDD were significantly different ($P < 0.0001$) between CWT (64.7 ± 0.3 cm) and GWT (53.5 ± 0.4 cm).

No significant differences were found ($P = 0.40$) in growth (TL) between clay (8.21 ± 0.09 mm) and algae paste (8.06 ± 0.07 mm) treatments (Figure 9-52). No significant differences were found ($P = 0.54$) in larval survival between clay (40.8 ± 2.7%) and algae paste (35.8 ± 7.2%) treatments (Figure 9-53).

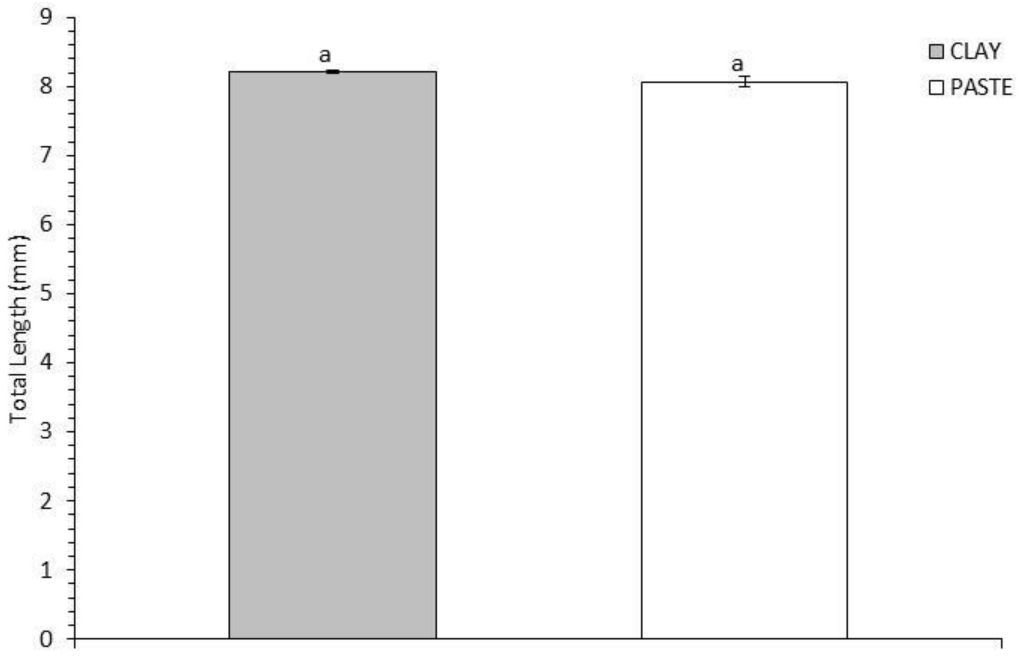

Figure 9-52: Final total length (TL) (13 dph) of cobia larvae reared in tanks with clay or paste.

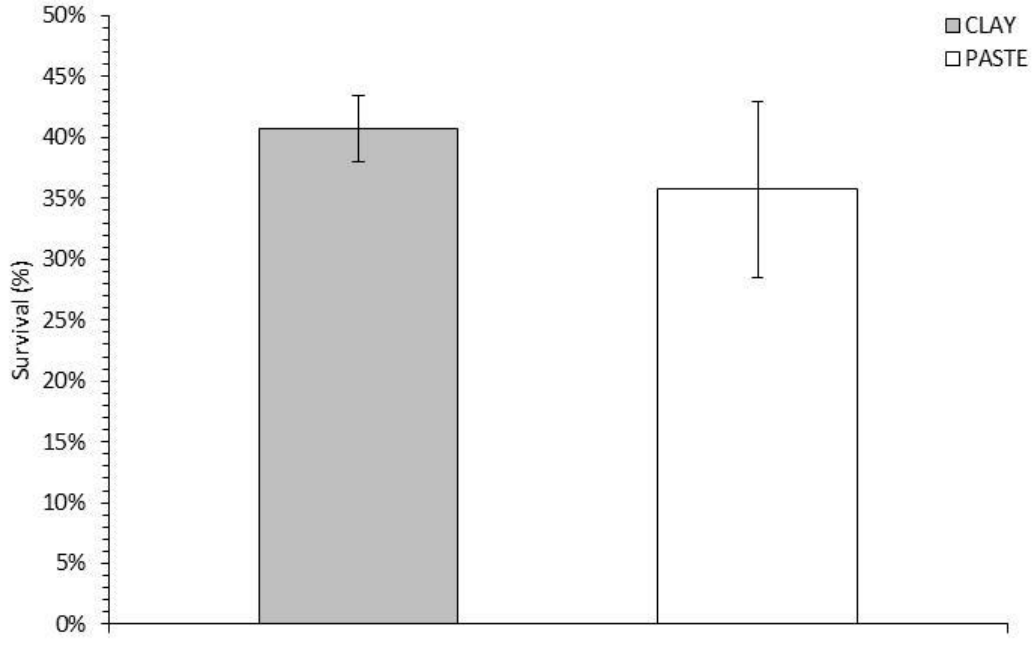

Figure 9-53: Survival of cobia larvae (13 dph) reared in tanks with clay or paste.

The number of *Vibrios* was always higher in the GWT compared with the CWT treatment (Figure 9-54). Despite a three-fold reduction in day 5 mean total *Vibrio* concentrations between GWT (32 ± 7 CFU/mL) and CWT (10 ± 9 CFU/mL), this difference between treatments was not significant ($P = 0.08$). However, significant differences in this parameter were found on day 7 ($P < 0.01$) and 10 ($P < 0.0001$) with the GWT (215 ± 44 and 598 ± 42 CFU/mL) having higher total *Vibrio* concentrations than the CWT (34 ± 4 and 17 ± 5 CFU/mL). Despite a 22 fold lower *Vibrio* count on day 12 in the CWT (14 ± 2 CFU/mL) relative to the GWT (320 ± 123 CFU/mL), this difference was not significant ($P = 0.06$) due to the high variation associated with the algae paste treatment.

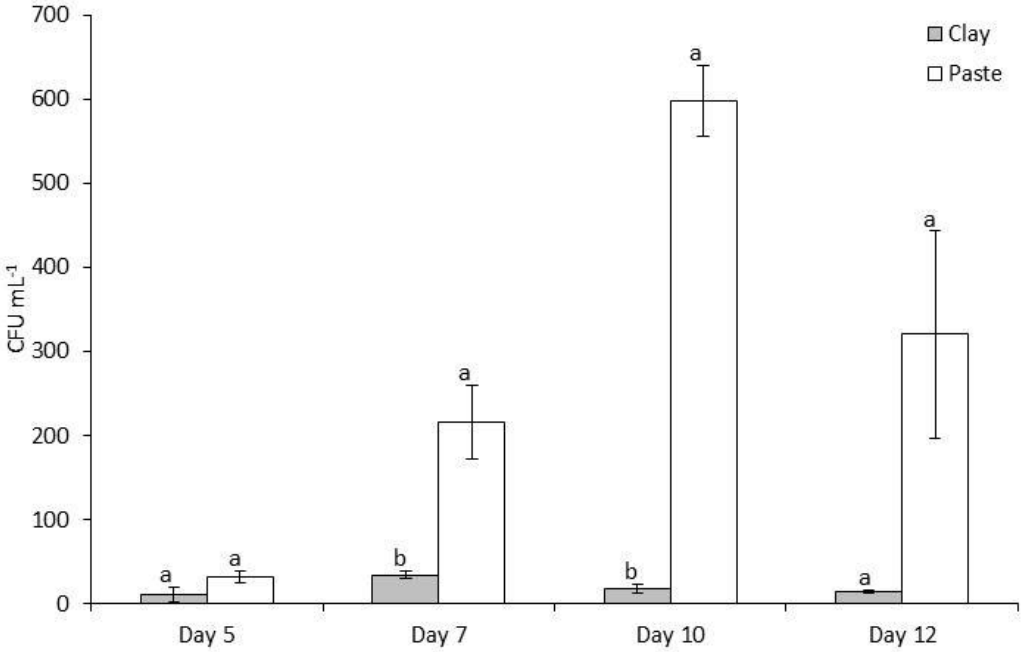

Figure 9-54: Total vibrios (CFU/mL) in cobia larval rearing water by day in tanks containing clay or paste.

9.4.2.4. Discussion

The methodology associated with transforming turbidity (NTU) into a relational Secchi depth (cm) developed for this study was found to be highly sensitive to the individual tank variations associated with additive dosages and daily exchange rates. Because of this sensitivity, there were significant differences

in transformed SDD between treatments; however, these differences were not apparent under visual observation and both treatments were within the target range of normal SDD for the larval rearing of cobia (55 to 65 cm). Although cobia foraging efficiency under different concentrations and sources of turbidity have not been studied, Gregory and Northcote (1993) show that juvenile chinook salmon *Oncorhynchus tshawytscha* possess the ability to select *Artemia* uniformly at turbidities ranging between 0 – 70 NTU indicating that foraging efficiency may not be significantly effected at such sensitive scales seen in this study.

Vibrio concentrations were found to be significantly lower in the clay treatment compared to the algae paste treatment. These results are in agreement with the findings of similar studies on clay's influence on *Vibrio* concentrations in marine larval culture (Attramadal et al., 2012b; Björnsdóttir et al., 2011). This study likely displayed much lower bacterial concentrations than those found by Attramadal et al. (2012b) because of the reduced stocking densities of larvae (100 /L versus 15 /L) and the associated reduction in organic inputs. Even though *Vibrio* concentrations were significantly reduced in CWT, there appeared to be no relationship with the observed larval performance when compared to GWT tanks. The effects of Vibriosis in cobia has been documented (Liu et al., 2004; McLean et al., 2008), but negative impacts to cobia larval rearing performance may be influenced more by optimal culture practices and adequate nutrition and less with the overall presence of *Vibrio* spp. within culture systems. The results suggest that either cobia possess resilience to the increased bacterial concentrations associated with typical greenwater aquaculture methods, or that potentially harmful *Vibrio* thresholds were not reached. Knowledge on the overall microbial composition and its effects on cobia larval performance is likely more important than observing only total opportunistic *Vibrio* concentrations (Attramadal et al., 2012b; Björnsdóttir, 2010; Munro et al., 1995; Vadstein et al., 2004, 1993; Verner-Jeffreys et al., 2004). Although no negative effects on survival with different levels of *Vibrio* were observed, clay turbidity may serve to prevent disease outbreaks associated with high levels of pathogenic and opportunistic bacteria.

Survival was similar between treatments as seen in halibut larvae (Björnsdóttir et al., 2011), however the GWT displayed a higher degree of variation. Although no significant differences were found in growth of cobia between additive treatments, CWT did possess slightly higher means with less variation across all replicated treatments. The observed stability could provide for more reliable production numbers when expanded to commercial scales. Although prey consumption was not directly measured during this study, a repeating pattern of clay treatment tanks requiring more rotifers to maintain 5 /mL prey densities was observed. This requirement for more prey items in CWT tanks during feeding periods may be attributed to increased rotifer mortality due to the prey's consumption of clay particles. In using clay strategies to replace algae, it is important to flush uneaten prey items from culture tanks to ensure only enriched prey items are being consumed (Stuart et al., 2013). Kirk and Gilbert (1990) have shown that a similar species of rotifer was unaffected by the presence of suspended sediments and were even capable of selecting algae cells when present in turbid environments. This indicates there may be potential benefits to an approach using a paste – clay additive combination that would both provide continued enrichment to rotifers and reduced bacterial loads. However, the presence of algae paste may override the buffering capacity for clay to mitigate opportunistic microbial communities.

9.4.3. Part 2 - Effects of an Inorganic Clay and Artificial Microalgae as an Algae Paste Substitute on the Larval Rearing Performance and Vibrio Community in the Culture of YTK.

9.4.3.1. Introduction

The purpose of this trial was to determine if the same benefits seen in the previous trial on cobia and which anecdotally exist for highfin amberjack could be achieved with YTK.

In addition to the same two treatments of microalgal paste and clay used in the previous trial, a third treatment was also compared in the current trial. ‘Sol-gel’ is an experimental artificial algae produced in Australia made from silica and containing chlorophyll.

9.4.3.2. Materials and Methods

The 3 treatments were compared with 4 replicates in the aforementioned ACAAR larval rearing research system (Section 9.2.2). Using very similar techniques used during the trial on cobia (see CRC report number 2012/70), each material was maintained at turbidity levels equating to Secchi disk depths (SDD) of 50 - 60 cm. The same previously described functions derived for clay and paste were used in the current trial (Equation 9-6 (a) and (b) in Section 9.4.2.3) and a new function was created for Sol-gel using the same methods (see Section 9.4.2.2).

Each treatment material was automatically dosed into the larval rearing tanks from header tanks in order to maintain the required NTU. The trial was conducted between 3 and 13 dph and larvae were fed on Spresso-enriched rotifers according to the hybrid method previously described. Growth, survival, rotifer ingestion and swim bladder inflation rates were assessed as per previous trials. Culture water from each larval rearing tank was sampled for

total bacterial counts (marine agar) and *Vibrio* sp. counts (TCBS) on 5, 9 and 12 dph.

9.4.3.3. Results and Discussion

The relationship between the Secchi disk depth and turbidity for Sol-gel is shown in Figure 9-55, along with the same previously derived relationships for clay and paste. The SDD-NTU relationship for Sol-gel is described in Equation 9-7:

$$SDD_{Sol-gel}\,(cm) = 382.9 \times T_{NTU}^{\;-0.89} \quad R^2 = 0.99$$

Equation 9-7: Relationship between Secchi disk depth and turbidity for Sol-gel.

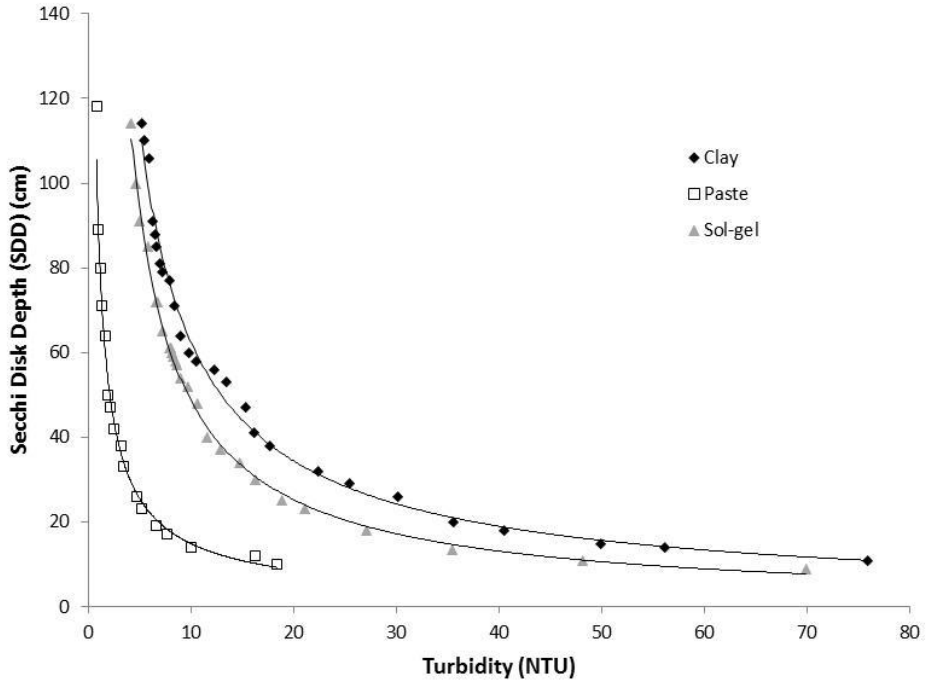

Figure 9-55: Relationship between Secchi disk depth and NTU for microalgal paste, clay and Sol-gel.

Bacteriology results revealed an early peak in *Vibro* within the microalgal based tanks, which subsequently stabilised, resulting in no significant differences in *Vibrio* numbers between treatments from 9 dph (Figure 9-56). Total bacteria numbers decreased in all tanks through time, however counts

in the paste and Sol-gel tanks were always higher than in the clay tanks (Figure 9-57), demonstrating the effectiveness of clay particles in adsorbing bacteria and removing them from the water column.

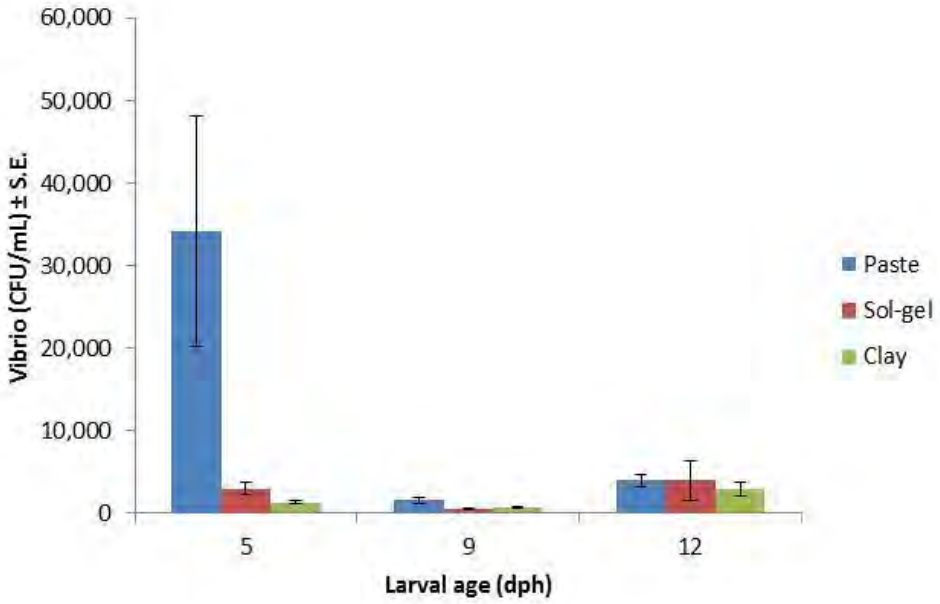

Figure 9-56: The effect of treatment and time on *Vibrio* counts in YTK larval rearing tanks.

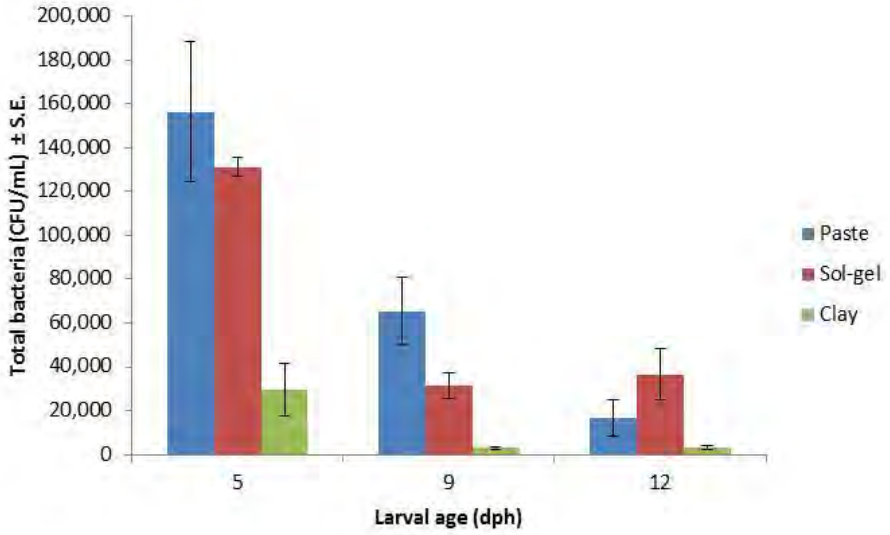

Figure 9-57: The effect of treatment and time on total bacteria counts in YTK larval rearing tanks.

Larval survival was significantly affected by treatment (P = 0.0002)(Figure 9-58). Those larvae cultured with microalgal paste had significantly higher

survival (21.6 ± 2.9%) than those in both the clay tanks (6.9 ± 1.0%) and Sol-gel tanks (2.3 ± 2.0%), which did not differ from each other.

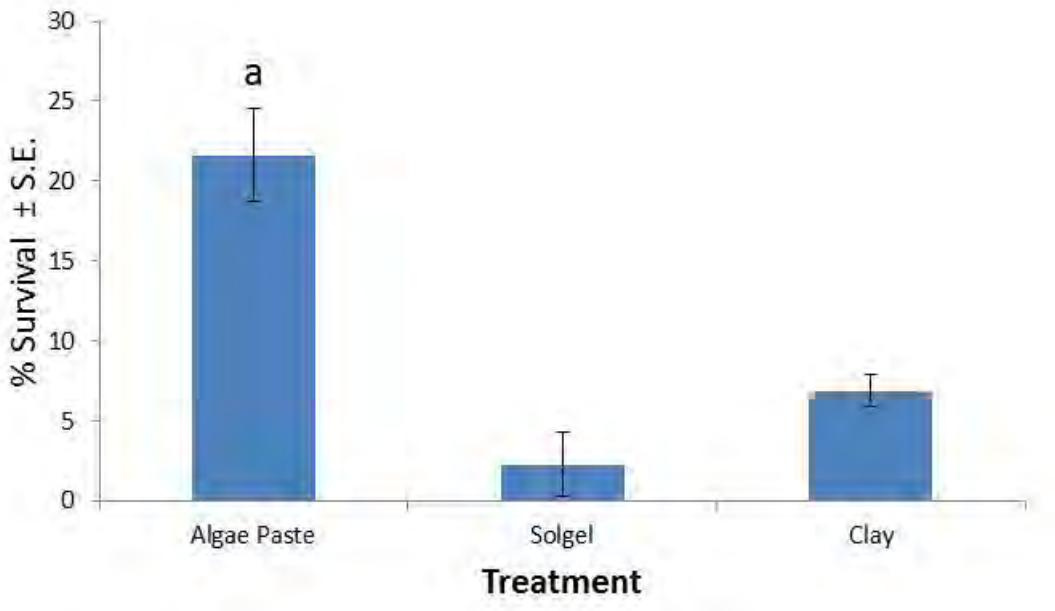

Figure 9-58: The effect of treatment on survival of YTK.

Swim bladder inflation in the Sol-gel tanks was 0%, due to the leaching of an oil-based component from the artificial microalgal cells which could not be effectively removed via skimming. Swim bladder inflation on 6 dph in the microalgae tanks (48 ± 8%) was significantly higher (P = 0.0007) than in the clay tanks (28 ± 5%)(Figure 9-59). Whilst the surface of the clay-based tanks remained relatively clean as a result of no leaching oils, we hypothesise that the poor swim bladder inflation in this treatment was due to the abnormal behavior and stress seen in the larvae in these larvae, as is described in further detail below.

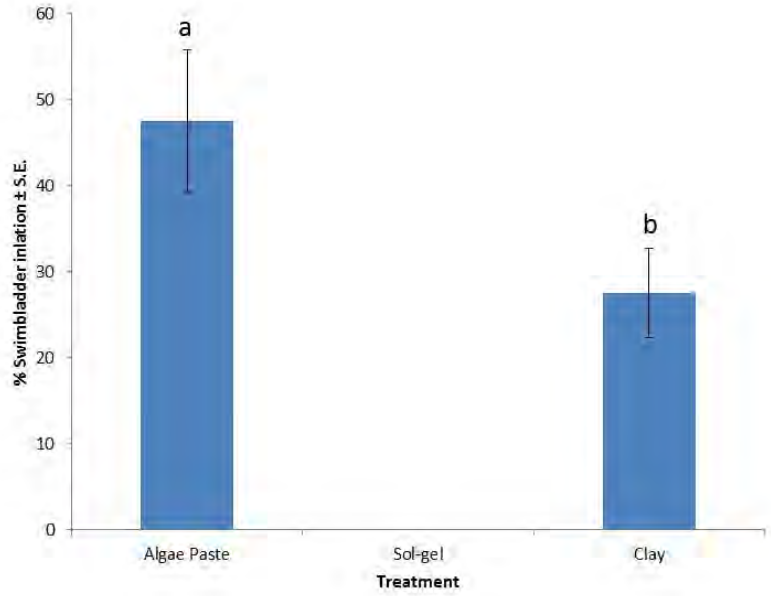

Figure 9-59: The effect of treatment on YTK swimbladder inflation on 6 dph.

Larval growth was also significantly affected by treatment (Figure 9-60). Those larvae grown in microalgae were significantly longer (SL 5.80 ± 0.08 mm) on 12 dph than those grown in the clay tanks (SL 5.40 ± 0.06 mm) but not different to those grown in Sol-gel tanks (SL 5.65 ± 0.03 mm).

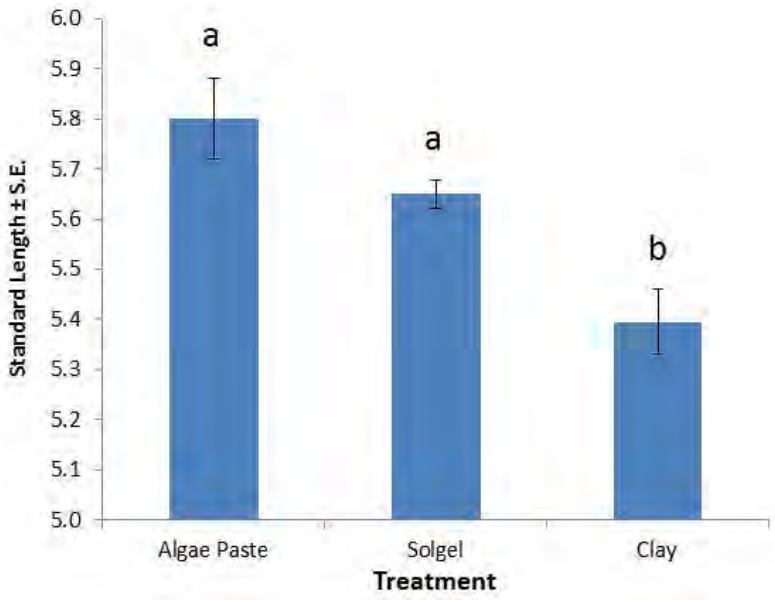

Figure 9-60: The effect of treatment on the standard length of YTK larvae on 12 dph.

Feeding incidence and rotifer ingestion rates were both significantly impaired in the clay and sol-gel tanks relative to the microalgal paste tanks (Figure 9-61

and Figure 9-62). Between 4 and 8 dph, feeding incidence in Sol-gel and clay based tanks were significantly lower than microalgae paste tanks. By 10 dph feeding incidence was equal across all tanks, most likely due to the fact that non-feeding larvae had starved by this time. On all days, larvae in microalgal paste tanks consumed significantly more rotifers than in the Sol-gel and clay-based tanks, which did not differ from each other.

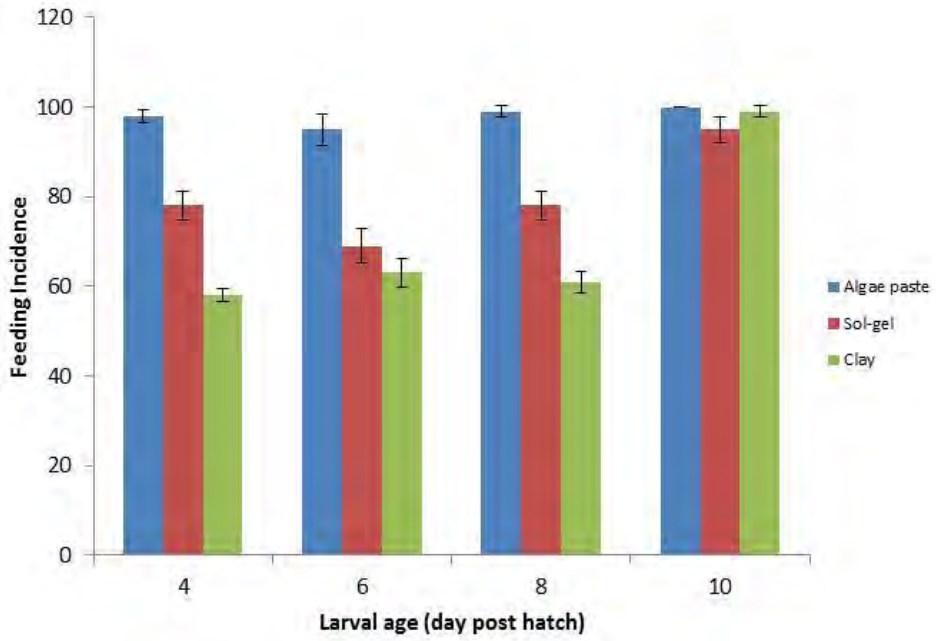

Figure 9-61: The effect of age and treatment on feeding incidence (% of larvae with food in their gut) of YTK larvae.

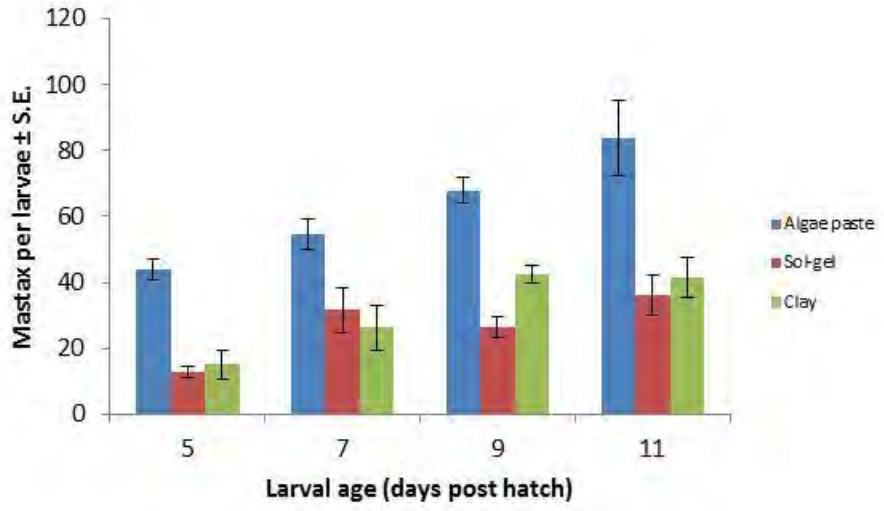

Figure 9-62: The effect of age and treatment on rotifer ingestion by YTK larvae.

We believe that the poor performance of the larvae in the clay tanks was due to an adverse effect on their behavior, as the larvae displayed erratic swimming behavior and disorientation; behaviors that have previously been attributed to light shock in YTK larvae. Whilst the light intensity at the surface of the water was equal across all tanks and treatments (P = 0.4)(49 ± 3 µE/m²/s), the white and reflective nature of the clay particles resulted in a significantly brighter downwards light intensity within the tank than all other treatments (measured underwater at the halfway point of the depth of the tank) (P < 0.0001) (clay = 9.2 ± 0.5 µE/m²/s and microalgae paste 5.7 ± 0.8 µE/m²/s)(Figure 9-63).

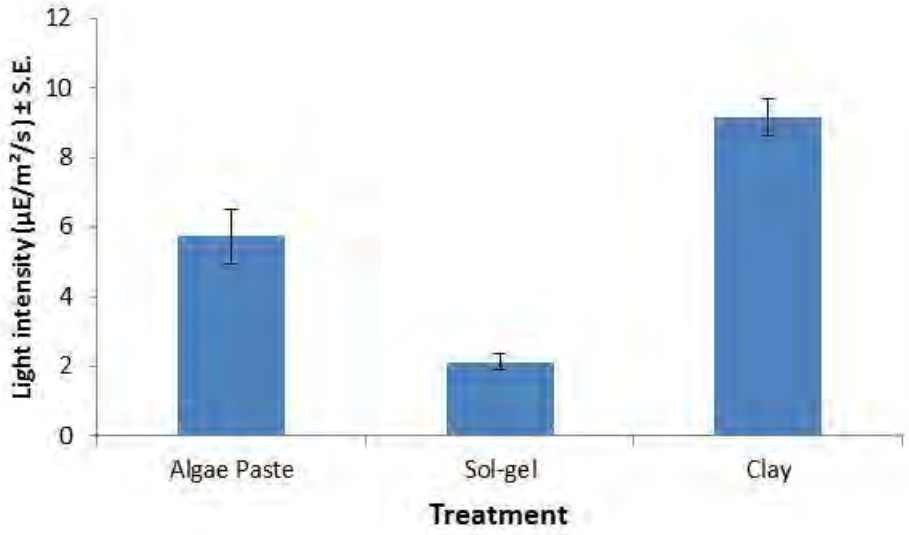

Figure 9-63: The effect of treatment on the downwards facing light intensity measured underwater at the mid-point in depth.

As YTK have been demonstrated to thrive under very high (surface) light intensity Stuart and Drawbridge (2011), we cannot attribute their poor performance or stress to only the surface light intensity, but the high degree of internal reflection within the water column may have resulted in the observed disorientation and stress in the larvae which subsequently resulted in poor swim bladder inflation, ingestion, growth and survival. Therefore, whilst high light intensities have been demonstrated to be effective for the rearing of YTK larvae in greenwater systems, further investigations into the interactive effect of light intensity and clay-induced turbidity will be required to determine if clay can be used effectively for the larviculture of YTK. In a subsequent trial testing

the effect of tank-base colour in clay tanks we effectively demonstrated that the downwards light intensity is much higher in tanks with white bases (i.e. the same tanks used in the aforementioned trials) than in black-based tanks (Figure 9-64) and that black-based tanks may therefore be more appropriate for use with clay. For example, within the NTU range corresponding to an equivalent Secchi disk depth of 50 – 60 cm (9.6 to 13.3 NTU), the downwards light intensity within the black based tank is reduced by ~90% (Figure X). Further research is therefore warranted in this area.

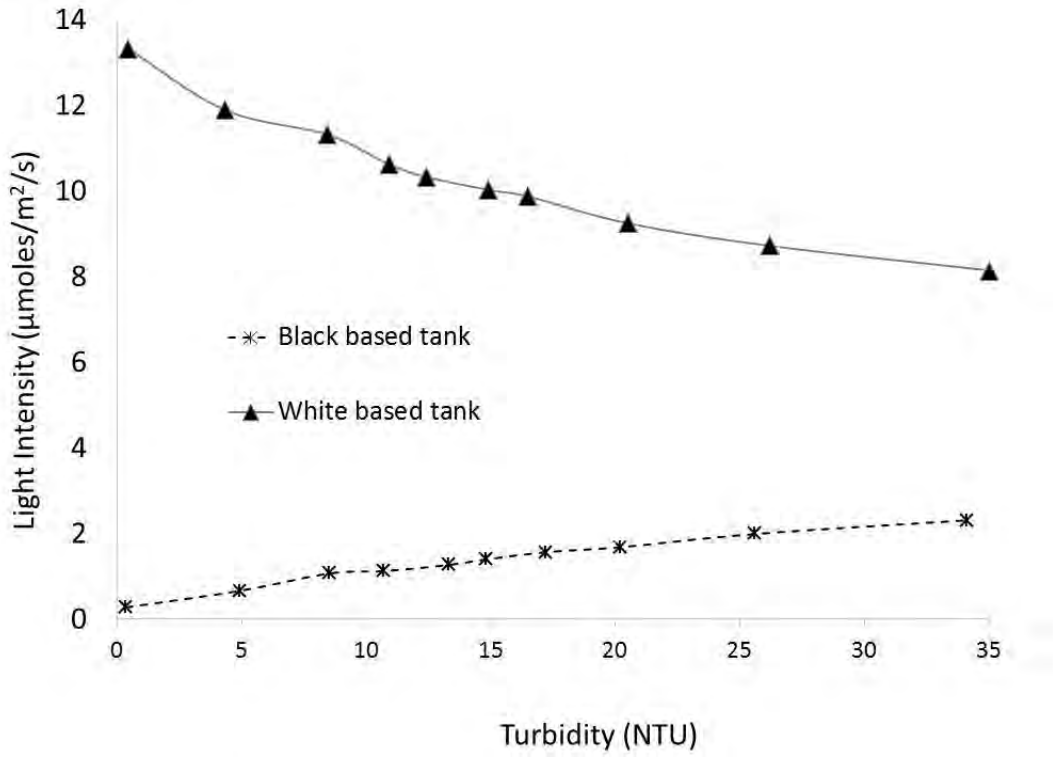

Figure 9-64: The effective of tank base colour and clay turbidity on downwards light intensity in 300 L YTK larval rearing tanks.

9.4.4. References

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9.5. Benefits and Adoption

Many of the findings presented in this report have already been adopted within ACAARs YTK hatchery. For example, ACAAR now feed rotifers under a hybrid feeding regime, resulting in significant savings in rotifer production costs. Rotifers are now routinely supplemented with selenium and further work is planned to capitilise on the data obtained during this project on taurine enrichment to further optimise the enrichment protocols for this nutrient and to determine optimum levels for YTK larvae.

The differences found in nutritional profiles between live feeds enriched on different products were used to confirm that the currently industry standard enrichment diet used for YTK larvae is the best of those tested and there are no indications that it should be changed. The significant differences in phospholipid DHA contents and DHA:EPA ratios in the phospholipid fractions between rotifers and *Artemia* have led to ACAAR investigating extending the rotifer feeding period.

9.6. Further Development

Throughout our report we have identified a number of areas that require further research. More research is required in the enrichment of live foods with taurine. In particular, the interactions between diet type, dose rate and enrichment time require more research. Once this work has been completed, further trials can then determine the optimum level of taurine in rotifers and *Artemia* for YTK. The supplementation of *Artemia* with taurine produced equivocal results in terms of malformation reductions and the interpretation of these data was complicated by differences in larval survival. Once the optimum level of taurine in enriched rotifers and *Artemia* is determined, the effect of taurine on malformation rates will be elucidated.

Further research is also required to determine if the stress and subsequent poor performance of larval YTK in the presence of clay can be overcome by changes in lighting and/or tank configuration.

9.7. Conclusions

Section 9.2 describes a study comparing various rotifer feeding regimes on the prey consumption, growth and survival of YTK larvae over the first 12 days post hatch (dph). The common practice of maintaining high densities of rotifers (10 – 30 /mL) in the rearing tank was compared to a pulse feeding technique, where lower densities of rotifers (5 – 8 /mL) were offered. A 'hybrid' feeding regime offered rotifers at the densities of the high density treatment until 5 dph and the lower pulse feeding densities thereafter. There was no significant difference in larval survival (hybrid: 28.9 ± 7 %, pulse: 17.3 ± 5 %, and high density: 17.2 ± 9 %,) or growth (hybrid: 6.12 ± 0.18 mm, pulse: 6.03 ± 0.10 mm, and high density: 6.11 ± 0.23 mm) between treatments. Rotifer ingestion was independent of rotifer density throughout the trial and increased with larval age, with larvae at 4 dph ingesting 22 ± 1.5 rotifers/larvae/hour and by 9 dph ingesting 59 ± 1.6 rotifers/larvae/hour during the first two hours of the day. These data demonstrate that even from first feeding, YTK larvae are efficient at capturing prey at the densities tested here and consequently significant savings in rotifer production costs as well as other benefits such as facilitation of early weaning and improved rotifer nutritional value can be obtained by utilising pulse or hybrid rotifer feeding regimes.

Section 9.3.2 describes the outcomes of the detailed biochemical composition analyses of rotifers enriched on different products. The enrichment diets tested were Spresso (current industry standard), N-Rich PL Plus (microalgal concentrate), Ori-Green (artificial diet), UM 'Standard' (a blend of enrichment products used in the commercial hatchery production of cobia) and UM Plus (UM Standard with the addition of selenium, taurine and vitamins C & E).

The biochemical analyses revealed some significant differences in certain aspects of the nutritional composition of the enriched rotifers that could potentially impact on growth, survival and malformation rates of YTK larvae. Rotifers enriched on all diets were deficient in taurine and selenium relative to wild zooplankton. We were able to successfully increase the level of both of these nutrients in the UM Plus diet. The current industry standard rotifer enrichment diet, Spresso, typically resulted in rotifers having better levels and ratios of essential fatty acids than N-Rich and Ori-Green enriched rotifers both in the total and phospholipid fractions, whilst the two UM diets had similar levels to those enriched on Spresso. Whilst N-Rich and Ori-Green had higher levels of total carotenoids, all diets had similar levels of astaxanthin. Spresso, Ori-Green and N-Rich enriched rotifers had similar levels of vitamins C and E, whilst UM Standard was lacking in both. The addition of lecithin, ascorbyl palmitate and alpha tocopherol was effective in increasing the levels of vitamins C and E in the UM Plus enriched rotifers. Whilst other studies have reported that AlgaMac-3050 (a major component of the UM diets) results in inferior performance of YTK larvae, are data demonstrate that the longer enrichment times employed in these studies increase the retro-conversion of DHA to EPA, thereby improving the fatty acid profile relative to those reported in other studies. Ori-Green was deficient in a number of components relative to the other diets. This may have been the result of the very short enrichment period that the manufacturers recommend for this product, which may result in only ‘gut loading’ rather than changes in the biochemical composition of the rotifers themselves.

The study comparing the performance of YTK fed rotifers enriched on Spresso, UM Standard and UM Plus showed no differences in larval survival or growth, despite the aforementioned differences in biochemical composition. Malformation rates were unable to be quantified at the end of the rotifer feeding phase due to the small size of larvae at this age. Comparing the taurine content of the enriched rotifers used in the current trial with other studies and with wild zooplankton, suggested that the taurine content of UM Plus enriched rotifers may have been too high. This was investigated in a subsequent trial in which the effects of different taurine enrichment dose rates

on the taurine content of rotifers and the subsequent effect these rotifers had on YTK was investigated (see Section 9.3.7).

Differences were also found in the biochemical composition of *Artemia* enriched on the various enrichment diets. *Artemia* were not supplemented with selenium on the basis of their naturally high levels. Taurine content of *Artemia* not receiving additional supplementation ranged from 0.68 to 0.99%DW, whilst those enriched with 4 g/L for 18 hours contained 8.24%DW, significantly higher than wild zooplankton. *Artemia* enriched on Spresso had significantly more astaxanthin (14.6 ± 1.8 mg/kg) than all other treatments, but still significantly lower than found in copepods (>400 mg/kg). Spresso enriched *Artemia* had the highest total lipid, but the lowest percentage of lipids in the polar lipid fraction. Despite having the lowest percentage in the polar lipid fraction, the high total lipid content of these *Artemia* resulted in them still having the highest absolute amount of DHA in the phospholipid fraction. Spresso enriched *Artemia* had a better DHA:EPA ratio in both the total and phospholipid fractions than all other treatments. The levels of vitamin C and E were similar in all treatments.

Of potential importance is a comparison between lipid contents of enriched rotifers and *Artemia*, from these studies, which demonstrate that enriched rotifers have considerably higher levels of phospholipid DHA and better DHA:EPA ratios within this fraction. All enriched rotifers had at least double the amount of DHA in the phospholipid fraction than the *Artemia* enriched on the same diets. Furthermore, most enriched rotifers had a DHA:EPA ratio in the phospholipid fraction close the optimum value of 2, whilst *Artemia* had ratios in this fraction of 0.3 at best. These data suggest that extending the rotifer feeding period and minimising the amount of *Artemia* fed to YTK larvae may be beneficial in terms of their nutrition and development, in addition to those benefits previously described by Woolley et al..

A larval rearing experiment during the *Artemia* phase compared two different enrichments, UM Standard and UM Plus. Those larvae offered UM Plus enriched *Artemia* grew significantly faster and had significantly fewer

malformations than those fed UM Standard *Artemia*, but survival was significantly lower, thereby complicating the interpretation of the data. Despite following published taurine enrichment protocols, analyses of the enriched *Artemia* and whole larval body suggested that the level of taurine enrichment may have been too high and a contributor to the lower survival seen in this treatment.

This was investigated in a subsequent trial which described the effects of feeding *Artemia* supplemented with various doses of taurine on the growth, survival, whole body taurine content and jaw malformation rate of larval YTK. Larvae were fed Spresso-enriched rotifers containing no supplemental taurine from 3 to 15 dph and Spresso-enriched *Artemia* co-enriched with taurine from 12 to 22 dph. *Artemia* were co-enriched at doses of either 0, 0.8, 1.6, 2.4, 3.2 or 4.0 grams of taurine per litre during the 18 hour HUFA enrichment process. Taurine content in the *Artemia* increased from 0.76 ± 0.04 %DW in those without supplementation to 3.95 ± 0.17% in those supplemented at 4 g/L. Survival rates of larval YTK were significantly lower in all taurine supplemented treatments compared to the unsupplemented control. Growth was significantly improved in those larvae fed taurine supplemented *Artemia*, however we cannot attribute this improvement solely to taurine, as improved growth may have been a function of the reduced survival in these treatments. The whole body taurine content of larvae fed unsupplemented *Artemia* was significantly lower (1.85 ± 0.03% DW) than those fed supplemented *Artemia*, which did not differ from each other (pooled average 2.48 ± 0.03% DW), suggesting an active excretion mechanism is in place, or a passive excretion mechanism past the whole body saturation point. Modeling suggested that in order to achieve the same whole body taurine content as seen in wild Japanese yellowtail (2.3%DW) that *Artemia* should have been enriched with a dose rate of 0.6 g/L for 18 hours, lower than our lowest tested dose rate. Jaw malformation rates were not affected by *Artemia* taurine content.

During these studies, indirect evidence suggested that a potential interaction between enrichment diet type and taurine content of enriched live feeds may exist. For example when *Artemia* were enriched with UM and taurine at 4 g/L

for 18 hours they contained 8.24%DW of taurine, whilst those enriched for the same time and with the same dose of taurine in the presence of Spresso contained only 3.95%DW. Furthermore, a comparison of the results obtained in the current trials with other studies suggests that the taurine content of the enriched live foods maybe highly sensitive to enrichment time (and this may be exacerbated at high dose rates). If this is proven to be the case then a protocol utilising a lower dose rate for a longer enrichment period, for example, may provide a safer method to deliver reliable and consistent target taurine levels in the enriched live feeds.

The results of investigations into the use of alternatives to microalgae paste for bacterial management in larval tanks are presented in 9.4. In a study with cobia larvae it was demonstrated that inorganic clay could successfully replace microalgae without any negative impacts on growth and survival. Such replacement significantly reduced the concentration of Vibrios in the larval rearing tank and resulted in significant savings due to the high cost of microalgal paste. The same study conducted with YTK larvae, however, resulted in low feeding incidence, poor growth, no swim bladder inflation and poor survival. Based on the behaviour of the larvae and subsequent lighting tests, we suggest that the scattering of light caused by the clay particles resulted in disorientation and stress to the larvae in the shallow, white-based tanks used in the study. We recommend that further studies be undertaken to determine if these issues can be overcome by different lighting or tank configurations to enable clay to be successfully incorporated into larval rearing tanks for YTK.

10. Appendix 1: Intellectual Property

No intellectual property issues.

11. Appendix 2: Staff

Part 1: Genetics

Dr. Nicholas Robinson

Mr. Shannon Loughton

Part 2: Larviculture

Dr. Gavin Partridge

Dr. Lindsey Woolley

Mr. Robert Michael

Mr. Zack Daugherty