Part I: Summary

PROJECT TITLE: **Optimizing the larval nutrition of marine finfish aquaculture species along the West Coast**

REPORT GIVEN IN YEAR 2015

REPORTING PERIOD: (July 2, 2011 to Sept 1, 2015)

AUTHOR: Mark Drawbridge

FUNDING LEVEL: $66,848 (per year for three years)

PARTICIPANTS:

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Principal Investigators: Dr. Michael Rust & Ron Johnson, NOAA Fisheries, Northwest Fisheries Science Ctr, 2725 Montlake Blvd E., Seattle, WA 98112

*Principal Investigator: Dr. Chris Langdon, Oregon State University, Coastal Oregon Marine Experiment Station, Hatfield Marine Science Center, Newport, Oregon 97365

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Indicate funded participants with an asterisk (*).

REASON for TERMINATION: Objectives completed and funds expended

PROJECT OBJECTIVES:

Objective 1 - Establish baseline indices for each species using current culture techniques as a benchmark for evaluating the project's success over time.

a. Culture Efficiency Index – transition points from one feed to another through the larval rearing phase. Less time on live feeds will ultimately translate into increased efficiency (e.g. reduced labor costs).

b. Biological Performance Indices – at the end of the larval phase measure: a) individual growth in length and weight, 2) survival as a percentage from the egg stage, and 3) quality using measures of morphology and/or stress tolerance.

Objective 2 - Refine and implement methods that allow assessment of larval feed intake and behavior to support research Objectives 3 and 4.

a. Employ diet "tracers" as a measurement tool for food choice and manipulative feeding trials –

b. Employ lipid spray beads (LSBs) as a delivery vehicle for feed supplements to live prey and complex feeds.

c. Employ video monitoring system and image analysis software for documenting larval feeding behaviors, kinetics and morphology under experimental feeding conditions; seek to couple this system with a feeder

Objective 3 – Increase survival and growth during the larval stage through optimization of live food types and enrichment formulations.

Objective 4 - Increase survival, fitness and growth during weaning from live foods to formulated feeds through the development and/or identification of appropriate formulated micro diets.

PRINCIPLE ACCOMPLISHMENTS:

Objective 1 - *Establish baseline indices for each species using current culture techniques as a benchmark for evaluating the project's success over time*. The baseline trials conducted in this project provided a reasonable means of assessing relative improvements in culture methods as improvements were made using a statistically valid experimental design. Key to this approach was including previous methods as treatment groups in order to make relative comparisons with the same cohort of fish given the high variability among cohorts. The relatively small tank size (320L) required for adequate and practical replication of treatments consistently yielded poorer performance (growth, survival, and quality) than observed in production tanks (~ 2-5m^3), which is a consideration that was not unexpected. Furthermore, the small tank size appeared to be better suited to WSB than CYT, presumably owing to the performance/activity differences of the two species.

Objective 2 - *Refine and implement methods that allow assessment of larval feed intake and behavior to support research Objectives 3 and 4*. The utility of several previously developed marking techniques was further validated in this project, especially as it relates to food choice trials and quantification of gut fullness (e.g. meal size). Novel marking techniques were developed that utilize fluorescently labeled lipid spray beads embedded in artificial diets. This technique provides researchers with a new and cost-effective method for evaluating feed intake by fish larvae and post-larvae.

Objective 3 – *Increase survival and growth during the larval stage through optimization of live food types and enrichment formulations*. We demonstrated that first instar *Artemia* is not required as an intermediate prey size for either CYT or WSB, which simplifies the culture process. We demonstrated the superiority of S.presso over other enrichment products, including a dramatic reduction in at least one malformation known to affect WSB. Both of these findings have been adopted into current culture practices. WSB performed the best when offered rotifers as a first feed but we have not yet determined if the improvement is enough to justify adding rotifers to the culture process given the added cost and complexity. OSU developed and evaluated the potential application of liposomes for delivering water-soluble nutrients to marine fish larvae. Using this technology, we were able to improve the efficiency of enriching live prey with taurine and demonstrate the enhanced nutritional value of the live prey for marine fish

larvae. Application of liposome technology to live prey enrichment will reduce costs and wastage of nutrients compared with the traditional enrichment method of immersing live prey in concentrated nutrient solutions.

Objective 4 - *Increase survival, fitness and growth during weaning from live foods to formulated feeds through the development and/or identification of appropriate formulated micro diets*. We successfully developed and tested an open formulation microdiet (OFM) that can be used by industry to develop custom feed formulations for each species. The OFM compared similarly in performance to Otohime, which is a current industry standard that is imported from Japan. Recently, due to modified import requirements, Otohime was unavailable in the USA, leaving customers scrambling for viable alternatives. This further emphasizes the need for US products that work as well or better. Development of an OFM allowed us to test specific ingredients and manufacturing processes in order to help optimize attraction, acceptance, formulation, etc.

Outreach and Evaluation Plan: Project photography including videos of the production of marine larval fish diets and macro diets were taken at the Oregon State University, Newport Marine Laboratory and the Bozeman, Montana, U.S. Fish and Wildlife Service laboratory. The videos and earlier still-photography taken at Hubbs-SeaWorld Research Institute (HSWRI) were used in the "Larval Feeds and Feeding Strategies for Marine Fish Workshop" held at HSWRI on August 25-27, 2015, and will be used in Articulate flash videos and other digital outreach pdf publications. Discussions are being conducted with project scientists to develop themes and concepts gained from the project's objectives, and the workshop, that best address in the digital outreach products. Following the termination report, the workgroup will make final decisions for materials to be included in the outreach products, graphic materials from the IAC/TC annual reports, final report, and workshop will be prepared for incorporation in the outreach digital presentations.

IMPACTS:

1. The practical results from this project have been transferred directly into hatchery protocols, especially with regards to live feeds and enrichments. This has resulted in greater operational efficiency and fish performance. Specifically this includes a more streamlined and cost-effective regime for live feeds production, as well as reduced malformations.

2. The research results from this project have allowed us to establish a foundation for future research, as well as highlighting directions for future research. Foundational examples include successful demonstration of an OFM; a novel marking method utilizing fluorescently labeled lipid spray beads; an effective delivery tool for water soluble nutrients in the form of liposomes; and a new particle type, alginate complex particles, which improves the nutrient delivery of low-molecular weight water-soluble compounds. Directional examples include development of custom (i.e. species-specific) microdiets, use of attractants, and micronutrient supplementation in both live feeds and microdiets.

3. The outreach results from this project were highlighted by a Marine Larval Nutrition (MLN) Workshop held at Hubbs-SeaWorld Research Institute from August 25-27, 2015. Information gleaned from the MLN WRAC project, including additional information from involved HSWRI, Oregon State University, USFWS and NOAA laboratories was extended to 27 invited nutritional

scientists; and representatives of feed mills and industry. In addition to the emphasis on larval nutrition, the MLN Workshop included information on broodstock nutrition and egg quality as a link to a second WRAC project that is currently in progress with some of the same workgroup partners.

Further impacts of the WRAC study will be assessed approximately 9-months post Termination Report to the WRAC Board to measure (1) how results of the WRAC project has influenced future direction of marine larval nutrition researchers, and (2) how results of the project has influenced future direction of feed companies involved in the manufacture of marine larval diets. The results of the survey will be presented in a special pdf document to WRAC for website posting.

RECOMMENDED FOLLOWUP ACTIVITIES:

Follow-on research can be focused on development of custom (i.e. species-specific) microdiets, use of attractants, and micronutrient supplementation in both live feeds and microdiets.

SUPPORT: Use the format shown below to indicate all sources of funding and additional other support, federal and non-federal, for this project. Specify the name of the "other" sources as a footnote to the table.

Year	WRAC-USDA Funding	OTHER SUPPORT				Total Support
		University	Other Federal	Other	Total	
2011-2015	$198,294		$21,480	$139,520	$161,000	$359,294
Total	$198,294		$21,480	$139,520	$161,000	$359,294

"Other" = HSWRI matching

PUBLICATIONS, WORKSHOPS, MANUSCRIPTS, OR PAPERS PRESENTED:

HSWRI bimonthly newsletter – March 2012, July 2013, November 2013, and Sept 2014

Stuart, Kevin, Federico Rotman, Mark Drawbridge. 2012. Larval rearing advancements for yellowtail amberjack Seriola lalandi in southern California. The 40th UJNR Scientific Symposium. Hatchery Technology for High Quality Juvenile Production, Honolulu, HI October 22 – 23.

Stuart, Kevin, Federico Rotman, Mark Drawbridge. 2013. Larval rearing advancements for yellowtail amberjack Seriola lalandi in southern California. Aquaculture 2013, Nashville, TN, February 21-25.

Rust, Michael B., Frederic T. Barrows, Mark Drawbridge, Emily R. Hart, Kevin Stuart, Ken Webb, Harold J. Barnett, Peter M. Nicklason, and Ronald B. Johnson. 2013. Characterization of several open formula reference diets for marine fish larvae. The 41st UJNR Scientific Symposium. Advanced Aquaculture Technologies, Sapporo, Japan October 9-10.

Stuart, K.R., F.J. Rotman, and M.A. Drawbridge. (2013). Larval rearing advancements for yellowtail amberjack (Seriola lalandi) in southern California. Rust, M., P. Olin, A. Bagwill and M. Fujitani (editors). Hatchery Technology for High Quality Juvenile Production: Proceedings of the 40th U.S.-Japan Aquaculture Panel Symposium, Honolulu, Hawaii, October 22-23, 2012. U.S. Dept. Commerce, NOAA Tech. Memo. NMFS-F/SPO-136.: 69 – 74.

Hawkyard, Matt, Ben Laurel, Yoav Barr, Kevin Stuart, Mark Drawbridge and Chris Langdon. 2014. The use of liposomes for the enrichment of rotifers with taurine and their subsequent effects on the growth of marine fish larvae. Aquaculture America 2014, Seattle, WA, February 9-12.

Stuart, Kevin, T. Barrows, Michael B. Rust, Ronald B. Johnson, and Mark Drawbridge. 2014. The use of experimental microdiets on two marine finfish species. Aquaculture America 2014, Seattle, WA, February 9-12.

Stuart, Kevin, T. Barrows, Michael B. Rust, Ronald B. Johnson, Matt Hawkyard, Chris Langdon and Mark Drawbridge. 2015. Evaluation of experimental microdiets with larvae of two marine finish species. Aquaculture America 2015, New Orleans, LA, February 19-22.

Matt Hawkyard, Ben Laurel, Kristin Hamre, Yoav Barr, Kevin Stuart, Mark Drawbridge & Chris Langdon. Challenges and solutions associated with the provision of water-soluble nutrients to marine fish larvae. Aquaculture America 2015, New Orleans, LA, February 19-22.

Hawkyard, M. Researchers develop better methods for delivery of water-soluble nutrients to marine fish larvae. Hatchery International. May/June 2015.

Hawkyard, M., Langdon C., Stuart, K., Drawbridge, M. Liposomes open new doors in larval fish nutrition. Global Aquaculture Alliance: Advocate. May/ June 2015.

Matt Hawkyard, Kevin Stuart, Chris Langdon & Mark Drawbridge. (2015) The enrichment of rotifers (*Brachionus plicatilis*) and *Artemia franciscana* with taurine-liposomes and their subsequent effects on the larval development of California yellowtail (*Seriola lalandi*). Aquaculture Nutrition. Accepted for publication.

WRAC-supported "Larval Feeds and Feeding Strategies for Marine Fish Workshop". 2015. Hubbs-SeaWorld Research Institute, August 25-27, San Diego, CA.

SUBMITTED BY: Sept 11, 2015

Title: (Work Group Chair or PI)/Date

APPROVED: Sept 11, 2015

Project Monitor/Date

Part II: Detail

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TECHNICAL SUMMARY AND ANALYSES:

Objective 1 - Establish baseline indices for each species using current culture techniques as a benchmark for evaluating the project's success over time.

At the end of Year 1, the WSB were fed 1st instar *Artemia* at 4 dph; 2nd instar *Artemia* at 8 dph; and the commercial weaning diet, Otohime, was introduced beginning at 15 dph. WSB grew to an average of 16.2 mm standard length (SL; 18.8 mg dry WT) at 35dph and 9% survived from the egg stage. A total of 7.5% of the juvenile WSB exhibited either jaw or opercular malformations at 35 dph (Table 1), with 6.3% and 1.3% of each, respectively. The CYT were offered rotifers at 2 dph; 1st instar *Artemia* at 6 dph; and 2nd instar *Artemia* at 8 dph. Otohime was again introduced at 15 dph and both species were fully weaned onto the dry diet by 35 dph. CYT grew to an average of 14.7 mm SL (14.0 mg dry WT) at 35dph and 12.1% survived from the egg stage. A total of 12.5% of the juvenile CYT exhibited either jaw or opercular malformations at 35 dph (Table 1), with 12.5% and 1.3% of each, respectively.

At the end of Year 2, the WSB baseline trial used three different treatments to help quantify the effects of the "optimal" feeding regime. Treatment 1 (1st – 2nd Easy DHA Selco) was the Year 1 feeding regime where larvae were fed 1st instar *Artemia* at 4 dph, enriched 2nd instar *Artemia* at 8 dph with Easy DHA Selco (INVE); and the commercial weaning diet, Otohime, was introduced beginning at 15 dph. Treatment 2 (1st – 2nd S.presso) – larvae were fed 1st instar *Artemia* at 4 dph, enriched 2nd instar *Artemia* at 8 dph with S.presso (INVE); and the commercial weaning diet, Otohime, was introduced beginning at 15 dph; Treatment 3 (Rots – 2nd S.presso) – larvae were fed rotifers enriched with ORI-GREEN (Skretting) at 2 dph, enriched 2nd instar *Artemia* at 6 dph with S.presso. The S.presso enrichment was used because it contains a higher DHA:EPA ratio, higher levels of Vitamin C, and Vitamin E, but lower Vitamin A and D3, when compared to Easy DHA Selco. This product was also recommended by several colleagues. Rotifers are not required as small first prey item but we wanted to see if there was an advantage to be able to feed fish earlier and with an enriched prey item. WSB were significantly larger at 40 dph in Treatment 3, 22.1 mm standard length (SL; 0.23 g wet WT). Malformation rates were significantly lower in Treatment 3 (15.5 ± 10.3%) than the other treatments, which went as high as 74.6% on average. Survival was not significantly different among the treatments (Table 2) but was low overall compared to the Year 1 baseline trial, which averaged 9%. This was

presumably due to batch differences in egg quality. Typically survival from egg to a juvenile fish on a production scale range from 20 – 35%.

The CYT Year-2 baseline trial had two treatments. The CYT Year-2 baseline trial had two treatments. Treatment 1 (Rots – 1st – 2nd) utilized the methods employed at the start of Year 1, larvae were offered rotifers enriched with ORI-GREEN at 2 dph; 1st instar Artemia at 6 dph; and 2nd instar Artemia enriched with Easy DHA Selco at 8 dph. Treatment 2 (Rots – 2^{nd}) represented the Year 2 baseline with improvements incorporated from Year 1. The larvae were offered rotifers enriched with ORI-GREEN at 2 dph, 2^{nd} instar *Artemia* enriched with S.presso at 5 dph. Otohime was again introduced at 15 dph for both treatments and larvae were fully weaned onto the dry diet by 35 dph. There were no significant differences in growth, survival, or malformation rates between treatments (Table 3). Survival and malformation rates were poor compared to the Year 1 baseline trial (survival: 12.1%; malformation rates: 12.5%), likely due to batch differences in larvae. The survival rates of larvae in the experimental tanks to 35 dph in Year 1 and 2 are not indicative of results seen in larger production tanks. Typically in production tanks CYT survival has ranged between 15 – 30 % from egg to 50 dph juvenile.

Results from both baseline trials in Year 2 confirmed that WSB and CYT can be reared effectively without the use of 1^{st} instar *Artemia*. This reduces the complexity of the culture process for CYT. In the case of WSB, eliminating 1^{st} instar *Artemia* would require replacement with rotifers, which increases the complexity of that operation if rotifers are not already part of the hatchery operation. A more detailed evaluation is required to justify that change. The enrichment product S.presso appeared to help reduce malformations among WSB but not CYT under our experimental conditions.

The final WSB baseline trial in Year 3 this project had four different treatments representing the progression of refinements over the three year project period. Treatment 1 (1st – 2nd Easy DHA Selco) represents the start of Year 1 feeding regime where larvae were fed 1st instar Artemia at 4 dph, enriched 2nd instar *Artemia* at 8 dph with Easy DHA Selco (INVE). Treatment 2 (1st – 2nd S.presso) – larvae were fed 1st instar *Artemia* at 4 dph, enriched 2nd instar *Artemia* at 8 dph with S.presso (INVE); Treatment 3 (Rots – 2nd S.presso) – larvae were fed rotifers enriched with ORI-GREEN (Skretting) at 2 dph, enriched 2nd instar *Artemia* at 6 dph with S.presso. Treatments 1-3 all applied co-feeding of the commercial diet, Otohime, beginning at 15 dph. Treatment 4 (1st – 2nd S.presso) - larvae were fed 1st instar *Artemia* at 4 dph, enriched 2nd instar *Artemia* at 8 dph with S.presso (INVE); and a Larval Extruded (LEX) weaning diet containing 3% taurine and a commercial attractant, was introduced beginning at 15 dph. The last treatment was a cumulative scheme of nutritional refinements made throughout the larval phase. Larvae were fully weaned onto their respective microdiets by 35 dph and this trial was concluded at 55 dph.

Growth and survival were similar among Treatments 1, 2, and 3 (Table 4). Growth was significantly less in Treatment 4, which would suggest that the LEX diet did not perform as well as the commercial diet. When looking at the growth over the course of the trial, notochord length was similar among the treatments until 30 dph when the larvae were shifting off of live prey. Malformation rates were significantly lower in Treatment 3 showing that feeding an enriched prey item through the early larval stages is important for early development.

Objective 2 - Refine and implement methods that allow assessment of larval feed intake and behavior to support research Objectives 3 and 4.

Task 2.1 *[OSU/HSWRI/NOAA/USDA]* – (determine leakage rates of tracers used in various complex particles). We assessed the leakage rates of two tracers in micro diets processed using two different methods during a 60 minute emersion (Figure 1). The results show no evidence of leakage with either combination. The increase in marker level at T_{10min} is most likely associated with increased water in the T_{0min} sample. A similar study will be performed on zein particles in Year 2, otherwise marker levels will be benchmarked at a single time point whenever they are used in trials.

Task 2.2 *[OSU/NOAA/USDA]* – (determine if tracers affect the ingestion rates of larvae). This task was not addressed in this project because NOAA addressed it separately.

Task 2.3 *[OSU]* – (determine leakage rates of sodium fluorescein and Poly-red for complex particles containing LSB and simple particles containing non-encapsulated sodium fluorescein and Poly-red).

Sodium fluorescein (SF) concentrations were determined for Particle-assisted rotational agglomeration (PARA) particles and resulted in higher SF concentrations in the particles when compared to the initial quantities added to the mash (Figure 2). We suspect that the higher-than-expected SF concentrations were due to water-loss during the preparation of PARA particles. Complex particles (LSB within PARA particles) did not show reduced SF leakage when compared to particles in which SF had been added directly to the mash (Figure 3). There are two possible explanations for this result: 1) LSBs were destroyed during the production of PARA particles; or 2) SF leached into the mash during production of PARA particles. Further investigation, utilizing poly-red dyed particles will be necessary to tease apart these possibilities. Poly-red does not leach from the LSBs, so the only way it can be released from the PARA particles is if the LSBs have been broken in the manufacturing process. Poly-red PARA particles have been produced and will be evaluated in further leakage trials.

When fed to 2-month-old Northern rock sole (*Lepidopsetta polyxystra*) larvae, liberated sodium fluorescein was evident in the gut of larvae for all three particles types (Figure 4). These results suggest that inclusion of water-soluble substances in the particle mash of PARA particles is sufficient for the delivery of these substances to marine fish larvae. If improved retention of water-soluble substances is desired, alternative strategies will need to be investigated, potentially utilizing liposomes in place of LSB, or lipid coating PARA particles.

Task 2.4 *[OSU/HSWRI/USDA]* – (determine sinking rates of microdiets developed using LSBs). We compared the sinking rates of the experimental diets (Micro-extrusion marumerization or MEM and PARA) as well as two commercial diets (Gemma and Otohime). MEM had the fastest sinking rate of 1.31 cm/sec, while Otohime had the slowest at 0.63 cm/sec. PARA had a sinking rate of 0.81 cm/sec, and Gemma had a sinking rate of 0.64 cm/sec.

Objective 3 – Increase survival and growth during the larval stage through optimization of live food types and enrichment formulations.

Task 3.1 *[OSU/HSWRI]* – (evaluate if micronutrient enriched live-prey increases the growth and survival of marine fish larvae). We conducted a trial to determine if live prey enriched with microparticles containing taurine increased the growth and survival of marine fish larvae when compared to live-prey that were not enriched with taurine. Rotifers were enriched using microparticles containing taurine with target concentrations equaling those previously measured in wild copepods. Control treatments utilized live-prey enriched with "empty microparticles" (without taurine). CYT larvae were fed for 5 days from 2 to 7 dph (typical rotifer period for CYT) on taurine-enriched rotifers or rotifers that were not enriched with taurine. Culture performance was evaluated based on larval growth (length and weight) and survival. CYT larvae fed taurine-microparticle enriched rotifers were significantly larger, on a dry weight basis, when compared to larvae fed rotifers, which were not enriched with taurine (Figures 5 and 6). These results suggest that CYT benefit from dietary taurine concentrations above those provided from rotifers not otherwise enriched with taurine. However, we also noted that the larvae lost weight from 3dph to 8dph and that the final weight at 8dph was much less than a typical dry weight for that age, which usually exceeds 120 μg per larva. The reason for these differences is unclear and further investigation is required.

We conducted a trial to determine if live prey enriched with microparticles containing taurine increased the growth and survival of marine fish larvae when compared to live-prey that were not enriched with taurine. The specific aims of this trial were to: 1) use microparticles (liposomes) to enrich rotifers and *Artemia* with the water-soluble nutrient taurine and 2) determine if the growth, survival and/or whole body taurine concentrations of CYT larvae were impacted by taurine-enriched live prey when compared to those fed taurine-unsupplemented live prey. CYT larvae were fed from 2 to 8 days post hatch (dph) with either taurine enriched rotifers or taurine unsupplemented rotifers. On 8 dph, larvae were transitioned to either taurine enriched *Artemia* or taurine-unsupplemented *Artemia* until 14 dph. Using this experimental design, we were able to determine if both rotifers and *Artemia* require taurine enrichment to meet the nutritional needs of CYT larvae. In addition, we were able to evaluate the interaction between early and late taurine supplementation, i.e. during the rotifer and/or *Artemia* phases, on the growth and body taurine concentrations of CYT larvae.

At the end of the rotifer phase CYT larvae fed taurine-microparticle enriched rotifers were larger, on a dry weight basis, when compared to larvae fed taurine-unsupplemented rotifers (Table 5). In addition, the whole body taurine concentrations were significantly higher when CYT larvae received taurine-enriched rotifers (Figure 7). These results suggest that CYT benefit from dietary taurine concentrations above those provided from rotifers not otherwise enriched with taurine. The positive growth effect observed in CYT as a result of microparticulate enrichment of rotifers provides a basis for future studies using microparticulate enrichment techniques. There were no differences in the growth of CYT larvae at the end or the *Artemia* phase (14 dph; Table 5). However, CYT larvae had the highest whole body taurine concentrations when larvae were fed taurine enriched *Artemia* (U-Rot:T-Artand T-Rot:T-Art), intermediate concentrations when fed taurine-enriched rotifers followed by taurine-unsupplemented *Artemia* (T-Rot:U-Art) and larvae had the lowest concentrations when fed

taurine-unsupplemented live prey (U-Rot:U-Art; Figure 8). Based on whole body taurine concentrations, it appears that the taurine concentrations of taurine-unsupplemented *Artemia* may be sub-optimal for CYT larvae. However, since growth indices were similar among treatments at 14 dph, it appears that taurine-unsupplemented *Artemia* do not present a major nutritional deficiency for CYT larvae. More research is needed to evaluate the delayed effects of taurine-enriched live prey on the growth and deformation rates of juvenile CYT. Cold storage of live feeds for three hours at 4-10º C resulted in a decrease of taurine for both rotifers and *Artemia*, but it was statistically significant only in the rotifers.

Task 3.2 *[HSWRI/NOAA]* – (gain a better understanding of appropriate transition phases between live feeds as well as the ingestion levels of these feeds over time). This task was completed for both WSB and CYT. Trials were performed by offering several live prey items (rotifers, 1st instar and 2nd instar *Artemia*) at equal densities (5 prey/ml) from 2 to ~20 dph. Prey consumed by larvae in a single feeding were enumerated using two methods – first by counting gut contents and secondly by measuring the levels of inert markers for each prey type in the larvae. WSB larvae consumed 2nd instar *Artemia* as early as 5 dph (Figure 9a), which is three days earlier than current HSWRI protocols. CYT trial also showed that larvae began to choose 2nd instar *Artemia* at 5 dph (Figure 9b), which is four days earlier than current HSWRI protocols. CYT larval consumption rates were similar to WSB larval consumption rates at 19 and 21 dph respectively, however consumption rates of WSB were much greater than CYT larvae at an early age. The differences in *Artemia* consumption between the species at similar ages suggests that WSB are more efficient predators of this prey type or that the species feed on different daily patterns.

As part of these trials, we also compared the *Artemia* consumption counts derived from the two methods we employed as shown in Figure 10. The inert marker method consistently underestimated the actual number of Artemia consumed by the larvae in a single feeding. The cause for this discrepancy needs further investigation.

Task 3.3 *[HSWRI]* – (evaluate if 1st instar *Artemia* can be removed from the current feeding regimes of marine fish larvae without impacting growth and survival). When we offered CYT 2nd instar *Artemia* at 5 dph or 7 dph, overlapping with rotifers but without the addition of 1st instar *Artemia*, no significant differences in growth or survival were observed when compared to the control feeding regime which included 1st instar *Artemia*. Because 1st instar *Artemia* are unable to be enriched (they have no ingestion mechanism), this finding will allow use of only enriched live prey items throughout development of CYT larvae. This has the potential to significantly increase growth and lower deformity rates as enrichment formulations are optimized.

The WSB trial showed faster growth (standard length) and lower malformation rate in larvae offered a feeding regime without using 1st *instar Artemia* (Table 6). This trial also showed an apparent beneficial effect of the S.presso enrichment over the traditionally used Easy DHA Selco relative to levels of certain malformations. This trial was conducted before the baseline trial and was one of the reasons S.presso has been integrated into rearing protocols for WSB. Survival rates were not reported because in this study design the larval survival was assessed at two time points. Having the ability to limit the live prey regime to two prey types both reduces live feeds labor and allows an enriched live prey item to be offered throughout the culture process. The similar trial proposed for CYT was completed in Year 1 and has been reported previously.

Task 3.4 *[HSWRI]* – (evaluate the effects of prey density on larval ingestion rates and associated performance). Three trials were completed with CYT looking at rotifer feed density and delivery method, and *Artemia* feed density. No significant differences were found in growth or survival when larvae were fed different densities of rotifers and *Artemia* (Tables 7 and 8). The CYT larvae did have an elevated feeding response when offered 20 rotifers/ml up to 6 dph (Figure 11). Typically, 6 dph is the time at which larvae are transitioned off of rotifers and onto 2nd Instar *Artemia*. Feeding rates were highest in CYT larvae when *Artemia* densities were at 1 *Artemia*/ml compared to higher food densities, which we cannot explain (Figure 12). However growth was lower at the low prey density compared to the other treatments (Table 9). Finally, two rotifer delivery techniques were tested - batch feeding and automatic feeding. Batch feeding was carried out by feeding rotifers four times throughout the day (7:00, 10:00, 13:00, 16:00) and automatic feeding was done through the use of peristaltic pumps, which maintained a constant feed density for up to 18hrs. We found that batch fed larvae were slightly larger than automatically fed larvae but survival was similar (Table 9). This result is different than what we typically see on a production level and we may be seeing a bias toward the batch fed tanks due to our experimental protocols, which exclude tank transfers as a bacterial mitigation approach.

Task 3.5 *[NOAA/HSWRI]* – (evaluate larval swimming behavior captured using the video image analysis system) – This task was dropped due to NOAA travel restrictions.

Task 3.6 *[NOAA/HSWRI]* – (evaluate larval behavioral feeding response to different feeding densities) - This task was dropped due to NOAA travel restrictions.

Objective 4 - Increase survival, fitness and growth during weaning from live foods to formulated feeds through the development and/or identification of appropriate formulated micro diets.

Task 4.1 *[OSU/HSWRI/USDA/NOAA]* – (evaluate how well fish larvae feed on zein, MEM and PARA particles as compared to the standard microdiet, Otohime, now used in production). In the first weaning trial using WSB, all performance measures were generally similar, although fish fed the commercial diet grew the best (Table 10). Survival was quite low but probably due largely to the experimental nature of the trial (e.g. low fish density, handling of larvae at stocking, small tank size). Our observation of feeding indicated that the MEM diet sank too rapidly and limited opportunity for consumption by the larvae. For this reason, we discontinued using it for subsequent trials. For CYT, the PARA formulation yielded the highest survival but lower growth than the commercial diets (Table 11). Malformation rates were generally high among all treatment groups at 40 dph, which is not uncommon for this species when described in the literature. Because the performance metrics were generally similar among all diets for both species, we can now proceed with customizing the weaning diets in subsequent trials.

Task 4.1 *[OSU/HSWRI/USDA/NOAA]* – (evaluate how well fish larvae feed on zein, MEM and PARA particles as compared to the standard microdiet, Otohime, now used in production).

In the first weaning trial using CYT, we tested two methods for producing microdiets, PARA and Flake. Both experimental diets used the same Montlake meal formulation. In this trial, the commercial diet (Otohime; OTO) out-performed both experimental diets (Table 12). Feeding incidence was similar for all diets (Figure 13), however the larvae fed the PARA treatment had a higher consumption rate than the Flake treatment. Results from this trial showed that the PARA method performed better than the Flake method. The second trial used WSB and because the Flake method did not perform well with CYT we used the PARA method for the experimental diets and tested two formulations, PARA (Clam) and PARA (Montlake). Again, the commercial diet showed higher survival and faster growth. The PARA (Montlake) diet showed significantly higher survival, faster growth and higher feed consumption when compared to the PARA (Clam) diet (Table 13 and Figure 14). Results from both trials are important steps to understanding the physical and dietary characteristics necessary for a successful microdiet.

In Years 1 and 2 multiple feeding trials were completed using WSB and CYT. These trials demonstrated that an open formulation PARA particle diet performed similarly to the leading commercial diet, Otohime. Although the PARA process has been proven to be effective, sufficient quantities could not be produced to meet the projects expanding needs. A different process was developed using cooking extrusion to produce a larval extruded feed (LEX) but with non-standard operating conditions. First, the product is cooked in the first three barrel sections with approximately 35% moisture. Then it is cooled in the next three sections so the water does not flash off when exiting the extruder. The wet extrudate is then rolled into flakes and dried at ambient temperature. A roller grinder is then used to reduce the particle size to the appropriate range. This approach results in more uniform particles after grinding than conventional extrusion, which has an edgy and porous appearance. This process also allows for large

quantities of product to be produced in a reasonable amount of time relative to particle-assisted rotational agglomeration (PARA) or micro-extrusion marumerization (MEM).

CYT larvae were used to test the open formula PARA against the open formula LEX in a trial that ran from 15 to 49 days post hatch (dph). No significant differences were observed in growth or survival (Table 14). However there were differences in the feeding incidence with larvae fed the LEX diet showing greater feeding incidence from 24 to 35 dph (Figure 15). Both particle types were marked with the inert marker yttrium to facilitate assessment of feed intake, but those results are pending laboratory analyses.

The next trial under this objective was also completed using CYT larvae. Five experimental diets were formulated to determine the effects of taurine concentration on fish performance. The microdiets were formulated and processed as PARA particles into separate treatment diets containing graded concentrations of taurine at inclusion levels of 0.4, 4.5, 9.3, and 12.2%. A fifth treatment was made containing 7.5% taurine as well as spirulina (Table 15). The trial was initiated with larvae at 15 dph and ended at 41 dph, with larvae being weaned onto the experimental diets at 35 dph. Feeding incidence for CYT between the treatments was similar until 31 dph, when diet YT 5 (7.5% taurine with spirulina) showed greater feeding incidence until 37 dph (Figure 16). Analysis of the whole body of the larvae after the trial showed a significant increase in the taurine concentrations of larvae fed experimental diets YT 2 through YT 5 (Table 16). This shows that an inclusion level of 4.5% taurine into the microdiet results in a corresponding increase in tissue taurine concentration, although the lower threshold concentration in the diet was not determined. Interestingly the diet with spirulina resulted in the highest concentration of taurine in the body of the larvae, although it was not significant. Growth was not significantly different between the control and all other treatment groups but the control values were less in all cases for both weight and length (Table 17). If the trial was allowed to run longer it is likely that statistical differences would have manifested. This trial supports existing information that taurine is essential for CYT larvae and is the first trial to incorporate taurine into an open formula weaning diet. Leaching rates of the diets in seawater showed that after 5 minutes approximately 40% of the taurine had leached from the diet. PARA particles sink between 0.6 and 1.7 cm/sec (Table 18). The water column in the experimental tank was 43 cm, which means that the food particles are available to the larvae for 25 to 73 seconds.

Task 4.2A-C *[OSU/HSWRI]* – (determine if attractants elicit an improved feeding response). Trials were conducted to determine if feed attractants could be used to increase the ingestion rates of artificial diets by WSB or CYT larvae. The purpose of this objective is to select nutrients for use as feed attractants and determine which mixtures affect the ingestion rates of artificial diets by fish larvae. These trials were completed in 10L clear aquariums.

In general, feeding rates were higher in larvae fed alginate-CP containing stimulants (Stim CP) when compared to those fed alginate-CP without stimulants (Empty CP) or empty alginate-CP along with stimulants (Empty CP + Stim) dissolved in the water (Figure 17 and Figure 18). Enhanced leakage of stimulants from alginate-CP due to freezing and thawing of particles (Ruptured CP) resulted in lower feeding rates when compared to the particles that were fully intact (Stim CP). Feeding incidence was higher for the commercial diet Otohime when compared to alginate-CP containing stimulants (Stim-CP). This difference may be due to several factors

including particle size, sinking rate, texture, taste or physical appearance (color). In addition, white seabass had been receiving Otohime as their primary food source prior to this trial and therefore may have been acclimated to this feed type. Particles containing powdered Qrill™ protein were also tested. However the addition of Qrill™ resulted in larger, faster sinking particles (Table 18) that were not eaten by the larvae and were therefore excluded from subsequent analyses. The treatments have been further tested with CYT larvae; however, these data are not fully analyzed and will be included in future reports.

WSB larvae consumed alginate complex particles at higher rates when glycine, L-alanine and betaine were included when compared to alginate-CP which contained only seawater. Alginate-CP were well suited for this experiment due to their simple chemical makeup (*i.e.* no need for complex macronutrient mixtures) and their ability to slow the release of feeding stimulants while in suspension. These results are promising because: 1) this is the first use of alginate-CP for feeding fish and will be the first description of this particle type in peer-reviewed literature; and 2) we have successfully selected several substances that stimulate feeding in white seabass larvae and we can move forward with refining these techniques; and 3) this is the first trial to utilize fluorescent markers to determine feeding incidence and gut fullness in fish larvae, allowing for evaluation of feeding rates, even when larval guts are heavily pigmented (Figure 19, images 3-6). If possible, we may further compare individual candidate stimulants to determine which substance has the greatest stimulatory effect. Once refined, we have proposed including the most effective stimulants in MEM particles, along with lanthanide markers, and comparing these particles in a similar fashion. Ultimately the goal of this objective is to develop particulate diets that may increasingly replace live prey during the culture of WSB and CYT.

CYT larvae were fed fluorescent-labeled complex alginate particles containing feed additives believed to have a stimulatory effect and were compared to empty alginate complex-particles Figure 20). The purpose of this trial was to determine if glycine, alanine and betaine, included individually or as a mixture, increased food particle consumption by CYT. In addition we wanted to know whether attractants were more effective when they were physically included in the food particles or when they were simply dissolved in the culture water. Fifteen CYT post-larvae were added to twenty four 10 L tanks and allowed to acclimate for 18 hours. Treatment assignments were as follows:

- "Empty CP" = empty alginate complex particles
- "Empty CP + stim" = empty alginate particles with a stimulant mixture of glycine, alanine and betaine dissolved in the culture water
- "Stim CP" = alginate complex particles containing the stimulant mixture
- "Glycine CP" = alginate complex particles containing glycine
- "Alanine CP" = alginate complex particles containing alanine
- "Betaine CP" = complex particles containing betaine

The results of this trial showed that feeding incidence of CYT larvae was greatest when larvae were fed the Stim CP treatment. When individual attractants were included in the particles, only glycine resulted in similar feeding incidence (Figure 21). Dissolving the stimulant mixture in the fish tanks did not result in increased feeding incidence when compared to the empty complex particles. This suggests that larvae may be accepting or rejecting particles on the basis of taste rather than smell. Gut fullness values (FUI) were not significantly different among treatments

suggesting that if fish chose to feed, they did so at similar rates independent of the inclusion of attractants (Figure 22). The total feeding index was highest, on average, when CYT were fed with alginate-CP containing the stimulant mixture and was the lowest when larvae were fed empty-CP. All other treatments showed intermediate values of TFI (Figure 23). The results of this trial are consistent with results we have obtained for white seabass. In previous trials, we have repeatedly found that a mixture glycine, alanine and betaine, included in alginate-CP, promotes the ingestion of particles by both WSB and CYT.

Task 4.2A *[OSU/HSWRI]* – (determine if attractants elicit an improved feeding response). Zein particles were produced with and without feed attractants. Zein particles included DiI[1] and DiO[2] fluorescent markers so that ingestion of particles could be easily observed with epifluorescent microscopy and measured with a fluorometer. Preliminary feeding trials suggest that white seabass larvae would not ingest zein particles even when feed attractants were used. It is likely that the small size of zein particles (100μm) precluded them from ingestion by the larvae. Given the purpose of trials 4.2 though 4.4 was to quickly screen the effectiveness of feed attractants, we propose switching to an alternative particle type which may be more effective and produced in larger particle sizes. Specifically, an alginate-based complex particle may be more suited to this application.

Task 4.3 *[OSU/HSWRI/NOAA/USDA]* (evaluate use of different feed delivery method of feed attractant on the ingestion rates of larvae). We conducted a trial using WSB larvae to determine if the attractant mix used in Task 4.2 could be effectively used in a PARA particle to increase feeding performance. A novel attractant currently in commercial development, ProMega 55, was also used as a treatment and two different weaning times where employed in a multi-factorial design. Larvae were reared from 15 to 47 dph, the diets used were as follows: Diet 1 – open formula PARA with no attractant; Diet 2 – open formula PARA with OSU stimulant mix (betaine, alanine, and glycine); Diet 3 – open formula PARA with ProMega 55 stimulant. Each diet was provided under two weaning schemes whereby *Artemia* co-feeding was discontinued at either 25 or 35 dph.

The results of this trial showed that larvae from all treatments that had attractants in the diets had significantly faster growth than the control (Table 19). Growth was also significantly faster in larvae fed Diet 3 (ProMega 55 stimulant) at the end of the trial regardless of weaning time. We showed that WSB could be successfully weaned 10 days sooner than the current standard of 35dph without sacrificing growth; however overall survival was slightly impaired (Table 19). The use of the ProMega 55 as an attractant showed significantly greater larval survival at 47 dph among the 25 dph treatments. This study shows that attractants are can be valuable in weaning of marine finfish larvae. Also the use of ProMega 55 showed significant improvements in larval performance for WSB.

[1] DiI = 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine perchlorate ('DiI'; DiIC18(3))
[2] Di0 = 3-octadecyl-2-[3-(3-octadecyl-2(3H)-benzoxazolylidene)-1-propenyl]-perchlorate

Outreach Objectives:

Project photography including videos of the production of marine larval diets and macro diets, were taken at the Oregon State University, Newport Marine Laboratory and the Bozeman, Montana, U.S. Fish and Wildlife Service laboratory. These videos and earlier still-photography of feeding trials and other marine larval nutrition related activities taken at Hubbs-SeaWorld Research Institute (HSWRI) were used in the Marine Larval Nutrition Workshop held at HSWRI in August 25-27, 2015. There were 27 invited nutritional scientists; and representatives of feed mills and industry in attendance. Following the workshop, the participants were provided a tour of the HSWRI, Carlsbad marine hatchery. A copy of the Larval Feeds and Feeding Strategies for Marine Fish Workshop program is attached in Appendix C.

Information extended at the Larval Feeds and Feeding Strategies for Marine Fish Workshop included system design for broodstock husbandry and spawning for Yellowtail, Sablefish, Drum and Flounder; broodstock feeds including preparation of wet feeds and formulated feeds; fish egg collection methods and assessing egg quality; and larval production methods as conducted by HSWRI and NOAA. Illustrated lectures were provided for live feed production and enrichment of rotifers and Artemia, other specialty foods, and bacterial management. Also included were larval production demonstrations for rotifers, Artemia, larval fish, including bacterial monitoring of cultures; weaning approaches; commercial diets; manufacturing processes and approaches to formulated diets and the move towards open formulations; commercial prospective on larval feeds and formulations and delivery methods for water-soluble nutrients; food markers; larval behavior and quality considerations. The PowerPoint presentations were converted to pdf files and transferred to thumbdrives for workshop participants. The pdfs will also be available to post on the WRAC website.

Discussions are now being held with project scientists to develop themes and concepts gained from the project's researched objectives, and the workshop, to be represented in the digital outreach products. Following the project's termination report, the workgroup will make final decisions for materials to be included in the digital outreach products detailed in the grant, which will include graphic materials from the IAC/TC annual reports, final report, and workshop; and will be prepared for incorporation in the outreach Articulate flash videos and .pdf digital publications. Outreach products will be prepared in 2016, submitted to the WRAC editorial process; and will include production, final editing and approval by the Marine Larval Nutrition Workgroup and the WRAC Editorial Committee.

IMPACTS:

Title: Advances in larval nutrition of marine fish

Relevance: Marine finfish farming is a fledgling industry in the United States but with great promise give available ocean waters and highly marketable native species. Regulatory hurdles remain the sole impediment to advancing the industry but progress is being made, especially in the Gulf of Mexico and southern California. Hatchery technologies have been developed over the last two decades for various marine species around the country. Without outlets to grow the fish out, much of this work has been done at a modest, experimental scale. In order to be immediately successful, commercial companies will need to rely on mass production of high quality juvenile fish to start the growout process. The larval phase is known to be the weakest link in this chain, and nutrition is known to be a cornerstone for health among all live stages. Therefore, the purpose of this project was to employ nutritional approaches to maximize larval survival and quality for commercial growout. The results of this project are also expected to have applicability to other fishes (e.g. freshwater) that are reared intensively.

Response: We assembled an expert team of scientists with experience in various facets of fish production and nutrition. We partitioned the project into discreet areas of focus through the larval stage in order to quantify stage-specific benefits (e.g. live feeds stage verses formulated feeds).

Results: We improved the consistency of larval survival and quality of two species of fish through nutritional manipulations, especially the adoption of a new commercially available enrichment. We discovered and/or validated several new techniques for packaging micro-nutrients into larval feeds; as well as developed several new processing techniques for formulated feeds. We also developed and validated an open formula microdiet that will allow the industry to customize diets for individual species in the future, as well as allow these diets to be manufactured in the United States. We held a workshop that included 27 industry professionals to transfer the findings of this project, as well as to communicate other related topics of interest.

Impact: As appropriate, research results were applied directly into hatchery protocols for yellowtail and white seabass, leading to more efficient production and more consistent larval survival and quality.

Collaborators: Hubbs-SeaWorld Research Institute, Oregon State University, US Department of Agriculture, National Oceanic and Atmospheric Administration, University of California, Davis.

PUBLICATIONS, WORKSHOPS, MANUSCRIPTS, OR PAPERS PRESENTED

HSWRI bimonthly newsletter – March 2012, July 2013, November 2013, and Sept 2014

Stuart, Kevin, Federico Rotman, Mark Drawbridge. 2012. Larval rearing advancements for yellowtail amberjack Seriola lalandi in southern California. The 40th UJNR Scientific Symposium. Hatchery Technology for High Quality Juvenile Production, Honolulu, HI October 22 – 23.

Stuart, Kevin, Federico Rotman, Mark Drawbridge. 2013. Larval rearing advancements for yellowtail amberjack Seriola lalandi in southern California. Aquaculture 2013, Nashville, TN, February 21-25.

Rust, Michael B., Frederic T. Barrows, Mark Drawbridge, Emily R. Hart, Kevin Stuart, Ken Webb, Harold J. Barnett, Peter M. Nicklason, and Ronald B. Johnson. 2013. Characterization of several open formula reference diets for marine fish larvae. The 41st UJNR Scientific Symposium. Advanced Aquaculture Technologies, Sapporo, Japan October 9-10.

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Stuart, Kevin, T. Barrows, Michael B. Rust, Ronald B. Johnson, and Mark Drawbridge. 2014. The use of experimental microdiets on two marine finfish species. Aquaculture America 2014, Seattle, WA, February 9-12.

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Hawkyard, M., Langdon C., Stuart, K., Drawbridge, M. Liposomes open new doors in larval fish nutrition. Global Aquaculture Alliance: Advocate. May/ June 2015.

Matt Hawkyard, Kevin Stuart, Chris Langdon & Mark Drawbridge. (2015) The enrichment of rotifers (*Brachionus plicatilis*) and *Artemia franciscana* with taurine-liposomes and their subsequent effects on the larval development of California yellowtail (*Seriola lalandi*). Aquaculture Nutrition. Accepted for publication.

WRAC-supported "Larval Feeds and Feeding Strategies for Marine Fish Workshop". 2015. Hubbs-SeaWorld Research Institute, August 25-27, San Diego, CA.

SUBMITTED BY: Sept 11, 2015

Title: (Work Group Chair or PI)/Date

APPROVED: Sept 11, 2015

Project Monitor/Date

APPENDIX A. TABLES

Table 1. Performance measures for each of two species collected at 35 dph when reared from an egg. Averages presented with standard deviation as applicable.

Species	SL (mm ± SD)	WT (g ± SD)	Survival (% ± SD)	Malformation (% ± SD)
WSB	16.2 (1.34)	18.7	9.0 (2.31)	7.5
CYT	14.7 (3.19)	14.0	12.1 (2.04)	12.5

Table 2. Performance measures for WSB collected at 40 dph when reared from an egg. Averages presented with standard deviation as applicable.

Treatment	SL (mm ± SD)	WT (g ± SD)	Survival (% ± SD)	Malfomation (% ± SD)
1st-2nd (Easy Selco)	17.29 ± 3.14^{b}	0.14 ± 0.17^{b}	1.3 ± 0.7	74.6 ± 25.3^{b}
1st-2nd (S. Presso)	20.95 ± 4.08^{a}	0.21 ± 0.10^{a}	1.3 ± 0.8	44.8 ± 12.6^{b}
Rots-2nd (ORI-GREEN/S. Presso)	22.05 ± 3.65^{a}	0.23 ± 0.11^{a}	2.9 ± 2.1	15.5 ± 10.3^{a}

Table 3. Performance measures for CYT collected at 40 dph when reared from an egg. Averages presented with standard deviation as applicable.

Treatment	SL (mm ± SD)	WT (g ± SD)	Survival (% ± SD)	Malfomation (% ± SD)
Rots-1st-2nd (ORI-GREEN/Easy Selco)	19.2 ± 3.8	0.17 ± 0.09	1.5 ± 1.6	37.5 ± 6.5
Rots-2nd (ORI-GREEN/S. Presso)	19.1 ± 3.5	0.16 ± 0.08	2.0 ± 1.0	37.5 ± 17.1

Table 4. Performance measures for WSB collected at 55 dph when reared from an egg. Averages presented with standard deviation as applicable.

Treatment	SL (mm ± SD)	Total Weight (g ± SD)	Survival (% ± SD)	Malformation (% ± SD)
1st-2nd (Easy Selco)/Otohime	25.1 ± 1.7^{a}	0.27 ± 0.03^{a}	4.8 ± 1.3	87.0 ± 10.5^{a}
1st-2nd (S. Presso)/Otohime	23.5 ± 0.8^{a}	0.25 ± 0.03^{a}	5.3 ± 0.6	75.0 ± 8.8^{a}
Rots-2nd (ORI-GREEN/S. Presso)/Otohime	24.7 ± 0.4^{a}	0.28 ± 0.03^{a}	5.4 ± 0.8	51.0 ± 10.5^{b}
Rots-2nd (ORI-GREEN/S. Presso)/LEX	19.7 ± 0.8^{b}	0.16 ± 0.02^{b}	3.9 ± 0.9	76.0 ± 9.8^{a}

Table 5. Mean dry weights (µg larva^{-1}), notochord lengths (NL) and survival of CYT larvae measured throughout the trial. Larvae had been fed either: 1) taurine-unsupplemented rotifers followed by taurine-unsupplemented *Artemia* (U-Rot:U-Art), 2) taurine-unsupplemented rotifers followed by taurine-enriched *Artemia* (U-Rot:T-Art), 3) taurine-enriched rotifers from followed by taurine-unsupplemented *Artemia* (T-Rot:U-Art) or 4) taurine-enriched rotifers followed by taurine-enriched *Artemia* (T-Rot:T-Art). Different letter denote significant differences. T-tests were used for comparisons between two groups and ANOVA followed by Tukeys's HSD were used for comparisons between 4 groups (significance level $p > 0.05$).

	U-Rot:U-Art	**U-Rot:T-Art**	**T-Rot:U-Art**	**T-Rot:T-Art**
Rotifer phase (2-8 dph)	Taurine unsupplemented rotifers		Taurine enriched rotifers	
Artemia phase (8-14 dph)	Taurine unsupplemented *Artemia*	Taurine enriched *Artemia*	Taurine unsupplemented *Artemia*	Taurine enriched *Artemia*
	Individual larval DW (ŭg larva^{-1})			
8 dph	99.0 ± 8^{a}		114.0 ± 9^{b}	
14 dph	411 ± 98^{a}	398 ± 99^{a}	389 ± 76^{a}	355 ± 52^{a}
	NL (mm)			
8 dph	5.08 ± 0.11^{a}		5.15 ± 0.14^{a}	
14 dph	6.53 ± 0.70^{a}	6.65 ± 0.61^{a}	6.30 ± 0.56^{a}	6.44 ± 0.59^{a}
	Survival (%)			
14 dph	6.8 ± 3.0^{a}	11.4 ± 9.0^{a}	9.3 ± 5.3^{a}	12.1 ± 13.2^{a}

Table 6. Standard length and malformation rate for WSB collected at 58 days post hatch (dph) when reared from an egg. Averages presented with standard deviation as applicable

Treatment	**Standard Length (mm ± SD)**	**Malformation (% ± SD)**
Rots - 2nd (S. presso)	48.43 ± 4.67^{a}	15.0 ± 3.5
1st - 2nd (S. presso)	47.66 ± 4.36^{a}	18.0 ± 13.2
1st - 2nd (Easy Selco)	45.33 ± 4.60^{b}	32.0 ± 10.7

Table 7. Larval performance measures for CYT at 7 dph fed at three rotifer densities.

Treatment (rots/ml)	SL (mm ± SD)	DW (µg ± SD)	Survival (% SD)
5	5.39 ± 0.44	95 ± 19	7.1 ± 2.9
10	5.30 ± 0.33	90 ± 12	5.3 ± 2.6
20	5.37 ± 0.36	100 ± 16	10.0 ± 3.3

Table 8. Larval performance measures for CYT at 16 dph fed at three *Artemia* densities.

Treatment (Artemia/ml)	SL (mm ± SD)	DW (µg ± SD)	Survival (% SD)
1	7.61 ± 0.57	1145 ± 60	86.6 ± 5.9
3	7.77 ± 0.74	1305 ± 221	90.4 ± 4.6
5	7.67 ± 0.56	1195 ± 138	86.2 ± 1.0

Table 9. Larval performance measures for CYT at 7 dph offered two different rotifer feed densities and two different delivery methods.

Treatment	SL (mm ± SD)	DW (µg ± SD)	Survival (% SD)
10 rotifers/ml Batch	5.55 ± 0.44	200 ± 59	4.8 ± 2.9
20 rotifers/ml Batch	5.55 ± 0.53	220 ± 85	9.7 ± 5.3
20 rotifers/ml Auto	5.38 ± 0.44	160 ± 37	5.6 ± 2.5

Table 10. Performance results for WSB fed two experimental diets and a commercial control (Otohime). Averages given with standard deviation in parenthesis. Treatments with different superscripts are significantly different from each other.

Diet	SL (mm)	Dry WT (mg)	Survival (%)	Malformation (%)
MEM	17.1 (1.95)^{b}	19.5	5.2 (0.9)	1.3 (2.5)
PARA	16.9 (1.75)^{b}	18.2	7.2 (1.1)	1.3 (2.5)
Otohime	18.3 (2.54)^{a}	24.1	6.2 (1.8)	2.5 (2.9)

Table 11. Performance results for CYT fed one experimental diets and two commercial controls (Gemma and Otohime). Averages given with standard deviation in parenthesis. Treatments with different superscripts are significantly different from each other.

Diet	SL (mm)	Dry WT (mg)	Survival (%)	Malformation (%)
Gemma	17.6 (3.09)[a]	27.0[a]	5.1 (0.5)[b]	27.5 (16.6)
PARA	14.8 (2.69)[b]	16.4[b]	8.1 (4.2)[a]	23.8 (7.5)
Otohime	16.4 (2.95)[a]	22.3[b]	3.3 (2.4)[b]	15.0 (14.7)

Table 12 Performance results for CYT fed two experimental diets and a commercial control (OTO). Experimental diets are formulated with Montlake meal. Treatments with different superscripts are significantly different from each other.

Treatment	WT (g ± SD)	SL (mm ± SD)	Survival (% ± SD)
Flake	0.59 ± 0.70[c]	27.5 ± 9.0[c]	0.4 ± 0.2[c]
PARA	0.88 ± 0.46[b]	31.9 ± 7.7[b]	2.1 ± 0.3[b]
OTO	1.31 ± 0.68[a]	39.6 ± 7.6[a]	7.6 ± 1.7[a]

Table 13. Performance results for WSB fed two experimental diets and a commercial control (OTO). Treatments with different superscripts are significantly different from each other.

Treatment	WT (g ± SD)	SL (mm ± SD)	Survival (% ± SD)
PARA (Montlake)	0.37 ± 0.21[b]	26.3 ± 4.9[b]	21.5 ± 5.8[b]
PARA (Clam)	0.20 ± 0.18[c]	21.9 ± 3.6[c]	7.4 ± 1.2[c]
OTO	0.62 ± 0.23[a]	32.3 ± 4.1[a]	51.6 ± 3.1[a]

Table 14 Performance results at 49 dph for CYT fed two experimental diets from 15 to 49 dph. Treatments with different superscripts are significantly different from each other.

Treatment	Individual Weight (g ± SD)	Standard Length (mm ± SD)	Survival (g ± SD)
LEX	0.35 ± 0.14	25.0 ± 3.5	43.8 ± 5.0
PARA	0.36 ± 0.14	25.4 ± 3.3	36.7 ± 5.8

Table 15. Treatment information for five experimental diets fed to CYT larvae.

Treatment	Target Taurine Inclustion (%)	Additional Ingredients	Actual Inclusion (Percent ± SD)
YT 1	0		0.4 ± 0.02
YT 2	3		4.5 ± 0.4
YT 3	6		9.3 ± 0.6
YT 4	10		12.2 ± 1.9
YT 5	6	spirulina	7.5 ± 0.8

Table 16. Whole fish taurine composition at 41 dph, diet composition, and leaching rate information at 5, 10, 15, and 30 minutes for five experimental diets fed to CYT larvae. Treatments with different superscripts are significantly different from each other.

	Taurine Level					
	Whole Fish	Diet	% Taurine Remaining After Each Time Interval (min)			
Treatment	(mg/g ± SD)	(mg/g ± SD)	5	10	15	30
YT1	1.87 ± 0.11^{a}	4.06 ± 0.18^{a}	35.9	62.8	80.5	79.1
YT2	5.12 ± 0.47^{b}	45.67 ± 4.06^{b}	37.4	65.8	86.2	93.8
YT3	5.13 ± 0.34^{b}	91.03 ± 6.93^{c}	41.6	73.0	94.4	97.6
YT4	5.06 ± 0.35^{b}	122.33 ± 18.50^{d}	43.4	76.1	97.9	98.6
YT5	5.81 ± 0.37^{b}	75.20 ± 7.58^{c}	43.4	75.9	97.6	97.2

Table 17. Growth, consumption, and survival data at 41 dph for CYT fed five experimental diets. Treatments with different superscripts are significantly different from each other.

Treatment	Individual Weight (g ± SD)	Standard Length (mm ± SD)	Food Consumption (µg/larvae ± SD)	Survival (Percent ± SD)
YT 1	0.24 ± 0.12	21.3 ± 3.7^{a}	29.8 ± 5.6^{a}	33.9 ± 4.3
YT 2	0.29 ± 0.12	24.5 ± 3.7^{b}	43.5 ± 3.3^{b}	31.3 ± 2.4
YT 3	0.28 ± 0.11	23.8 ± 3.5^{ab}	34.6 ± 6.2^{b}	34.5 ± 3.1
YT 4	0.26 ± 0.11	23.4 ± 3.5^{ab}	42.2 ± 3.7^{b}	30.1 ± 3.2
YT 5	0.30 ± 0.12	24.8 ± 3.6^{b}	64.3 ± 13.8^{b}	37.7 ± 4.2

Table 18. Sinking rates of various particle types associated with the WRAC project.

	Particle Size (μm)		Average Sinking Rate	
Diet	Min	Max	(cm/sec)	SD
Oto - B1	250	360	0.53	0.13
Oto - B2	360	650	0.93	0.15
Oto - C1	580	840	1.21	0.18
Oto - C2	840	1410	2.20	0.39
Gemma	50	100	0.23	0.09
Gemma	100	200	0.29	0.07
Gemma	200	400	0.64	0.17
Empty Alginate			0.78	0.13
Alginate with Amino Acid Mix			0.71	0.08
Empty Alginate with Qrill			1.09	0.14
Alginate with Amino Acid and Qrill			1.04	0.14
PARA no LSB	355	500	0.59	0.18
PARA with LSB	355	500	0.59	0.11
PARA Flake	150	425	0.78	0.10
PARA Flake	425	710	1.45	0.24
PARA Flake	800	800	1.73	0.23
PARA Clam	125	425	0.69	0.14
PARA Clam	425	710	1.44	0.22
PARA Clam	800	800	1.94	0.30
LEX	250	500	0.54	0.03
LEX	500	750	1.11	0.07
PARA	250	500	0.90	0.10
PARA	500	750	1.32	0.31

Table 19. Performance measures for WSB at 47 dph following several dietary treatments and weaning strategies, values within columns sharing lowercase superscripts are not significantly different.

	Treatment Factor Combinations			
Treatment ID	Stimulant	Weaning Age (dph)	Weight (g ± SD)	Survival (% ± SD)
WSB 1	none	25	0.277 ± 0.035^{ab}	31.1 ± 6.8^{a}
WSB 2	Gly/Ala/Bet	25	0.233 ± 0.039^{a}	37.7 ± 4.1^{ab}
WSB 3	ProMega 55	25	0.301 ± 0.033^{b}	48.2 ± 3.2^{bc}
WSB 4	none	35	0.244 ± 0.038^{a}	57.4 ± 3.7^{c}
WSB 5	Gly/Ala/Bet	35	0.234 ± 0.021^{a}	59.1 ± 7.4^{c}
WSB 6	ProMega 55	35	0.315 ± 0.032^{b}	59.1 ± 5.2^{c}

APPENDIX B. FIGURES

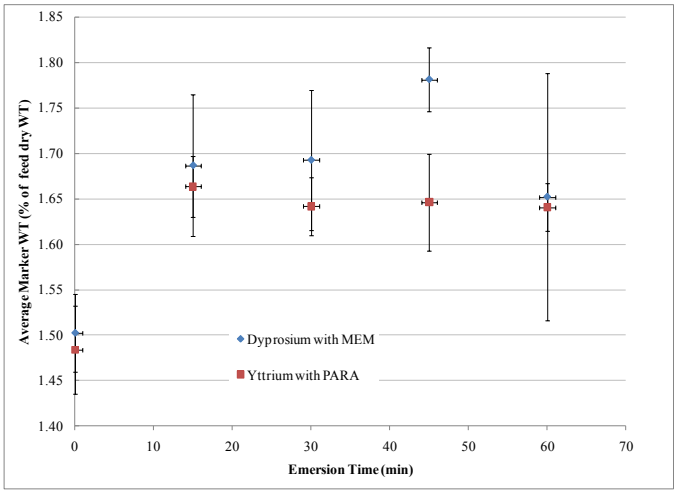

Figure 1. Relationship between time of emersion and marker concentration for two marker types in each of two separate feed types.

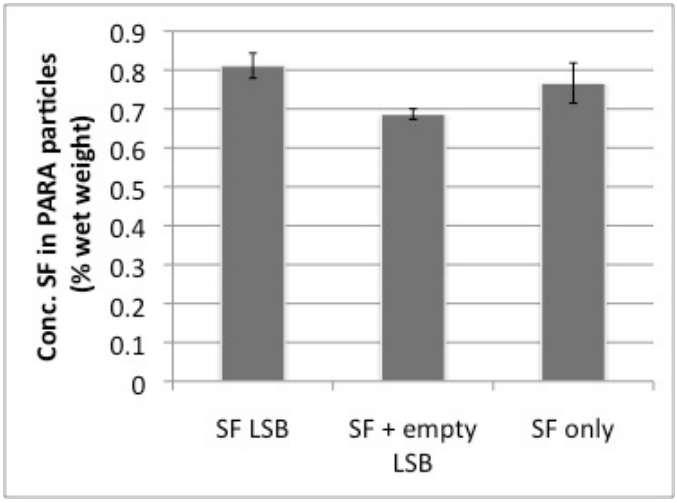

Figure 2. Concentration of sodium fluorescein (SF) in PARA particles produced with 1) lipid spray beads containing sodium fluorescein, "SF LSB", 2) crystalline sodium fluorescein and empty lipid spray beads, "SF + empty LSB" or 3) crystalline sodium fluorescein. Prior to agglomeration, SF was added to the mash at 0.5% of the mash by wet weight.

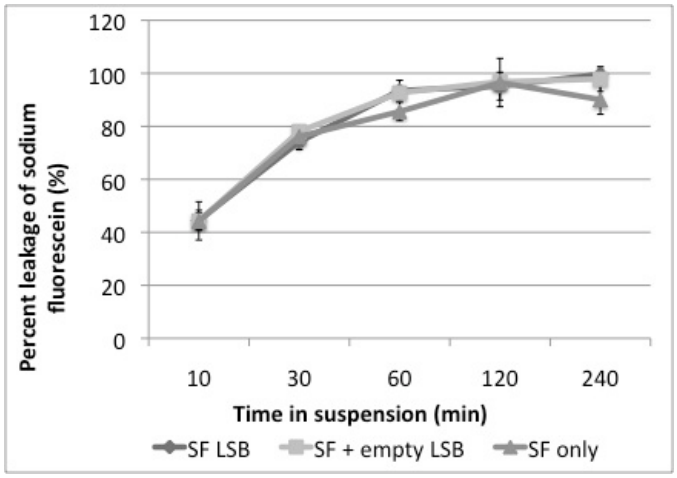

Figure 3. Leakage of sodium fluorescein from PARA particles produced with 1) lipid spray beads containing sodium fluorescein, "SF LSB", 2) crystalline sodium fluorescein and empty wax spray beads, "SF + empty LSB" or 3) crystalline sodium fluorescein, "SF only". PARA particles were suspended in 10 µm-filtered seawater for the duration of the leakage trial.

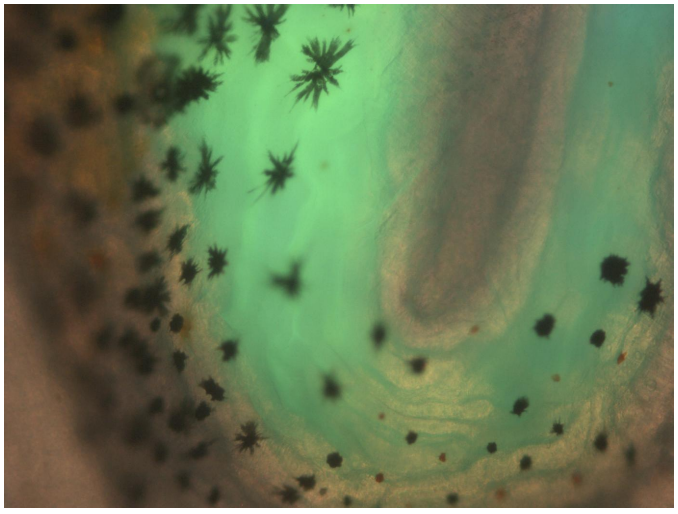

Figure 4. Epifluorescent image of Northern rock sole larval gut taken with a 10X objective. Larva had been fed PARA particles with sodium fluorescein included in the mash (SF + empty LSB). Green fluorescence was seen throughout the larval gut suggesting that fluorescein had been released from PARA particles. Similar images were taken of larvae fed each of the three experimental diets.

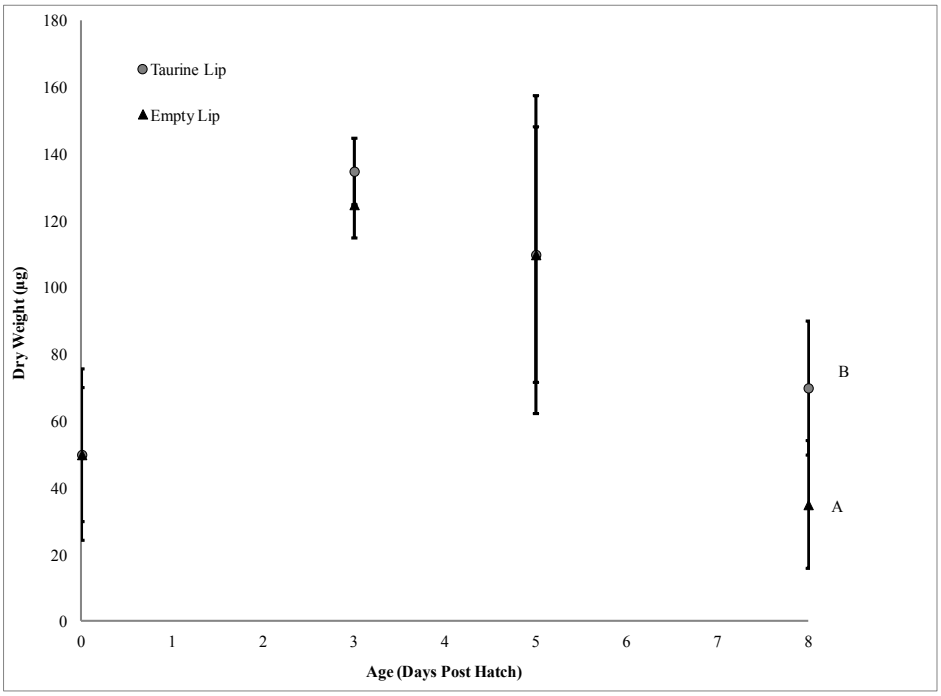

Figure 5. Mean dry weights (µg larva^{-1}) of CYT larvae measured throughout the rotifer phase. Larvae had been fed either rotifers enriched with microparticles containing taurine (Taurine Lip) or rotifers enriched with empty liposomes (Empty Lip). Different letter denote significant differences (Tukeys's HSD; significance level $p > 0.05$).

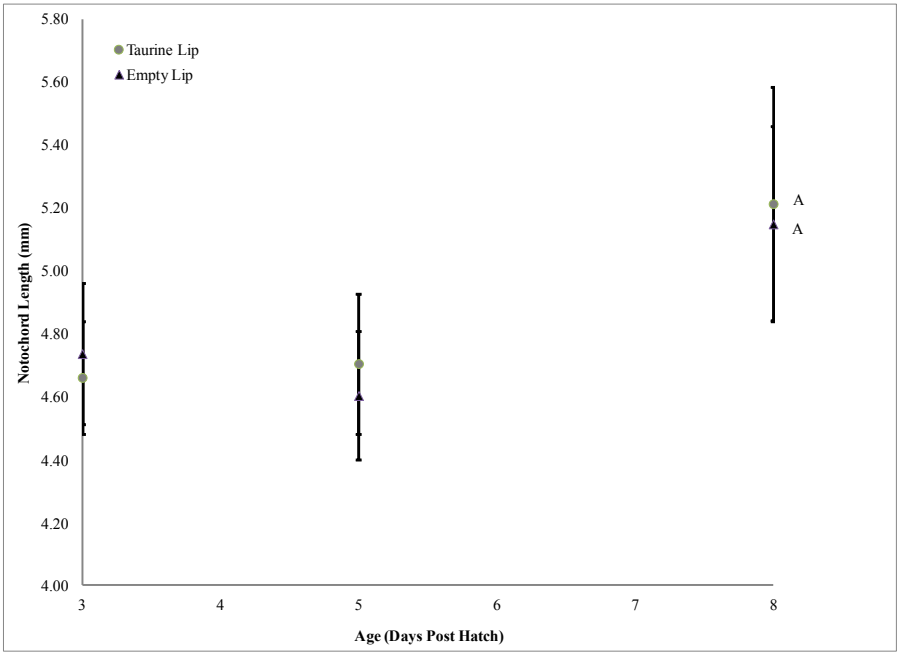

Figure 6. Notochord lengths (mm) of CYT larvae measured throughout the rotifer phase of CYT. Larvae had been fed either rotifers enriched with microparticles containing taurine (Taurine Lip) or rotifers enriched with empty liposomes (Empty Lip). (Tukeys's HSD; significance level $p > 0.05$).

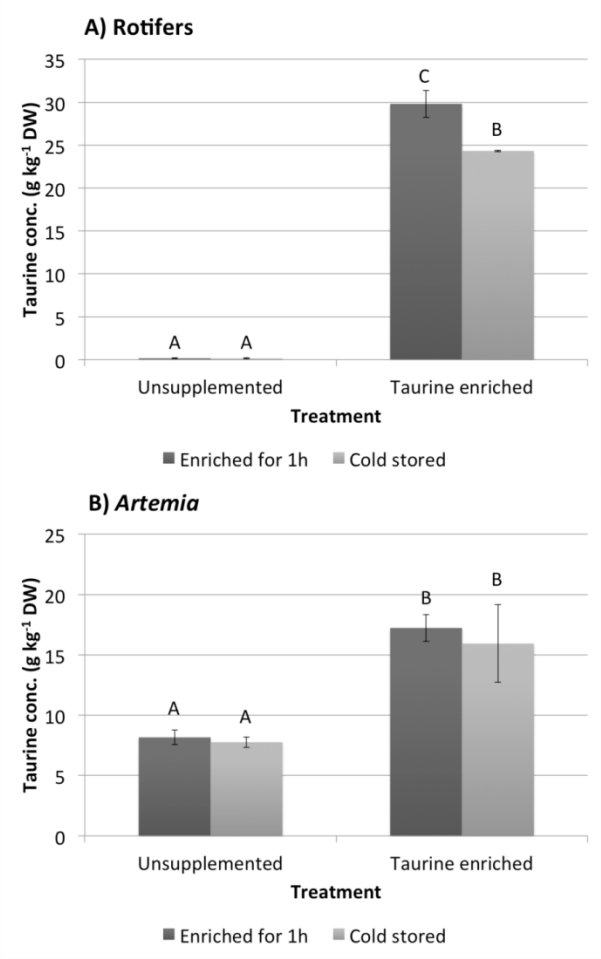

Figure 7. Taurine concentrations (g kg-1 DW) in A) rotifers and B) Artemia following enrichment with liposomes for 1 h ("Enriched for 1h) and after 3 hours of cold storage ("Cold stored"; 4-10° C). Different letter denote significant differences between treatments (Tukeys's HSD, significance level $p > 0.05$).

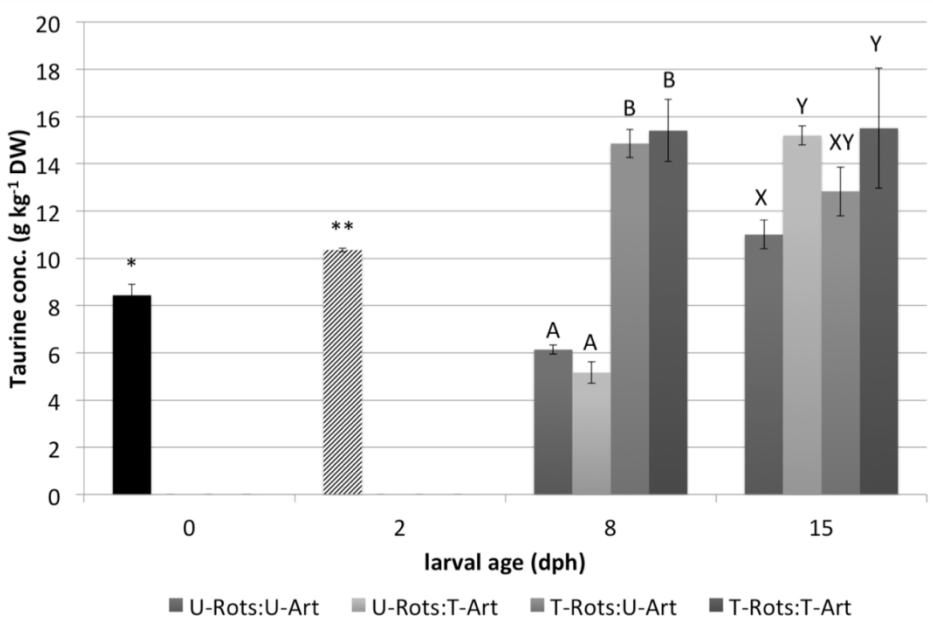

Figure 8. Whole body taurine concentrations of CYT larvae immediately after hatching (0 dph), prior to first-feeding (2 dph) and at the end of the rotifer phase (8 dph) and at the end of the *Artemia* phase (15 dph). Larvae had been fed either: 1) taurine-unsupplemented rotifers followed by taurine-unsupplemented *Artemia* (U-Rot:U-Art), 2) taurine-unsupplemented rotifers followed by taurine-enriched *Artemia* (U-Rot:T-Art), 3) taurine-enriched rotifers from followed by taurine-unsupplemented *Artemia* (T-Rot:U-Art) or 4) taurine-enriched rotifers followed by taurine-enriched *Artemia* (T-Rot:T-Art). Different letter denote significant differences. T-tests were used for comparisons between two groups and ANOVA followed by Tukeys's HSD were used for comparisons between 4 groups (significance level $p > 0.05$).

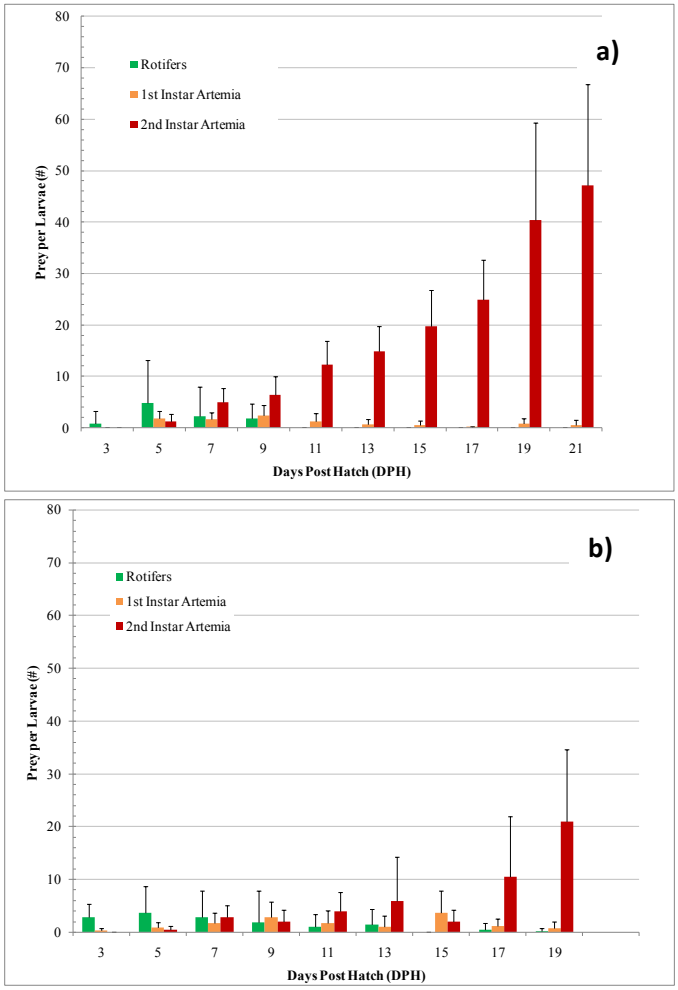

Figure 9 Relationship between number of prey consumed and age of larvae for a) WSB and b) CYT. Each prey type was offered simultaneously at 5 prey/ml. Prey counts are direct visual counts after dissection.

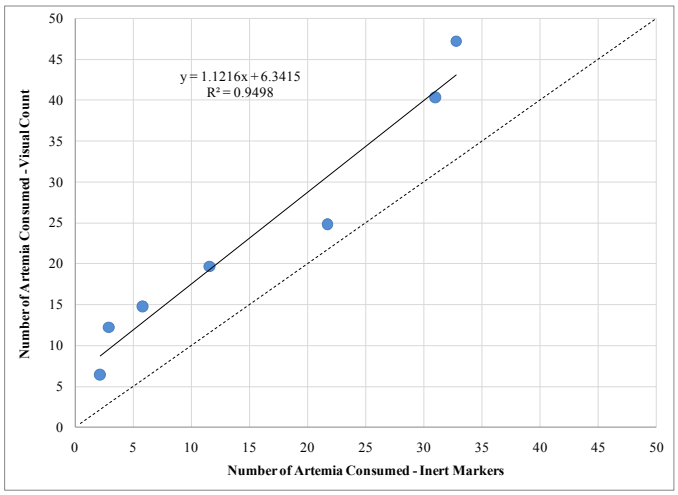

Figure 10. Relationship between number of *Artemia* consumed using two different methodologies.

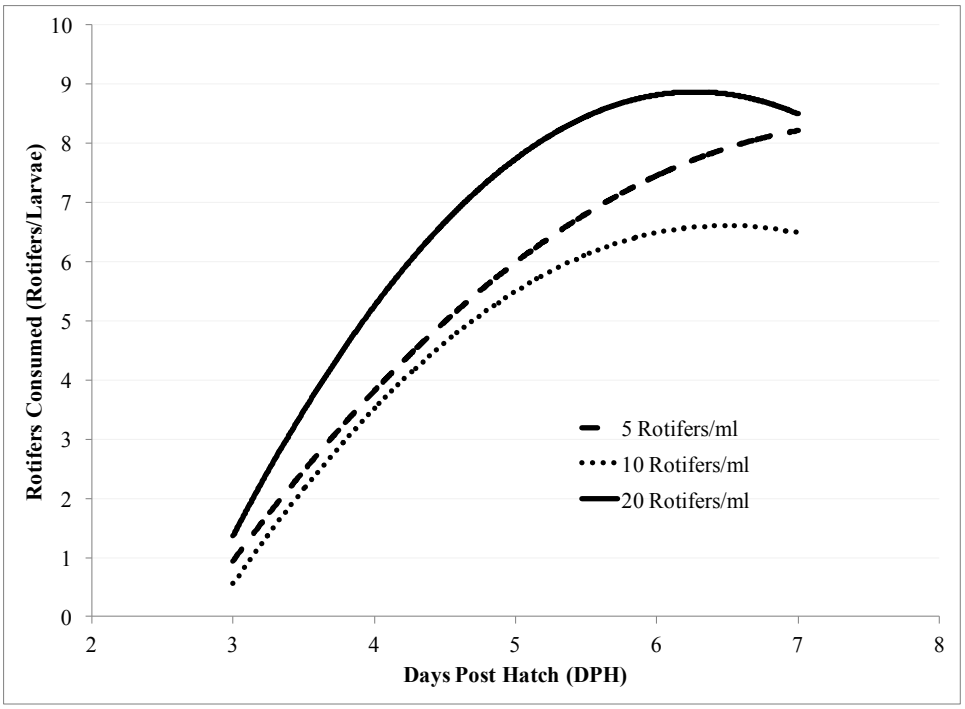

Figure 11. Rotifer consumption by CYT larvae offered 5, 10, and 20 rotifers/ml.

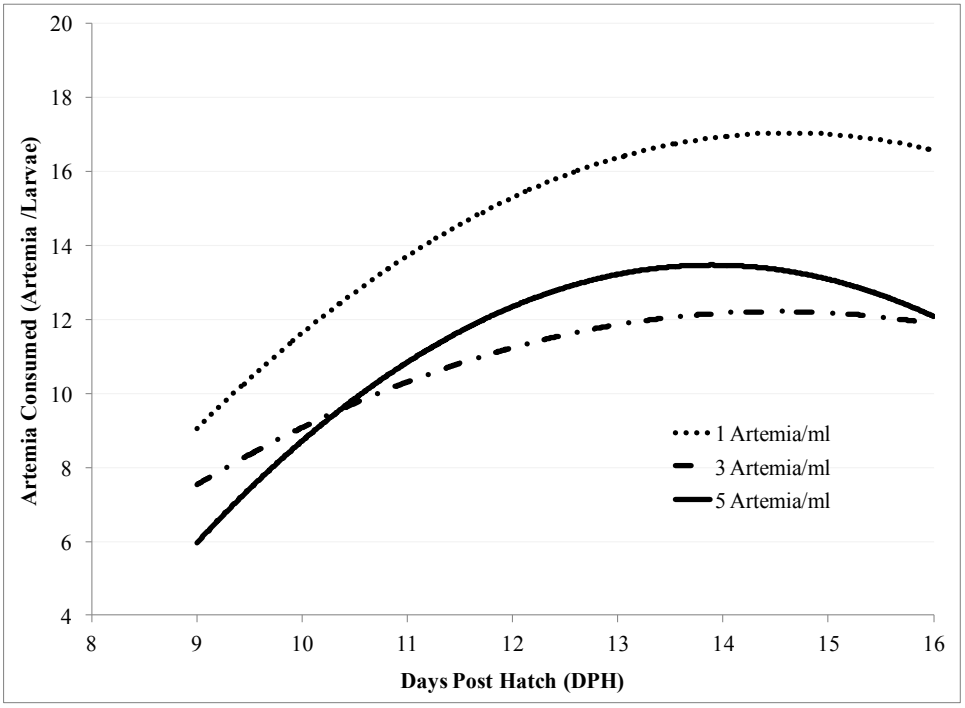

Figure 12. *Artemia* consumption of CYT fed *Artemia* densities for 1, 3, and 5 *Artemia*/ml.

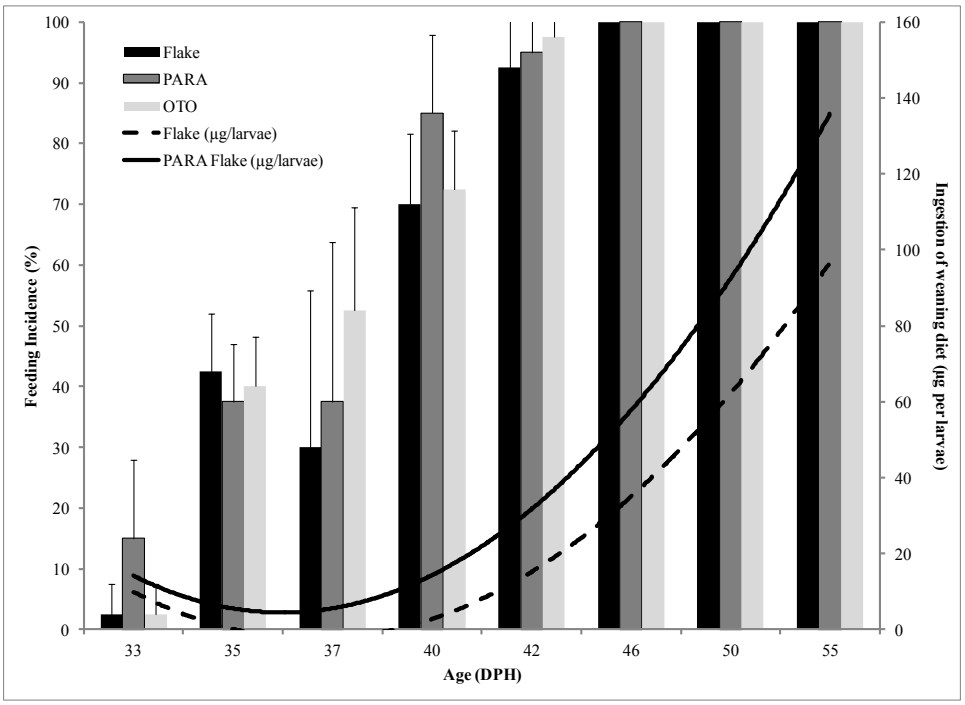

Figure 13. Feeding incidence (bars) measured using gut contents and feed consumption (lines) measured using inert markers for CYT larvae fed two experimental diets. There is no consumption curve for OTO because we are unable to mark it.

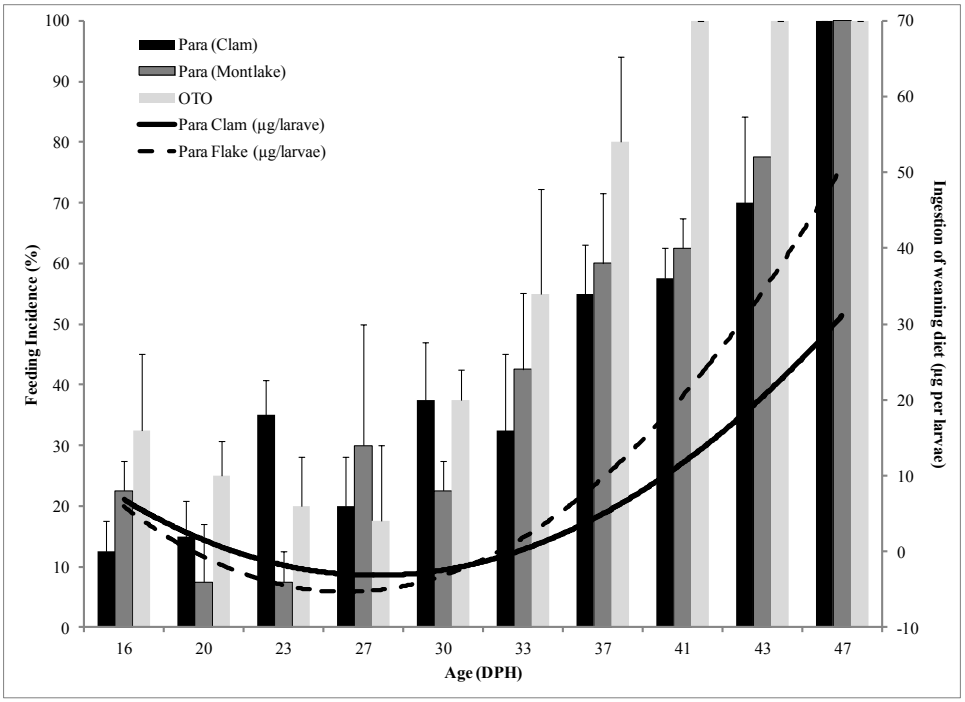

Figure 14. Feeding incidence and consumption for WSB larvae fed two experimental diets. There is no consumption curve for OTO because this diet is unable to be tracked with an inert marker.

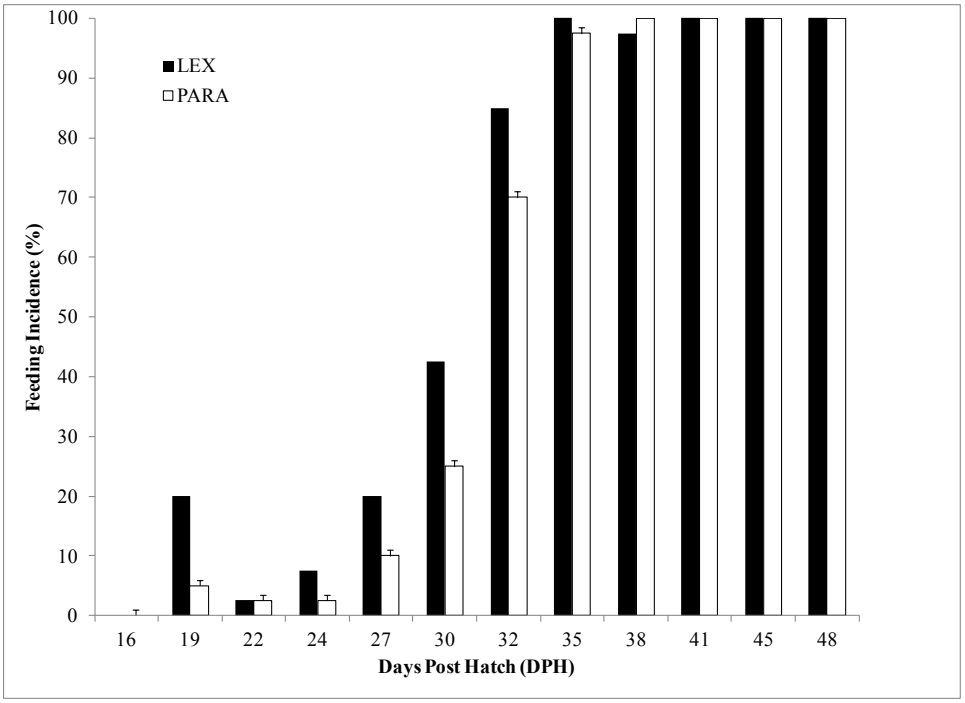

Figure 15. Feeding incidence for CYT larvae fed two experimental weaning diets.

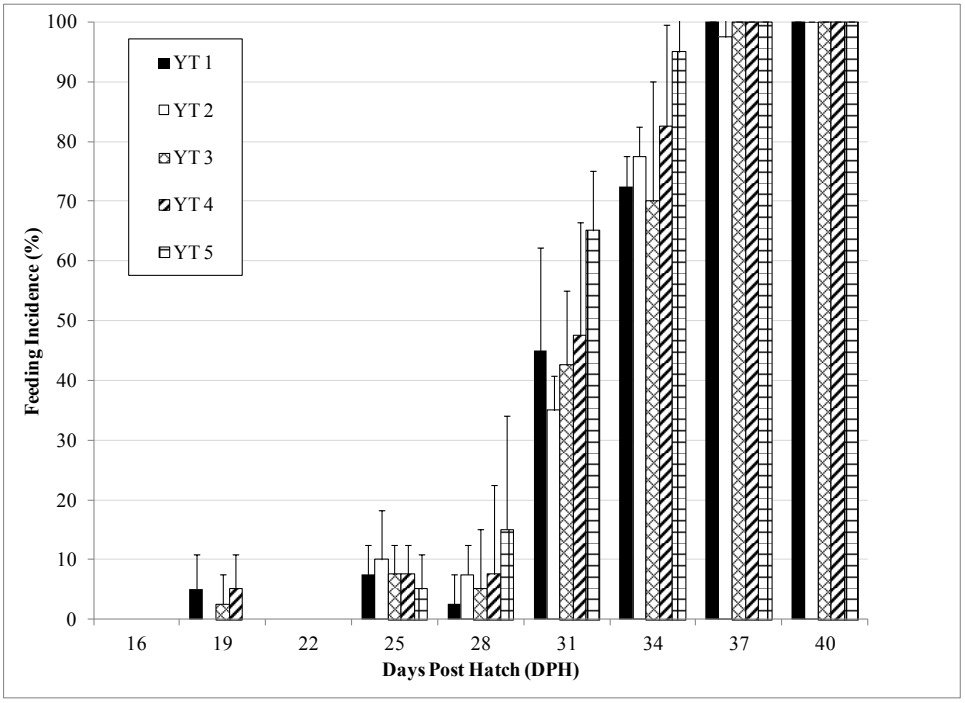

Figure 16. Feeding incidence for CYT fed five different experimental diets from 15 to 41 dph.

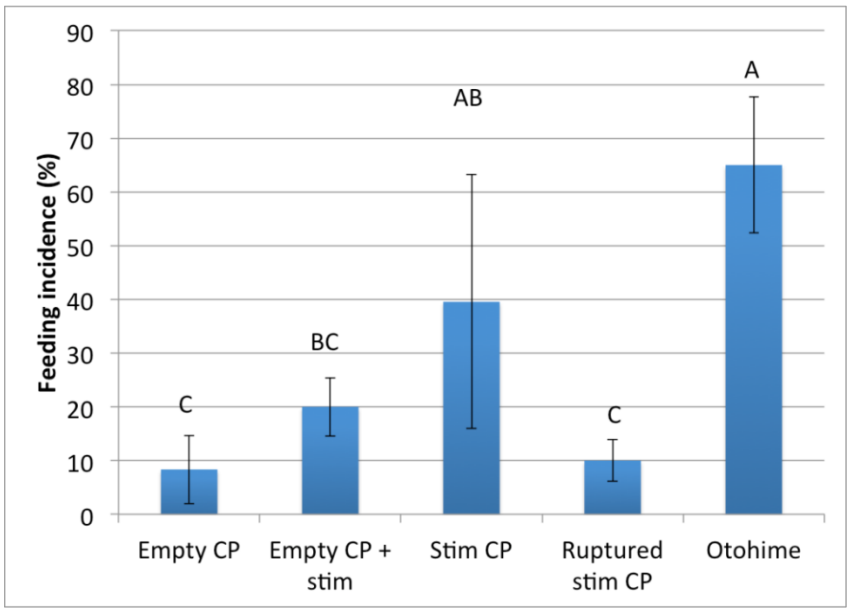

Figure 17. Feeding incidence (% of the total number of fish with food in the gut) of white seabass fed various experimental diets. Treatment descriptions are given above. Data were arcsin-square root transformed for statistical analyses; untransformed data are shown. Different letters denote significant differences between treatments (Tukey's HSD, significance level $p < 0.05$).

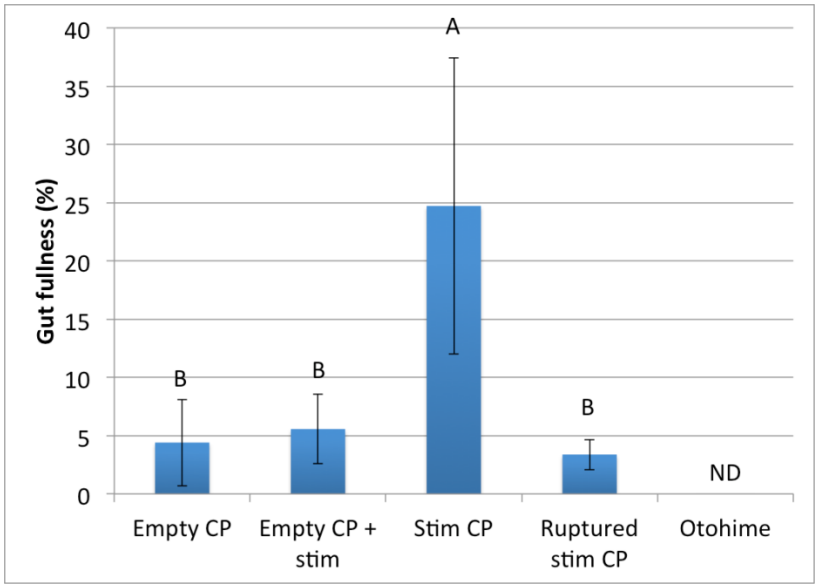

Figure 18. Gut fullness (%) of white seabass fed various experimental diets. Gut fullness was calculated as the number of red-spectrum pixels in each image divided by number of red-spectrum pixels in the most fluorescent image (100% full) X 100. Data were arcsin-square root transformed for statistical analyses; untransformed data are shown. Different letter denote significant differences between treatments (Tukey's HSD, significance level $p < 0.05$). Gut fullness values of larvae fed Otohime diet were not determined (ND).

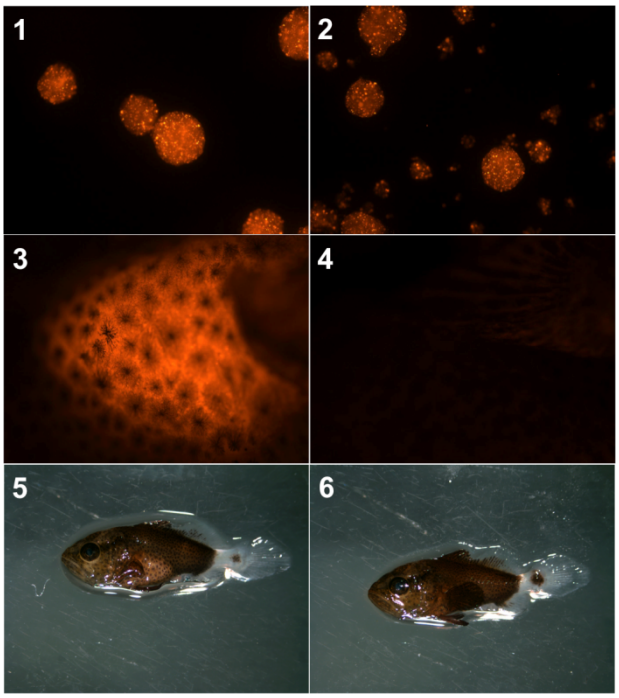

Figure 19. Digital images of 1) alginate complex particles (alginate-CP) with candidate feeding stimulants (glycine, alanine, betaine), 2) alginate complex particles without feeding stimulants, 3) gut of white seabass containing alginate-CP with stimulants, 4) gut of white seabass with alginate-CP without stimulants "No stimulants", 5) white seabass fed alginate-CP with stimulants (from image 3), 6) WSB fed alginate-CP without stimulants (from image 4). Images 1-4 were taken at with an epifluorescent microscope with a 4X objective. Images 5 and 6 were taken with a dissecting microscope with a 0.63X objective.

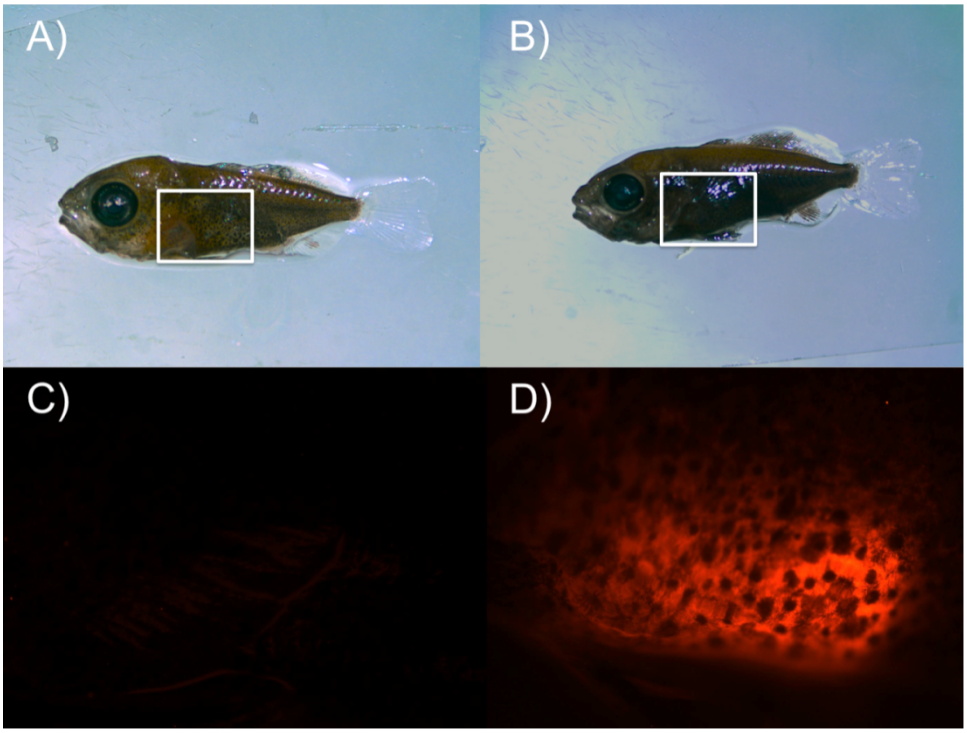

Figure 20. Digital images of CYT post-larvae fed fluorescent alginate complex-particles and photographed under a stereoscope (0.63X objective; images A and B) and an epifluorescent microscope with a 4x objective (C and D). The white squares in images A and B highlight the area of the fish photographed in images C and D. The fish larva in figures A and C was fed alginate particles without attractants (empty-CP) and has no evidence (indicated by lack of orange fluorescence) of CP particles in the gut. The fish larva in images B and D was fed alginate particles containing a glycine, betaine and alanine and it had eaten one or more alginate particles (indicated by orange fluorescence).

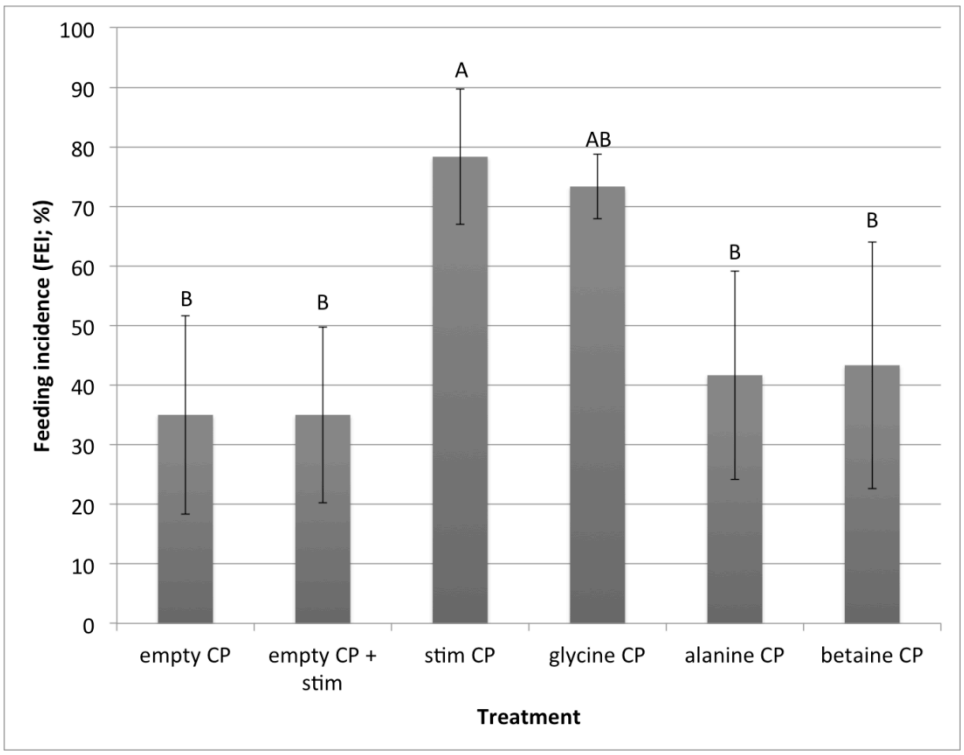

Figure 21. Feeding incidence (FEI): the percentage of larvae that had fed on fluorescent-labeled alginate particles in a given treatment (15 fish tank^{-1}; n = 4). Ingestion rates were determined from digital images of the larval gut region taken with an epifluorescent microscope. "Feeding" occurred when >5% of the pixels in a given image were colored red. Data analysis was performed on arc-sin square-root transformed data, raw data shown. Different letters denote significant differences (Tukey's HSD; significance level $p < 0.05$).

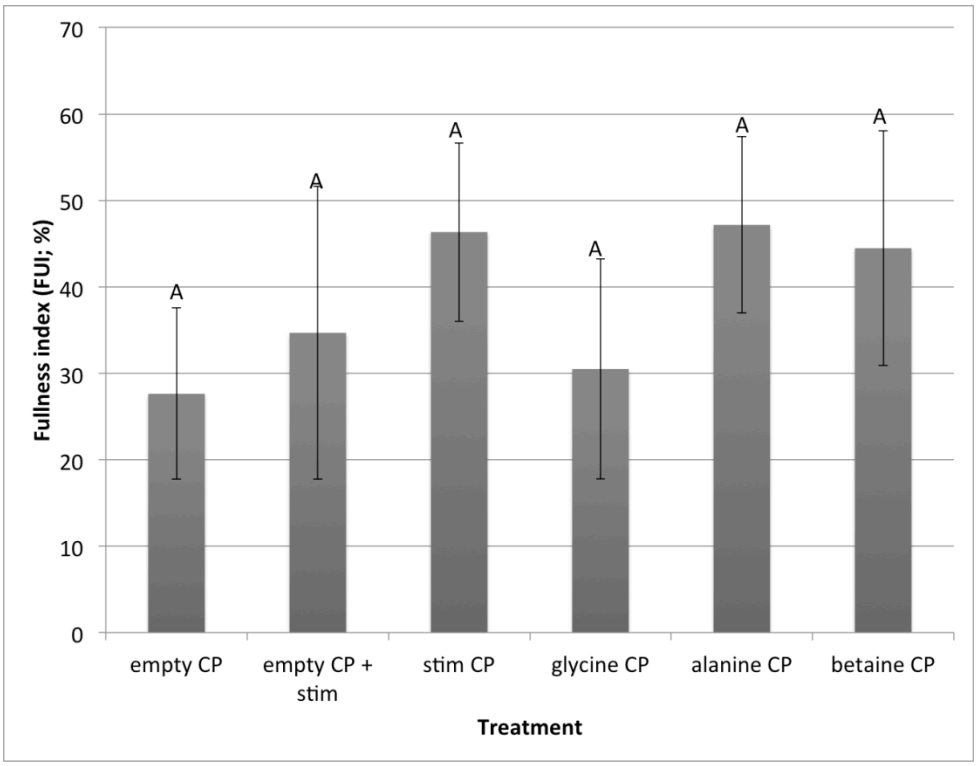

Figure 22. Fullness index (FUI; %) measured in CYT larvae fed. Digital images were taken of larval guts using an epifluorescent microscope and were analyzed with Image Pro software. Gut fullness was determined by analyzing the percent coverage of red spectrum pixels per image. Fullness index serves as a proxy for gut fullness and indicates how full the larvae were with alginate complex particles. Data analysis was performed on arc-sin square-root transformed data, raw data shown. Different letters denote significant differences (Tukey's HSD; significance level $p < 0.05$).

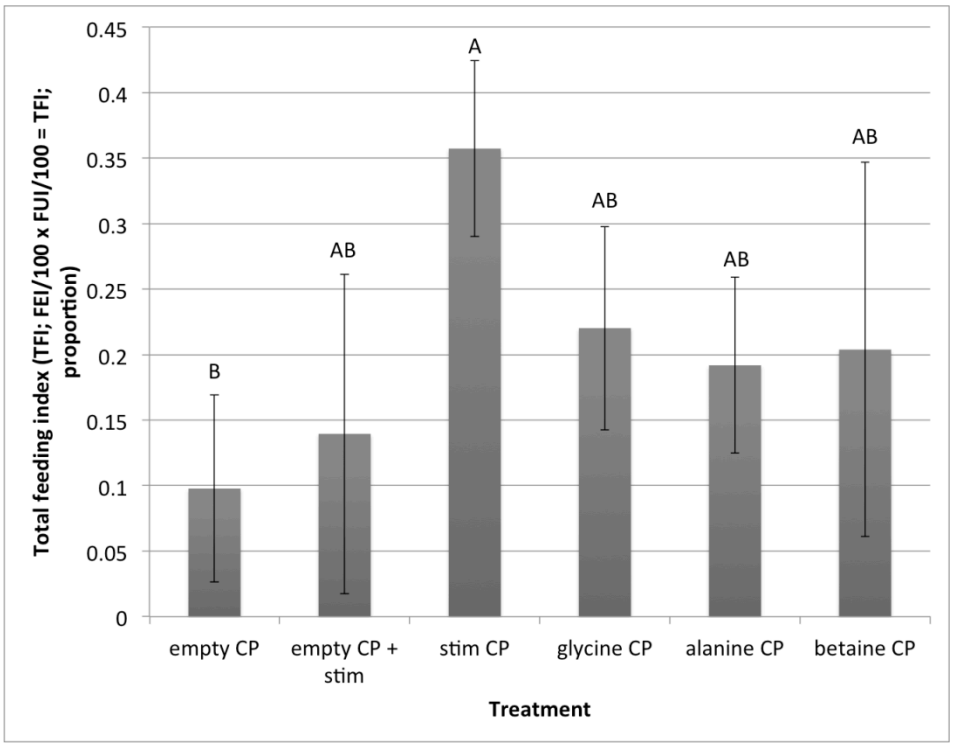

Figure 23. Total feeding index (TFI): Feeding incidence (FEI) x fullness index (FUI) = TFI. Data analysis was performed on arc-sin square-root transformed data, raw data shown. Different letters denote significant differences (Tukey's HSD; significance level $p < 0.05$).

APPENDIX B. WORKSHOP AGENDA

WRAC Western Regional Aquaculture Center

Alaska • Arizona • California • Colorado • Idaho • Montana • Nevada • New Mexico • Oregon • Utah • Washington • Wyoming

USDA United States Department of Agriculture Agricultural Research Service

UC DAVIS UNIVERSITY OF CALIFORNIA

NOAA National Oceanic and Atmospheric Administration U.S. Department of Commerce

Hubbs-SeaWorld Research Institute

OSU Oregon State University

Larval Feeds and Feeding Strategies for Marine Fish

August 25-27, 2015

Hubbs-SeaWorld Research Institute

San Diego, CA

This workshop is designed as an education, training and discussion platform focused on feeds and feeding strategies for larval marine fish with applicability to other marine and freshwater organisms that are fed. It is being organized by experts from the National Oceanic and Atmospheric Administration (NOAA), United States Department of Agriculture (USDA), Oregon State University (OSU), University of California Davis (UCD), and Hubbs-SeaWorld Research Institute (HSWRI). The workshop represents an extension component of a recently-completed three year collaborative research project funded by the Western Regional Aquaculture Center (WRAC). The workshop will incorporate lectures with hands-on learning activities with marine fish of different life stages from eggs to juveniles.

AGENDA

Tuesday, August 25th

1. **Introduction**
    a. 8:30 - 9:00: Welcoming session [Drawbridge and Conte]

b. 9:00 - 9:30: Tour of HSWRI Aquaculture Facilities [HSWRI Staff]

2. **Broodstock Husbandry and Spawning Presentations**
    a. 9:30 - 10:00: System Design Considerations [Rotman]

10:00-10:30 [Break]

b. 10:30 - 11:00: Yellowtail [Stuart]
c. 11:00 - 11:30: Sablefish [Massee]
d. 11:30 - 12:00: Drum and Flounder [Faulk]

12:00 – 1:00 pm [Lunch]

3. **Broodstock Demos [1:00 – 3:00pm]** *four stations for 5-6 participants each*
    a. Preparation of wet feeds: 20 min – Food prep room – [Maull]
    b. Preparation of formulated feeds: 20 min – Wet lab - [Appel]
    c. Egg collection and enumeration methods: 20 min – T7 area – [Rotman]
    d. Assessment of egg quality: 20 min – Ecology Lab/Wet lab – [Stuart]
    e. Reconvene and discuss: 20 min [ALL]

3:00 – 3:15 pm [Break]

4. **Larval Production and Live Feeds Presentations**
    a. Larval production methods
        i. 3:30 - 4:00: HSWRI methods [Rotman/Stuart]
        ii. 4:00 - 4:30: NOAA methods [Cook]

4:30-5:30 pm [Social hour and discussion with or without guest presentation; sponsored by Skretting]

Dinner on your own with suggestions for group

Wednesday, August 26th

b. Live feeds production and enrichment
    i. 8:30 - 9:00: Rotifers and Artemia [Rotman and Maull]
    ii. 9:00 - 9:30: Other specialty foods [Eric Cassiano]
    iii. 9:30 - 10:00: Bacterial management [Rotman]

10:00-10:30 [Break-Discussion-Overrun Buffer]

5. **Larval Production Demos [10:30 – 12:30]** *four stations for 5-6 participants each*
    a. Rotifers: 20 min – [Maull]

b. Artemia: 20 min – [Mauser]
c. Larval Rearing: 20 min – [Stuart]
d. Bacterial Monitoring: 20 min – [Rotman]
e. Reconvene and discuss – 20 min [ALL]

12:30 – 1:30 pm [Lunch]

6. Formulated Feeds

a. 1:30 – 2:00: Weaning approaches and commercial diets [Rotman and Stuart]
b. 2:00-2:30: Manufacturing processes and formulation approaches [Barrows]
c. 2:30-3:00: Moving towards open formulation [Johnson]

3:00 – 3:15 pm [Break]

d. 3:30-4:00: Commercial perspectives on larval feeds and manufacturing [Zimmerman]

7. Larval Fish Nutrition Research

a. 4:00 – 4:30: Delivery methods for water-soluble nutrients [Hawkyard]

4:30-5:30 pm [Social hour and discussion with or without guest presentation; sponsored by Skretting]

Dinner on your own with suggestions for group

Thursday, August 27th

a. 8:30-9:00: Food markers [Cook and Hawkyard]
b. 9:00-9:30: Larval behavior [Lee]
c. 9:30-10:00: Quality considerations [Silbernagel]

10:00-10:30 [Break – Feeder Demonstration]

8. Group discussion and wrap up [Moderated by Stuart and Hawkyard]

a. 10:30 - 11:00: Research gaps in N. American industry
b. 11:00 - 11:30: Potential collaborative efforts
c. 11:30 - 12:00: Summary and Conclusions

Workshop officially over – THANK YOU!

9. Optional [1:00-4:00pm]

a. Field trip to HSWRI WSB hatchery in Carlsbad, CA
b. More detailed or repeated hands-on exercises at San Diego Laboratory