Hatchery Technology for High Quality Juvenile Production

Proceedings of the 40th U.S.-Japan Aquaculture Panel Symposium

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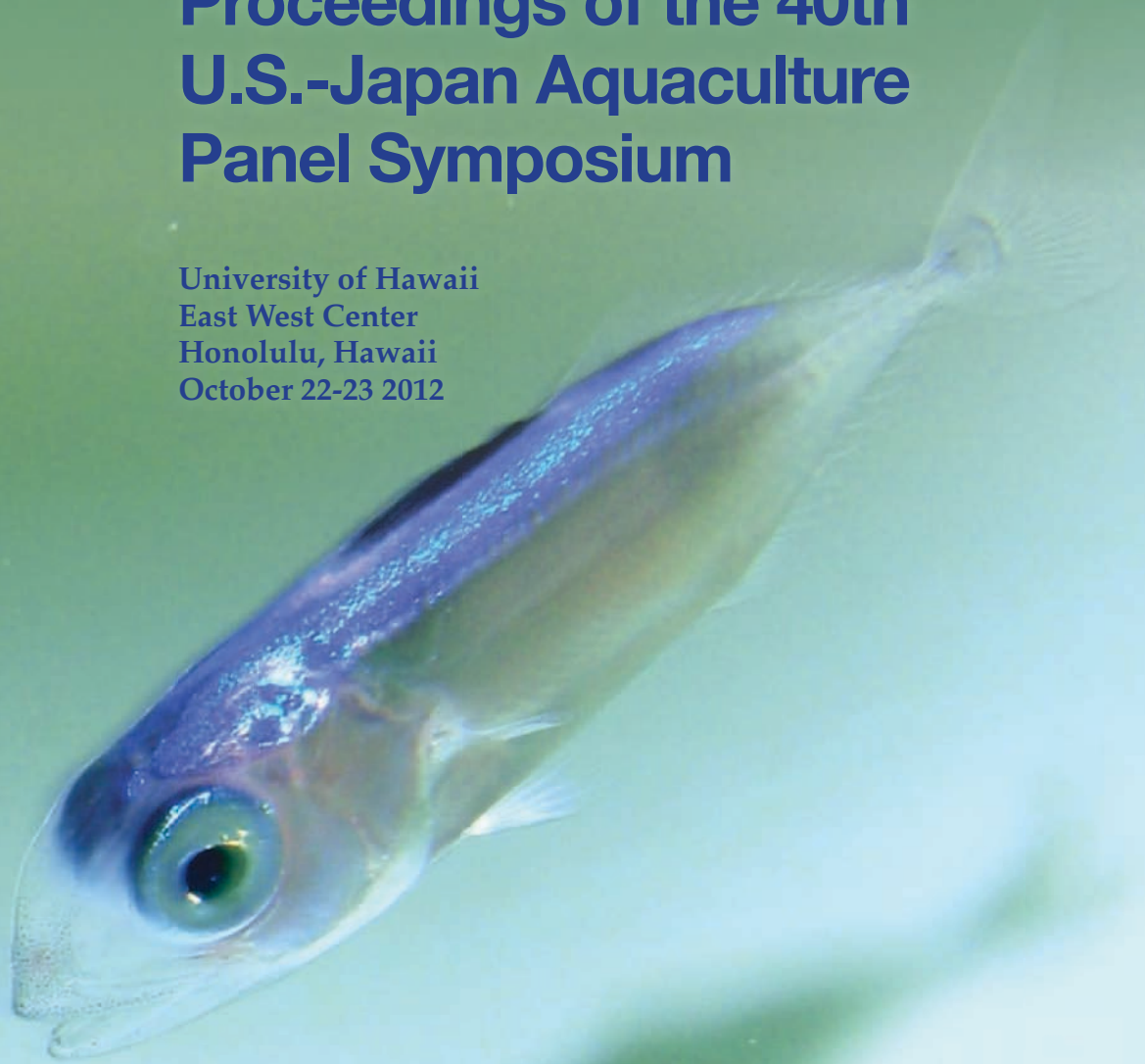**Honolulu, Hawaii**
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Cover photo of juvenile Yellowfin courtesy of Dan Benetti, Aquaculture Program Director at the University of Miami.

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Introduction

The 40th United States-Japan Natural Resources Panel on Aquaculture Science Symposium and these proceedings are dedicated to the strength and compassion the Japanese people exhibited in the aftermath of the tragic earthquake and tsunami that began at 2:46 pm on March 11, 2011. This meeting was postponed for a year as Japan dealt with the devastating impacts in the aftermath. Let me first extend the condolences of the entire U.S. delegation for the tragic loss of life that resulted. We wish Japan and her people a swift recovery as we dedicate this meeting and proceedings to them.

These events and others like them remind us of how uncertain the future is. Though on a much smaller scale, the U.S. has also been dealing with setbacks in its aquaculture sector from uncommon events. Shellfish production in the Gulf of Mexico was still reeling from the impacts of the 2010 Deep Water Horizon oil spill when it was hit by flooding in 2011. Extreme run-off from the Mississippi river lowered salinity enough to kill a large percentage of the recovering shellfish crop. On the U.S. West coast, ocean acidification caused catastrophic die-offs in shellfish hatcheries in the states of Oregon and Washington until it was diagnosed and mitigation measures could be employed. Concurrently, recruitment failures in natural populations of Pacific oysters have been documented over the past seven years; this worrisome phenomenon may also be attributable to ocean acidification.   In finfish aquaculture, drought in the agricultural heart of the United States in 2012 raised feed prices for the catfish, trout, and salmon industries. While these events are minor compared to the tsunami and earthquake in Japan, what all these occurrences have in common is that they are largely out of our control.

One key role of research is to develop information and technology needed in the future. However, as these events illustrate, predicting the future - much less its information and technology needs, - is a formidable task.

Peter F. Drucker, a noted political economist and author (including at least one Japanese anime) is quoted as saying, *"The best way to predict the future is to create it."* I think he is mostly right. I think the future has two parts: the part we create and the part that happens to us. Clearly a part of our future is random, but the story of human progress supports the notion that we do exercise some control, which can have a profound impact in guiding our future.

If you accept this paradigm, then I argue that the better job we do creating the future, the less we are at the mercy of those parts we cannot control. It is our role as scientists to make the best future we can and to be ready for the part of the future we cannot control.

The advances in research and technology we share in these proceedings are great examples of creating our future. Understanding ocean acidification and adaptively managing shellfish hatcheries made it possible to supply oyster seed to farmers in the Pacific Northwest and kept the industry going, despite the failure of natural sets. Even if not explicitly stated, the idea

of creating our future through enhanced knowledge is a common thread among the papers in these proceedings. The cutting-edge technologies discussed here are building a future of increased efficiency, sustainability, and species variety in aquaculture.

Many people contributed to the success of this meeting and the production of the proceedings. I would like to thank our organizing committee, Cheng Sheng Lee, Alan Everson, and U.S. Vice Chair Paul Olin, and especially the one who did most of the work, April Bagwill. Marie Fujitani, April, and Paul also deserve thanks for co-editing the proceedings, as well as Su Kim for creating the document layout.

Michael B. Rust, US Panel Chairman.

Genetic Breeding for Parasite Resistance *(Benedenia seriolae)* Using Genomics Information in Yellowtail *(Seriola quinqueradiata)*

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ABSTRACT

Benedenia disease caused by the ectoparasite *Benedenia seriolae* is a serious parasitic disease in yellowtail aquaculture, leading to secondary viral or bacterial infections. Benedenia disease is difficult to prevent in marine aquaculture systems. To evaluate the genetic basis of Benedenia disease resistance in yellowtail, a genome-wide and chromosome-wide linkage analysis was initiated using F_1 yellowtail families. Two major quantitative trait loci (QTL) regions on linkage groups Squ2 and Squ20 were identified and then confirmed in F_1 families. These QTL regions explained 32.9–35.5% of the phenotypic variance. This is the first genetic evidence detailing phenotypic resistance to Benedenia disease, and the results will help resolve the mechanism of resistance to this important disease of yellowtail.

INTRODUCTION

In Japan, the name yellowtail is commonly used for three species, *Seriola quinqueradiata, S. lalandi* and *S. dumerili*. The combined production of cultured yellowtail in Japan was approximately 152,800 tons in 2009, which accounted for 59% of marine finfish aquaculture in Japan (MAFF of Japan 2009). Yellowtail have been cultured in the southern areas of Japan using juveniles caught in natural waters and sometimes imported from other countries as seedlings. Harvest of wild juveniles has varied from year to year, but in recent years it has declined, likely due to decreasing wild populations (Nakada 2008). Capture-based aquaculture (CBA) is practiced worldwide on a variety of marine species, with important environmental, social and economic implications. A problem of CBA is that it has a negative impact on wild stocks of both targeted species and non-targeted species. Therefore, there has been an increased interest in artificial seedling production. Advances have been made in artificial seedling production of yellowtail (Mushiake et al. 1994; 1998), and it is expected that artificially produced seedlings will eventually replace seedlings caught from wild sources. Although research on disease, nutrition and pond management has supported the development of the yellowtail aquaculture industry, genetic improvement programs leading to improved yellowtail lines are still nascent. Genetic linkage maps play a prominent role in many areas of genetics, including quantitative trait locus (QTL) analysis, marker-assisted selection (MAS), positional candidate or positional cloning of genes approach, and comparative genomics. Linkage maps are essential tools to study QTL. Low resolution genetic linkage maps were first constructed for yellowtail by Ohara et al. (2005), and recently, the second generation maps that span the genome at a higher resolution have been completed (Fuji et al. 2012). The map contains several hundred markers with microsatellites associated with candidate genes. The map will facilitate the genome mapping efforts in *S. quinqueradiata*, and other related species. The mapping data could be compared to reference species and utilized for QTL analyses and further MAS breeding programs of yellowtail.

Benedenia disease, caused by *Benedenia seriolae,* is a serious parasitic disease for yellowtail in aquaculture, and often leads to secondary infection due to viral or bacterial disease. Because fish rub their bodies against the fish cage to remove the parasite, the mortality can be quite high, particularly with juveniles. Although the method of removing the parasite is generally to soak affected fish in a freshwater bath, this method takes a lot of time, cost, and effort. Thus, Benedenia disease is difficult and costly to treat in marine aquaculture systems. In addition, for wildlife conservation, yellowtail aquaculture is considered a potential vector of parasite transmission (Hutson

et al. 2007). Managing these risks to wild stocks is an important consideration for the long-term sustainability of the aquaculture industry.

S. quinqueradiata is generally regarded to have an inherent higher resistance to *B. seriolae* than other kinds of yellowtail (e.g. *S. lalandi* and *S. dumerili*; Nagakura et al. 2006; 2010). Also Nagakura et al. (2010) observed some degree of heritable variation in the levels of infestation among individual *S. quinqueradiata*. These results confirm earlier evidence of genetic variation for susceptibility to *B. seriolae* in *S. quinqueradiata* and suggested that host genes play a significant role in determining infection levels.

Previous genetic studies of parasite resistance include a study of *Myxobolus cerebralis* in rainbow trout, primarily focused on QTL (Baerwald et al. 2011) and a study on *Lepeophtheirus salmonis* in Atlantic salmon focused on candidate genes (Gharbi et al. 2009). Based on QTL and candidate gene expression changes in response to infection, these studies have allowed us to gain insights into potential genes and pathways that may be differentially regulated between resistant and susceptible strains. This is an important step towards understanding host responses to infection. However, much remains to be learned about the genetic basis of the immunological response to parasitic infection, and gene expression studies alone are not capable of discovering the genetic regions that directly contribute to host infection outcomes.

In this study, we analyzed QTLs using wild F_1 strains of *S. quinqueradiata* to elucidate the genetic basis of resistance to Benedenia disease. Following the construction of a high-density linkage map by microsatellite and SNP, we identified two major QTLs contributing to the Benedenia disease infection response of *S. quinqueradiata*. Additionally, we revealed the relationship between QTLs for susceptibility to *B. seriolae* and QTLs for fish body size, and also explored QTLs contributing to variations in growth. The discovery of a large QTL effect for Benedenia disease resistance has broad implications for improving our general understanding of external parasitic diseases and host-pathogen interactions.

MATERIALS AND METHODS
Fish families and samples for QTL analysis
Juvenile *S. quinqueradiata* (100-120mm total length) were captured in coastal waters off of Goto Fukue island (Tsushima Strait, Nagasaki Prefecture). One thousand juveniles were kept in a fish pen for two years. Out of these, two hundred three-year-old fish were pit tagged and the number of *B. seriolae* on each were counted five times. Individuals were assigned to familial groups based on the counts of *B. seriolae* on each individual, and the selected fish were reared to maturity. We prepared two F_1 families for QTL analysis. To evaluate Benedenia disease resistance, artificial infections of *B. seriolae* were performed in about 100 progeny in each F_1 family. For genome-wide linkage analyses, 90 individuals of family A were used for finding candidate QTLs. Then 93 individuals of family B were used to confirm the QTLs found in family A.

Parasite collection and artificial infection experiment
B. seriolae used for artificial infection were collected from adult fish in grow-out fish pens. Mesh nets were hung in the pens containing adult fish parasitized by *B. seriolae*. *B. seriolae* parasitizing adult fish released eggs that then became entangled in the nets for removal. This method has been confirmed to reproducibility yield *B. seriolae* for infection experiments (Nagakura et al. 2010). Collected *B. seriolae* eggs could be induced to hatch by exposure to fluorescent light for fifteen minutes. Hatched *B. seriolae* larvae were kept in a shaded tank before infection experiments. Two days before infection experiments, experimental fish were soaked in a freshwater bath for five minutes to remove any parasites, and transferred to experimental tanks. The two day wait period allowed fish to recover from fresh water stress before artificial infection. Hatched *B. seriolae* larvae were introduced to experimental fish tanks in controlled quantities such that each fish was exposed to two hundred larvae. Water temperature was kept at 25.3-25.6 °C during the infection experiment for 10 days until the larvae reached the adult stage and were large enough to be counted.

Phenotypic measurement of external parasitism resistance
Ten days after exposure to *B. seriolae*, each fish was individually dipped into a freshwater bottle to remove any parasites. We recorded the total number of *B. seriolae* found on each fish. Benedenia disease frequency conformed to a normal phenotypic distribution as shown in Figure 1. Phenotypic information was used as quantitative trait values after linkage analysis. The total number of *B. seriolae* per fish was used as phenotypic information for QTL analysis. The number of parasites infecting individual fish was normally distributed for both families (Shapiro–Wilk test; family A: P=0873; family B: P=0.331), and individual Z-scores were calculated.
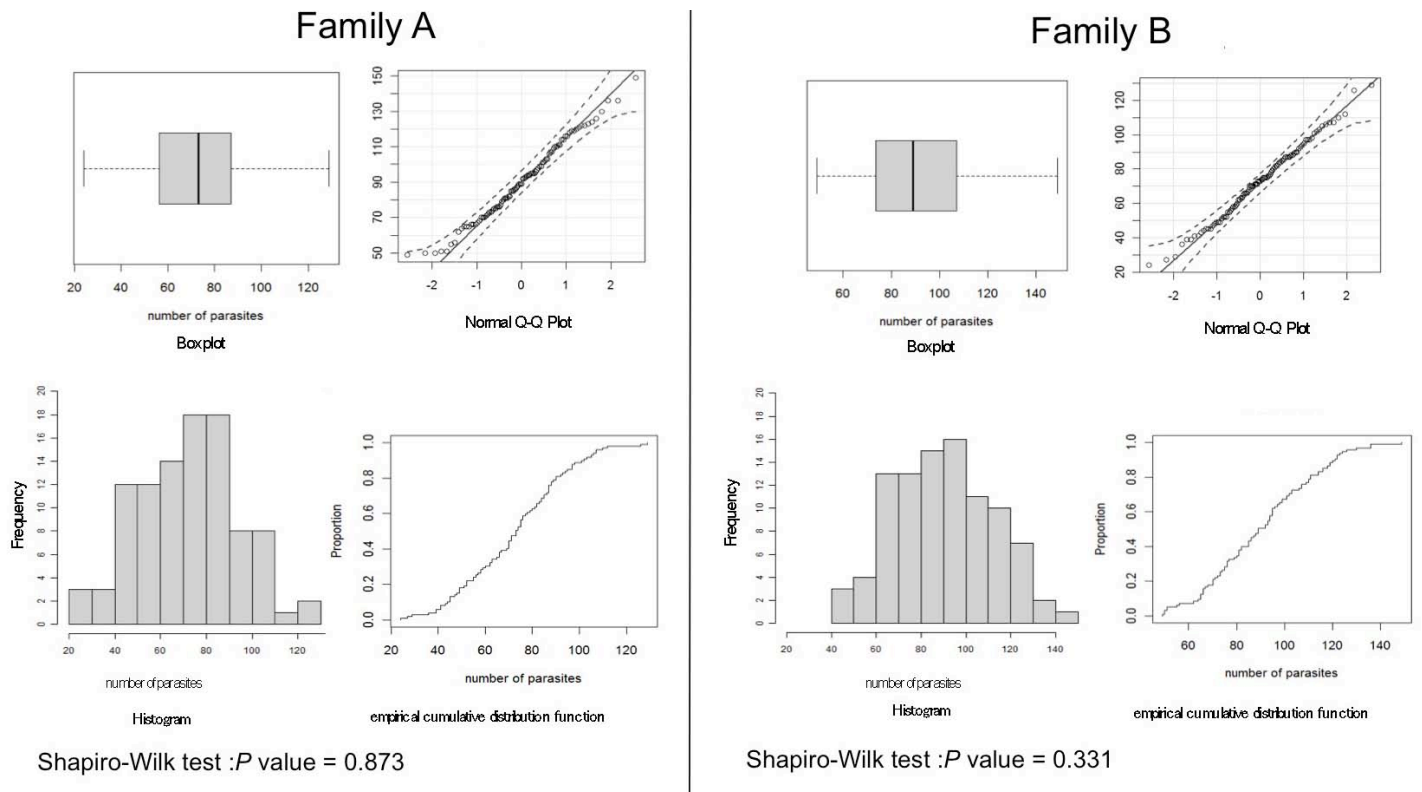
Figure 1. Benedenia disease frequency conformed to a normal phenotypic distribution (Shapiro-Wilk test).

Phenotypic measurement of total length, body length, body weight, and surface area recorded

We measured total length, body length, and body weight for each individual, which conformed to a normal phenotypic distribution as shown in Figure 1. The average length and weight across all F_1 progeny were 185±5mm and 65±10g, respectively, and the surface area was calculated (surface area = 0.109 × 2 × (total length)$^{2.113}$; Ohno et al. 2008). We investigated the correlation of these factors with the number of *B. seriolae* parasites. The phenotypic information on fish size was used as quantitative trait values for genome-wide analysis.

Data collection and genotyping

Microsatellite genotyping was performed in a 10 μl reaction volume containing 0.5 pmol/μl of unlabeled primer, 0.05 pmol/μl of fluorescence-end-labeled primer with [5'-TET], plus 1× buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTP, 1% BSA, 0.025 U of Taq DNA polymerase (Takara: Ex-Taq), and 25 ng of template DNA. Suitable annealing temperatures for each microsatellite marker were used. PCR was performed on a MJ PTC-100 (Bio-Rad, Hercules, CA, USA), and the program conditions were 95 °C for 2 min for initial denaturation, followed by 35 cycles of 30 seconds each at 95 °C, 1 min at the annealing temperature (56–58 °C), 1 min at 72 °C, and 3 min at 72 °C for final extension. Amplification products were mixed with an equal volume of loading buffer [98% formamide, 10 mM EDTA (pH 8.0), 0.05% bromophenol blue], heated for 5 min at 95 °C and then immediately cooled on ice. The mixture was loaded onto a 6% PAGE-PLUS gel (Amresco, Solon, OH, USA) containing 7 M urea and 0.5× TBE buffer. Electrophoresis was performed in 0.5× TBE buffer at 1800 V constant voltage for 1.5 h. After electrophoresis, the gel was scanned and imaged using an FMBIO III Multi-View fluorescence image analyzer (Hitachi-soft, Tokyo, Japan).

Single Nucleotide Polymorphism (SNP) loci from new expressed sequence tag (EST) sequences of *S. quinqueradiata* are known from previous research (Kai et al., unpublished data). To identify the polymorphism on SNP sites, we directly sequenced the PCR products of SNP regions in the P_1 individuals of mapping family A using a Sanger sequencer. The regions of polymorphic SNPs in the P_1 were also sequenced in 90 F_1 progeny of family A.

Genotype scoring was performed by using LINKMFEX ver. 2.3 software package (Danzmann 2006). The software can separate the allele genotypes which come from males or females, and check the accuracy of genotypes in their progeny from parental male and female alleles to avoid genotype scoring errors. Alleles were tested for goodness-of-fit for Mendelian segregation distortion using χ^2-analysis.

The marker loci order was also confirmed to be correct, checked by double recombination events with Map Manager QTX (Manly et al. 2001)

Linkage analysis and QTL detection
A total of 1002 polymorphic markers in 24 linkage groups were used for family A using 860 microsatellite loci and 142 SNP markers. These marker locations and groups likely encompass all chromosomes for this cross, though the *S. quinqueradiata* chromosome number is variable across strains. Nomenclature for the linkage groups was based on chromosome names using the linkage map generated by Fuji et al. (2012). The present map would be useful in some molecular studies for genome-wide linkage analysis, because the average inter-marker distances of both maps (4.8cM and 4.2cM in the female and male maps, respectively) offer sufficient marker density for QTL studies.

Estimation of QTL region of Benedenia disease resistance
QTL analysis was conducted using MapQTL 5 software (Van Ooijen 2004) and R/qtl (Broman et al. 2003; Arends et al. 2010). The frequency distribution of the number of *B. seriolae* on fish bodies conformed to a normal phenotypic distribution, indicating variation in the infection rate. Simple interval mapping was also checked to identify the location of significant LOD max position. Co-factor selection was performed, which is based on backward elimination. Finally, multiple QTL model mapping was applied to detect significant loci excluding ghost QTL (Martinez and Curnow 1992).

Significance thresholds and confidence intervals
Experiment-wide "genome-wide and chromosome-wide" significance thresholds were derived from permutation estimates by dividing the nominal p-value by the total number of chromosomes examined in the study (Churchill and Doerge 1994). Permutation tests were performed (n = 10,000 permutations) to determine the threshold for LOD with type I error rates of $P = 0.05$ and $P = 0.01$. Significant QTLs and regions were graphically visualized using the software MapChart 2.1 (Voorrips 2002) and MapQTL 5 (Van Ooijen 2004). QTL confidence intervals were estimated using a 1.8-LOD support interval with 95% confidence interval probability coverage (Manichaikul et al. 2006).

Estimation of other QTL regions related to total length, body length, body weight, and surface area
QTL analyses for total length, body length, body weight, and surface area were also performed using simple interval mapping, the same method of linkage analysis used to detect QTL regions for Benedenia disease resistance.

RESULTS
Phenotypic trait correlation with fish size and number of parasites *(B. seriolae)*
Pearson correlation coefficients for total length, body length, body weight, surface area, and number of pathogens are shown in Table 1. Weight and length were positively correlated with each other, and to a lesser degree, the number of pathogens and fish sizes were negatively correlated in family A. But in family B, the number of pathogens was marginally ($\alpha = 0.001$) significantly correlated with body size.

Table 1. Pairwise Pearson correlations between fish size and number of parasites.

Family A

	Total length	Body length	Body weight	Surface area	Number of *B. seriolae*
Total length		0.967	0.932	0.999	0.050
Body length			0.947	0.963	0.041
Weight				0.933	0.094
Surface area					0.050
Number of *B. seriolae*					

Family B

	Total length	Body length	Weight	Surface area	Number of *B. seriolae*
Total length		0.964	0.933	0.999	0.389
Body length			0.946	0.964	0.386
Weight				0.935	0.429
Surface area					0.390
Number of *B. seriolae*					

Values in bold are different at a significance level α=0.001

Table 2. Simple interval mapping results of the significant markers for Benedenia disease resistance in linkage group 2, 8, and 20 with two families.

Linkage Group	Locus	Family A		Family B	
		LOD	% Var.	LOD	% Var.
Squ2	Sequ0171BAC	1.61	7.9	NS	
	Sequ0172TUF	2.04	9.9	NS	
	Sequ0672TUF	3.15	14.9	NS	
	Sequ0125TUF	4.15	19.1	NS	
	Sequ1065TUF	4.15	19.1	NS	
	Sequ1295BAC	**4.71**	**21.4**	NS	
	Sequ1066TUF	4.38	20.1	NS	
	Sequ0979BAC	4.38	20.1	NS	
	Sequ1067TUF	3.6	16.8	NS	
	Sequ1068TUF	2	9.7	NS	
	Sequ1069TUF	2.24	10.8	NS	
	Sequ1070TUF	1.74	8.5	NS	
	Sequ0788TUF	1.17	5.8	NS	
Squ8F	Sequ0074TUF	2.17	10.5	NS	
	Sequ0409TUF	2.17	10.5	NS	
	Sequ0431TUF	2.17	10.5	NS	
	Sequ0851TUF	2.17	10.5	NS	
	Sequ0906TUF	2.17	10.5	NS	
	Sequ3175BAC	2.17	10.5	NS	
	Sequ2078BAC	2.17	10.5	NS	
	Sequ1769BAC	2.41	11.6	NS	
	Sequ0670BAC	**2.45**	**11.8**	NS	
	Sequ0503TUF	**2.45**	**11.8**	NS	
	Sequ0610TUF	**2.45**	**11.8**	NS	
	Sequ0101TUF	**2.45**	**11.8**	NS	
	Sequ2536BAC	**2.45**	**11.8**	NS	
	Sequ2198BAC	**2.45**	**11.8**	NS	
	Sequ0985TUF	**2.45**	**11.8**	NS	
	Sequ1013TUF	**2.45**	**11.8**	NS	
	Sequ0507BAC	**2.45**	**11.8**	NS	
	Sequ0575BAC	**2.45**	**11.8**	NS	
	Sequ2218BAC	**2.45**	**11.8**	NS	
	Sequ3193BAC	**2.45**	**11.8**	NS	
	Sequ3288BAC	**2.45**	**11.8**	NS	
	Sequ00955SNP	**2.45**	**11.8**	NS	
	Sequ01036SNP	**2.45**	**11.8**	NS	
	Sequ02608SNP	**2.45**	**11.8**	NS	
	Sequ02777SNP	**2.45**	**11.8**	NS	
Squ20F	Sequ1071TUF	**2.83**	**13.5**	NS	
	Sequ0439TUF	**2.83**	**13.5**	NS	
	Sequ2134BAC	**2.83**	**13.5**	NS	
	Sequ1100BAC	**2.83**	**13.5**	NS	
	Sequ01056SNP	**2.83**	**13.5**	NS	
	Sequ02679SNP	**2.83**	**13.5**	NS	
	Sequ1072TUF	2.51	12	1.91	9
	Sequ3071BAC	2.51	12	**2.24**	**10.5**
	Sequ0938TUF	2.51	12	**2.24**	**10.5**
	Sequ0719TUF	2.19	10.6	1.96	9.3
	Sequ1074TUF	2.19	10.6	1.96	9.3
	Sequ1075TUF	2.19	10.6	1.96	9.3
	Sequ00695SNP	2.19	10.6	1.96	9.3
	Sequ02734SNP	2.19	10.6	1.96	9.3
	Sequ2569BAC	2.19	10.6	1.96	9.3
	Sequ1073TUF	2.19	10.6	1.96	9.3
	Sequ2645BAC	2.19	10.6	NS	
	Sequ1076TUF	2.19	10.6	NS	
	Sequ0537TUF	2.19	10.6	1.66	7.9
	Sequ0596BAC	2.19	10.6	**1.91**	**9**
	Sequ0829TUF	2.19	10.6	**1.91**	**9**
	Sequ0836TUF	2.19	10.6	**1.91**	**9**
	Sequ1989BAC	2.19	10.6	1.48	7.1
	Sequ2312BAC	2.19	10.6	1.48	7.1
	Sequ0017BAC	2.19	10.6	1.39	6.6
	Sequ0730TUF	2.19	10.6	1.39	6.6
	Sequ1077TUF	2.25	10.9	1.39	6.7
	Sequ1078TUF	2.56	12.3	1.39	6.7
	Sequ0808TUF	**2.98**	**14.1**	1.39	6.6
	Sequ1079TUF	**2.98**	**14.1**	1.39	6.6
	Sequ0288TUF	2.17	10.5	1.39	6.6

Locus; marker name, LOD; Lod scores, % Var; percent of variance explained, NS; not significant,

Values in bold are LOD max in Peak of each QTL, marker position and each value.

Simple interval mapping results in family A about all linkage groups and Squ2, Squ8, Squ20 chromosomal-wide in family B

We show the interval mapping results for Benedenia disease resistance in family A for all linkage groups in Figure 2. The results from three significant loci in linkage groups Squ2, Squ8, Squ20 for families A and B are given in Table 2.

Simple interval mapping results for family A detected a QTL (tentatively termed *BDR-1*) in the same chromosomal region in the Squ2 linkage group. The peak LOD value of sequ1295BAC (LOD = 4.71) was substantially higher than the genome-wide LOD significance threshold value of 2.9 determined by permutation testing ($Pg < 0.05$; Pg: P value genome-wide LOD). Linkage groups in Squ2 QTL region (*BDR-1*) were observed as a high single peak of genome-wide LOD significance level ($Pg < 0.001$) in interval mapping (Figure 3-a).
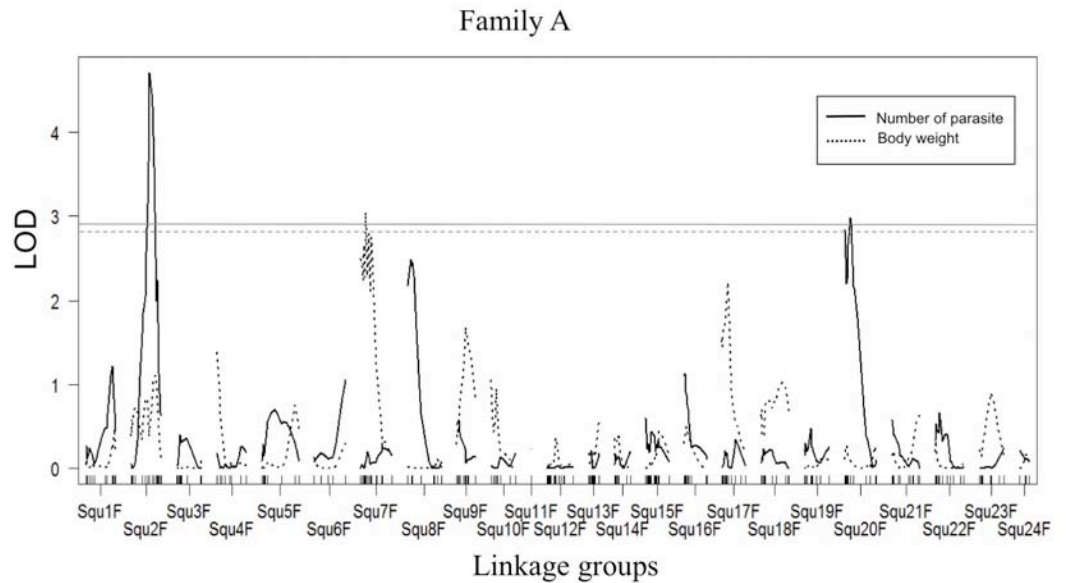
Figure 2. Simple interval mapping results for Benedenia disease resistance and body weight in all linkage groups. This figure was described using by R/qtl. Number of parasites: *Pg* <0.05 significant threshold is indicated as a solid line. Body weight: *Pg* <0.05 significant threshold is indicated as a dotted line.
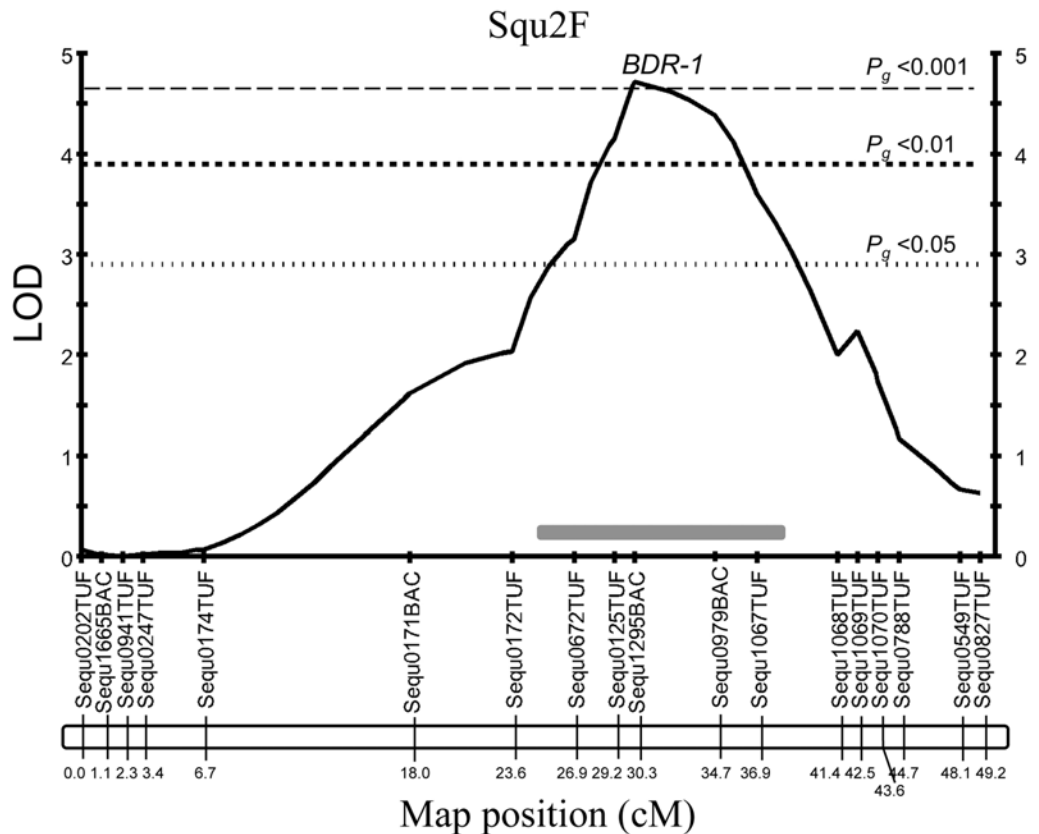
Figure 3-a. Localization of significant markers for Benedenia disease resistance in linkage group Squ2F with family A. Squ(linkage group)F; marker distance in female map, Squ(linkage group)M; marker distance in male map. Map positions and LOD scores (LOD) are based on a simple interval mapping QTL analysis using the software MapQTL 5. Marker absolute map distances are given in (cM). 95% confidence probability LOD support interval was indicated as a gray bold line. Horizontal lines across each plot indicate LOD significance threshold, P_g; genome-wide significance threshold, P_c; chomosome-wide significance threshold. This figure was described using by Map QTL 5.
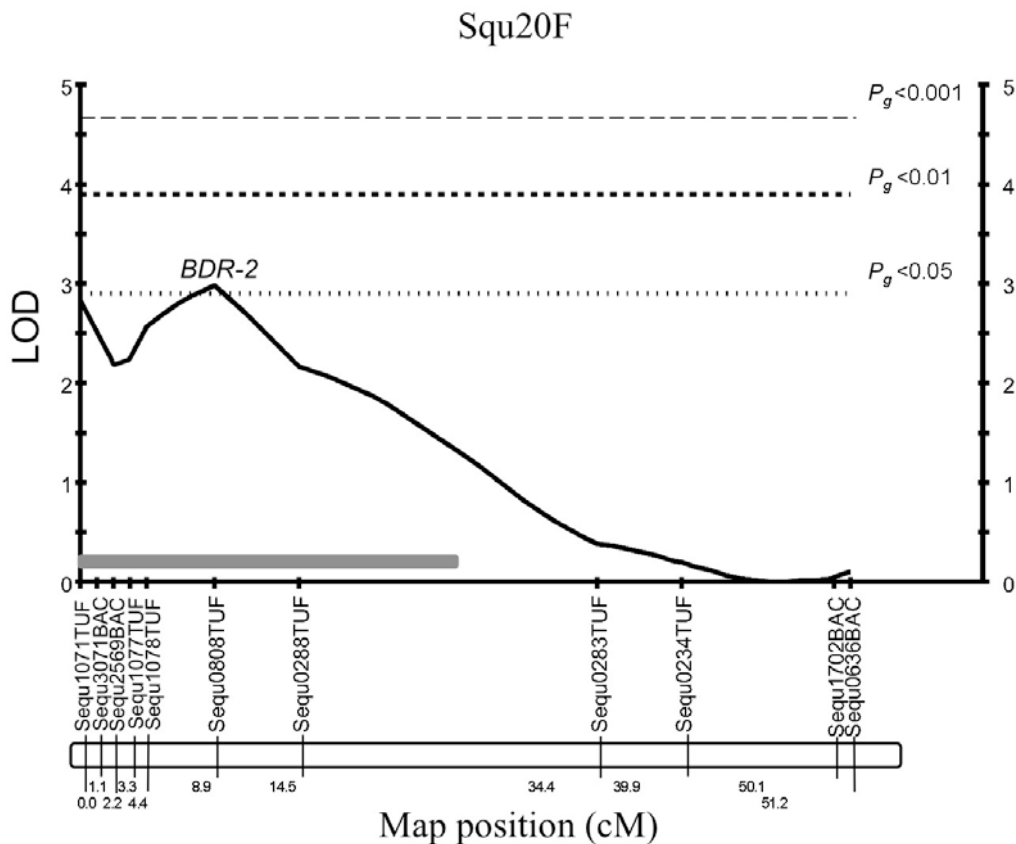
Figure 3-b. Significant markers for Benedenia disease resistance simple interval mapping in linkage group Squ20F with family A.
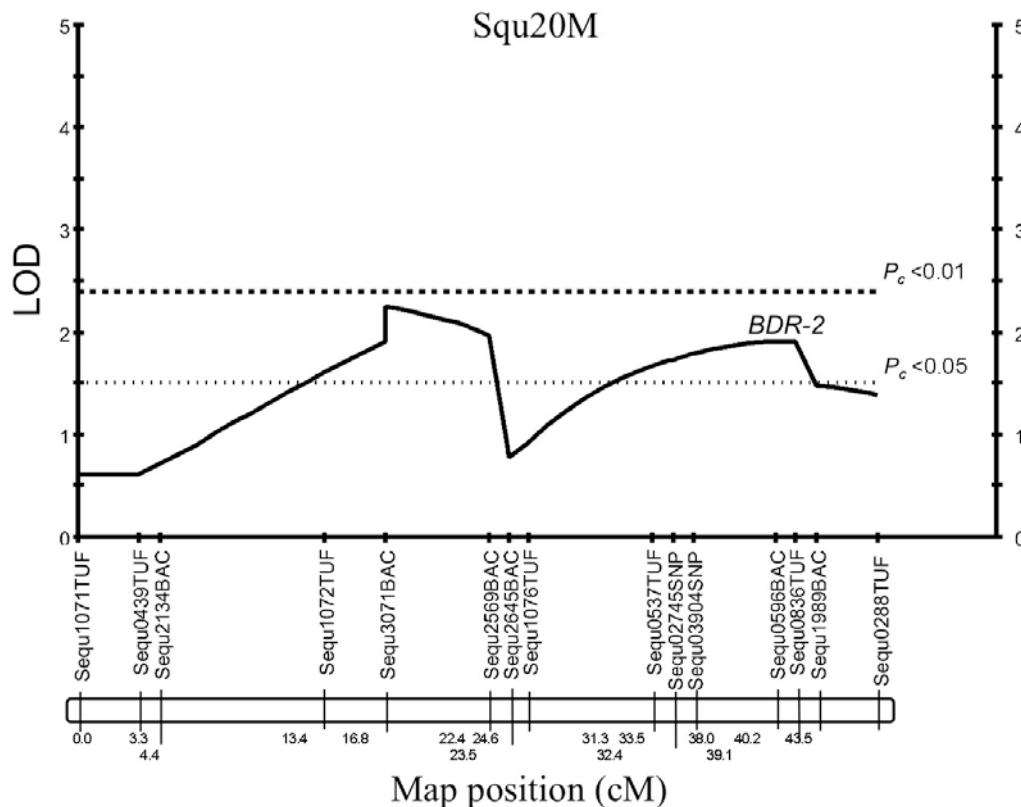
Figure 3-c. Significant markers for Benedenia disease resistance simple interval mapping in linkage group Squ20M with family B.

The markers of chromosomal region of Squ8 linkage group, for example sequ0670BAC (LOD=2.45), was less than the genome-wide LOD significance level ($Pg < 0.05$).

The markers of chromosomal region of Squ20 linkage group in family A, for example Sequ0808TUF (LOD=2.98), had slightly exceeded the genome-wide LOD significance level ($Pg < 0.05$) in family A (Figure 3-b). The candidate QTL region in Squ20 linkage group with LOD score 2.24 in family B exceeded the threshold for a confirmed reproducible family chromosome-wide LOD significance level ($Pc < 0.05$, Pc: P value chromosome-wide LOD) by interval mapping in chromosome Squ20 (Figure 3-c). Results for both families can be seen as two peaks of significance in linkage group Squ20. One of the peaks is tentatively named the *BDR-2* significant region, based on the rules of nomenclature for QTL (Ishikawa 2010; Members of the Complex Trait Consortium 2003).

Cofactor selection and multiple QTL model mapping about significance loci in linkage groups Squ2 and Squ20.

After simple interval mapping co-factor selection was performed. The marker locus sequ1295BAC in peak of value QTL region (*BDR-1*) on linkage group Squ2, and marker locus Sequ1071TUF and Sequ0808TUF in QTL region (*BDR-2*) on linkage group Squ20, were presumed as co-factors. These loci were selected because they were confirmed as highly significant on the genome-wide cutoff level level or produced reproducible results for chromosome-wide significance level in analysis of families.

Multiple QTL model mapping was applied to detect significance loci with the exception of ghost QTL, which is based on backward elimination. Therefore significant regions in linkage group Squ8 (ex. Sequ0670BAC) were rejected as QTL regions in this

step. Map positions and LOD scores were determined from Multiple QTL model analysis using the software MapQTL 5. The results of Multiple QTL model mapping were shown (Table 3) and (Figure 4-a, b). Peak LOD score were higher than simple interval mapping results, for example Sequ1295BAC on Squ2 was indicated as LOD = 5.21, and Sequ0808TUFon Squ20 was indicated as LOD=3.47. However, the marker locus Sequ1071TUF (LOD 2.89) was less than the genome-wide LOD significance level ($Pg < 0.05$) in the edge of Squ20 linkage group. In the results in multiple QTL model test, the interaction of Squ2 region and Squ20 were estimated as independent QTL effects.

Table 3. Multiple QTL model mapping results of the significant markers for Benedenia disease resistance in linkage group 2 and 20 in family A.

Linkage Group	Locus	LOD	% Var.
		Family A	
Squ2F	Sequ1065TUF	4.75	18.5
Squ2F	Sequ1295BAC	**5.21**	**20.1**
Squ20F	Sequ1071TUF	**2.89**	**10.8**
Squ20F	Sequ1072TUF	2.66	10
Squ20F	Sequ3071BAC	2.66	10
Squ20F	Sequ0938TUF	2.66	10
Squ20F	Sequ2569BAC	2.5	9.4
Squ20F	Sequ0719TUF	2.5	9.4
Squ20F	Sequ1074TUF	2.5	9.4
Squ20F	Sequ1075TUF	2.5	9.4
Squ20F	Sequ00695SNP	2.5	9.4
Squ20F	Sequ02734SNP	2.5	9.4
Squ20F	Sequ1073TUF	2.5	9.4
Squ20F	Sequ2645BAC	2.5	9.4
Squ20F	Sequ1076TUF	2.5	9.4
Squ20F	Sequ0537TUF	2.5	9.4
Squ20F	Sequ0596BAC	2.5	9.4
Squ20F	Sequ0829TUF	2.5	9.4
Squ20F	Sequ0836TUF	2.5	9.4
Squ20F	Sequ1989BAC	2.5	9.4
Squ20F	Sequ2312BAC	2.5	9.4
Squ20F	Sequ0017BAC	2.5	9.4
Squ20F	Sequ0730TUF	2.5	9.4
Squ20F	Sequ1077TUF	2.43	9.2
Squ20F	Sequ1078TUF	2.66	10
Squ20F	Sequ0808TUF	**3.47**	**12.8**
Squ20F	Sequ1079TUF	3.47	12.8
Squ20F	Sequ0288TUF	2.61	9.8

Locus; marker name, LOD; Lod scores, % Var; percent of variance explained,

Values in bold are LOD max in Peak of each QTL marker position and each value.

Figure 4-a. Significant markers for Benedenia disease resistance multiple-QTL model mapping in linkage group Squ2F with family A. This figure was described using by MapQTL 5.

Figure 4-b. Significant markers for Benedenia disease resistance multiple-QTL model mapping in linkage group Squ20F with family A.
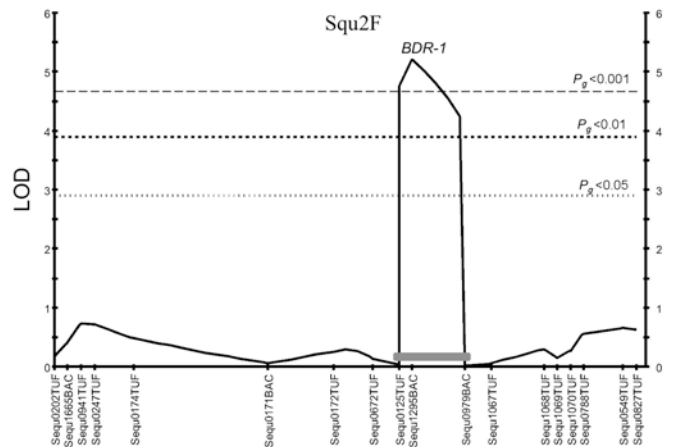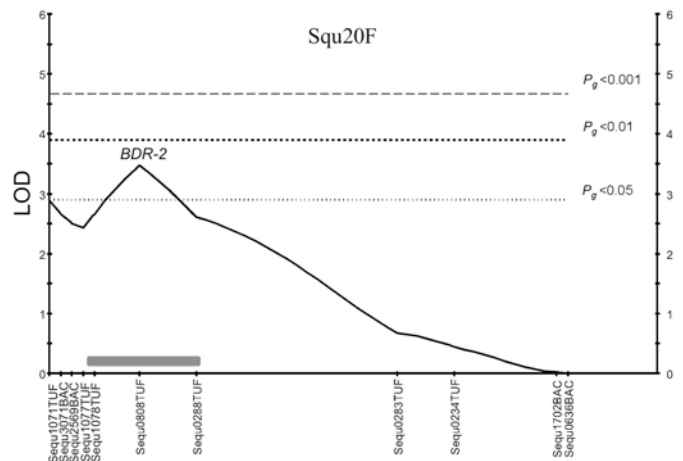
Percentage of phenotypic variance explained by candidate QTL region

Each of the LOD peaks, Squ2 (Sequ1295BAC), Squ8 (Sequ0670BAC), Squ20 (Sequ1071TUF), and Squ20 (Sequ0808TUF), can explain phenotypic variance ranging from 11.8 to 21.4% by simple interval mapping. When LOD peaks were combined into simple interval mapping results, two loci (Sequ1295BAC; Squ2, Sequ0808T TUF; Squ20) could explain 35.5% of phenotypic variance. If the LOD peak of Squ20 (Sequ1071TUF) and Squ8 (Sequ0670BAC) are added, these four loci were responsible for 60.8% of the total phenotypic variation in family A. The remaining two loci (Sequ1295BAC; Squ2, Sequ0808T TUF; Squ20) could explain 32.9% of phenotypic variance .

Linkage analysis estimation of QTL region for fish size.

We show interval mapping results for body weight QTL in family A for all linkage groups in Figure 4. Significant loci involving fish size (total length, body length, body weight, surface area) in linkage groups Squ7 and Squ17 are shown in Table 4. The markers of chromosomal region Squ7 linkage group, for example Sequ0582TUF involving body weight, had values exceeding (LOD=3.04) the genome-wide LOD significance level (LOD 2.8, $Pg < 0.05$) in family A (Figure 5). The region of LOD maximum locus (Sequ0582TUF) could explain 14.4% of phenotypic variance of of body weight. Taking size measurements into account with QTL candidate regions in these family analysis results, the number of pathogens were negatively correlated with fish sizes in agreement with the previous analysis of correlation coefficients.

Table 4. Simple interval mapping results of the fish sizes in linkage group 7 and 17 in family A.

Linkage Group	Locus	total length LOD	total length % Var.	body length LOD	body length % Var.	body weight LOD	body weight % Var.	surface area LOD	surface area % Var.
Squ7F	Sequ1041TUF	2.46	11.8	2.62	12.6	2.5	12	2.46	11.8
Squ7F	Sequ1041BAC	2.65	12.7	2.67	12.8	2.43	11.7	2.65	12.7
Squ7F	Sequ3208BAC	2.06	10	2.5	12	2.24	10.8	2.06	10
Squ7F	Sequ0447TUF	2.48	11.9	2.9	13.8	2.66	12.7	2.48	11.9
Squ7F	Sequ0582TUF	**2.57**	**12.3**	**2.92**	**13.9**	**3.04**	**14.4**	**2.57**	**12.3**
Squ7F	Sequ0623TUF	2.12	10.3	2.22	10.7	2.27	11	2.12	10.3
Squ7F	Sequ2990BAC	2.5	12	2.62	12.5	2.78	13.3	2.5	12
Squ7F	Sequ00662SNP	1.83	8.9	2.02	9.8	2.1	10.2	1.83	8.9
Squ7F	Sequ0416TUF	2.49	12	2.72	13	2.74	13.1	2.49	12
Squ7F	Sequ0784TUF	1.39	6.8	1.62	7.9	1.22	6.1	1.39	6.8
Squ7F	Sequ1421BAC	1.23	6.1	1.47	7.2	1.15	5.7	1.23	6.1
Squ7F	Sequ0382BAC	0.65	3.3	0.81	4.1	0.48	2.5	0.65	3.3
Squ7F	Sequ1628BAC	0.44	2.2	0.53	2.7	0.27	1.4	0.44	2.2
Squ7F	Sequ00355_1SNP	0.52	2.6	0.66	3.3	0.34	1.7	0.52	2.6
Squ7F	Sequ1700BAC	0.44	2.2	0.45	2.3	0.15	0.8	0.44	2.2
Squ17F	Sequ1016TUF	1.3	6.4	1.84	9	1.53	7.6	1.3	6.4
Squ17F	Sequ0025TUF	1.1	5.5	1.68	8.2	1.46	7.2	1.1	5.5
Squ17F	Sequ0895TUF	1.3	6.4	1.82	8.9	1.68	8.3	1.3	6.4
Squ17F	Sequ0228TUF	1.35	6.7	1.78	8.7	1.7	8.3	1.35	6.7
Squ17F	Sequ3088BAC	1.59	7.8	1.94	9.4	1.78	8.7	1.59	7.8
Squ17F	Sequ1690BAC	1.73	8.5	2.15	10.4	2.01	9.8	1.73	8.5
Squ17F	Sequ01964SNP	**1.79**	**8.8**	**2.3**	**11.1**	**2.22**	**10.8**	**1.79**	**8.8**
Squ17F	Sequ0716TUF	0.86	4.3	1.05	5.2	0.92	4.6	0.86	4.3
Squ17F	Sequ2051BAC	0.45	2.3	0.6	3	0.56	2.8	0.45	2.3
Squ17F	Sequ2269BAC	0.12	0.6	0.18	0.9	0.34	1.7	0.12	0.6
Squ17F	Sequ2942BAC	0.02	0.1	0.02	0.1	0.16	0.8	0.02	0.1

Locus; marker name, LOD; Lod scores, % Var; percent of variance explained, Values in bold are LOD max in Peak of each QTL, marker position, and each value.

DISCUSSION

This study is the first evidence to report the detection and positioning of major loci affecting resistance to external parasites in yellowtail. We identified in yellowtail two chromosomal regions containing QTL (*BDR-1, BDR-2*) that were associated with Benedenia disease resistance. Two putative QTL associations, with medium to large effect on Benedenia disease resistance, were localized to linkage groups Squ2 and Squ20.

On Squ2, simple interval and multiple model interval mapping results indicate the example marker loci Sequ1295BACcan explain 20.1 to 21.4% of phenotypic variation. On Squ20, both mapping methods show the example marker loci Sequ0808TUF can explain 12.8 to 14.1% of phenotypic variation. These two loci were responsible for from 32.9 to 35.5% of the total phenotypic variation in family A. If the marker Sequ1071TUF on the tips of Squ20 can be considered as significant loci, they explain 10.8% to 13.5% of phenotypic variation. In addition, suggestive level markers Sequ0670BAC on Squ8 can explain the phenotypic variance of about 11.8%. In total, these four
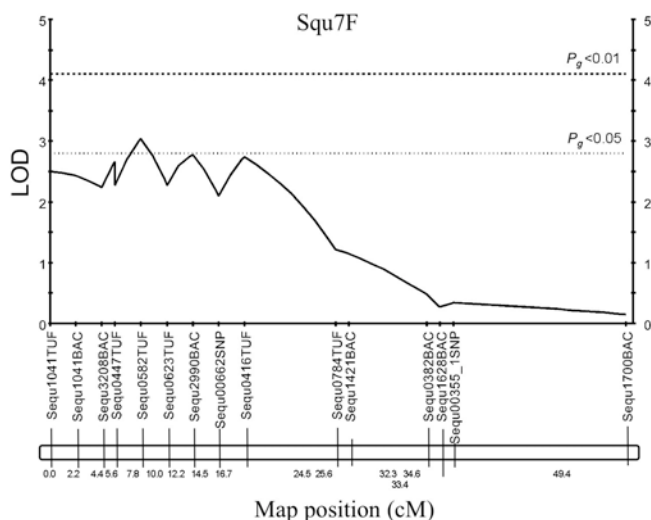

Figure 5. Significant markers for body weight simple interval mapping in linkage group Squ7F with family A. This figure was described using by MapQTL 5.

loci can explain 55.5% to 60.8% of phenotypic variation in family A. However, the latter two additional QTL were only significant at the chromosome-wide level and should be regarded as tentative until other family results are confirmed.

Family B was not used in the main genome-wide analysis. The number of pathogens was marginally significantly correlated with fish sizes. Family B was analyzed in limited linkage groups with chromosome-wide significance levels to confirm that the candidate QTL regions are reproducible. Candidate QTL regions in the Squ20 linkage group were confirmed to have a significant value in family B. However, a highly significant region in family A in linkage group Squ2 were rejected as significant in family B. A suggestive region in family A on linkage group Squ8 was also rejected in family B. We speculate that this is due to differences in the P_1 individuals of family B compared to family A, as the F1 progeny of family A showed much susceptibility to the parasite than the F1 generation of family A (Figure 6). Further, from cofactor selection and multiple QTL model results in family A, a QTL epistasis is thought to exist between the Squ2 region and the Squ8 region (data not shown).

The most important finding of this study was a single peak of QTL (*BDR-1*) associated with Benedenia disease resistance within the proximal region of linkage group Squ2. The QTL peak (Sequ1295BAC) was located at position 30.3 cM, with a 95% confidence interval that the QTL region lies within 10 cM of the most proximal marker from Sequ0672TUF to Sequ1067TUF. The 10 cM QTL confidence interval is narrow and should be considered a fine approximation, given the large QTL effect and high recombination rate found in yellowtail females. The QTL peak marker Sequ1295BAC was isolated from BAC library end sequencing (Fuji et al. 2012), and adjacent markers Sequ0979BAC were also isolated from BAC library end sequencing. These physical sequences give rise to the possibility of identifying positional candidate genes or positional cloning of genes for resistance of external parasitism. But before determining initiative sequences of contignation from BAC libraries, it would be beneficial to further refine the QTL region by increasing the marker density around the QTL peak with EST based SNP markers. It is also necessary to map additional families using near-isogenic lines, which are separated from other QTL effects on other linkage groups, and successive generations with larger progeny sizes to increase the total recombination events. Future QTL studies in yellowtail should focus on fine-mapping the QTL identified on Squ2 as well as searching for additional QTL on other linkage groups.

On the other hand, the QTL (*BDR-2*) significant region would contain multiple QTL on Squ20, because results of A and B families are detected as two peaks around the significant region in linkage group Squ20. In addition, this QTL significant region was confirmed with Benedenia disease resistance in hybrid lines between *S. quinqueradiata* and *S. lalandi* (Kubota et al. 2009) in yellowtail. Therefore, it is possible that this linkage group is enriched with external parasitism disease resistance genes. However, without a sequenced genome, this is highly speculative.

Information on genetic variation will contribute to our understanding of the Benedenia resistance phenotype in different families or even across species. Finding this QTL region lends support to the potential success of marker-assisted selection (MAS) for this disease. However, it
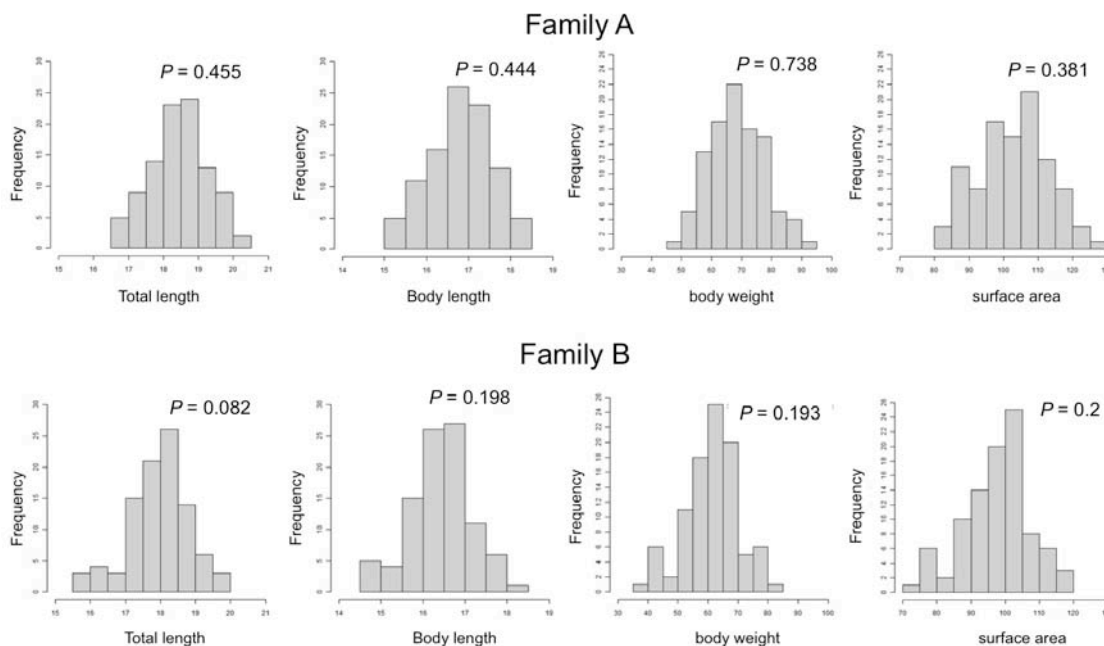

Figure 6. Fish Total length, body length, body weight and surface area conformed normal to a phenotypic distribution (Shapiro-Wilk test)

is difficult to evaluate each QTL effect separate from these two peaks. Utilization of this QTL has to pay attention to the broad region on Squ20, which should be integrated into the next generation for MAS.

Our results suggest that fish size is not responsible for the resistance of *B. seriolae* in yellowtail, because the number of parasites and fish size were negatively correlated in pairwise Pearson correlation factors with family A. Although we cannot exclude the possibility that multiple fish growth loci are present within the currently identified *B. seriolae* resistance QTL region in Squ2 and Squ20, the results of significant loci concerning fish size (total length, body length, body weight, surface area) were identified in different linkage groups in Squ7 by the three month old stage. The results from our current study revealed a negative correlation between growth traits and the number of parasites. However, given the effect of the discovered QTL region on the Benedenia disease phenotype, it is unlikely that growth has a major role in conferring parasite disease resistance. In the case of whirling disease, caused by the pathogen *Myxobolus cerebralis*, fish age and size were found to be key factors influencing the severity of whirling disease in experimentally infected rainbow trout (Ryce et al. 2004; 2005). In this disease, rainbow trout growing at faster rates may more rapidly become resistant to both clinical signs of whirling disease and high numbers of myxospores in their skeletal elements. In our study, Fish size QTL were identified for different linkage groups in Squ7. It might be necessary to measure the phenotypic differences in various development stages by time series analysis in order to identify growth QTLs.

Sex was not directly correlated with *B. seriolae* in the fish used in this study. But sex-linked markers have already been identified on the linkage group Squ12 within several families (Fuji et al. 2010). Our results suggested that there are no differences between males and females. This result is similar to the QTL study on *Lepeophtheirus salmonis* in Atlantic salmon (Gharbi et al. 2009). No differences were observed between males and females, although fish size, which is known to vary between sex has been found to be an important factor in determining lice abundance (Glover et al. 2005).

In this study we explored the potential of MAS and marker-assisted introgression (MAI) for disease risk management of marine aquaculture. Wild aquatic species are not selected and still maintain high genetic diversities. Individuals have high potential for genetic breeding with large pools of available phenotypic variation. Natural populations will be more appropriate to contribute to those genetic resources and maintain large QTL effects than strain populations for supplies of fingerlings for aquaculture.

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The sex-determining system of yellowtail (Japanese amberjack), *Seriola quinqueradiata*, is not known. In this study, we identified the sex-linked locus in yellowtail and we characterized the sex-determining system by genetic linkage analysis conducted on 19 female and 19 male progenies from a single family. The associations between phenotypic sex and genotypic data of 71 microsatellite markers selected from yellowtail genetic linkage map were tested. The putative sex-determining locus is located between locus Sequ21 and locus Sequ17 in LG12, and the sex-linked alleles were inherited from the female parent. This result suggests that yellowtail has a ZZ–ZW sex-determining system, and that it would be possible to use these sex-linked markers to discriminate the sexes.

Kanako Fuji, Takashi Koyama, Wataru Kai, Satoshi Kubota, Kazunori Yoshida, Akiyuki Ozaki, Junya Aoki, Yumi Kawabata, Kazuo Araki, Tatsuo Tsuzaki, Nobuaki Okamoto, and Takashi Sakamoto (2013). Construction of a high-coverage bacterial artificial chromosome library and comprehensive genetic linkage map of yellowtail (*Seriola quinqueradiata*). *BMC Genomics: submitting*

The Japanese amberjack/yellowtail (*Seriola quinqueradiata)* is a popular marine cultured fish in Japan. Presently, the growing fish pens are stocked with wild-caught juveniles but using cultured brood fish for seed would have less environmental impact. Information on genetic markers and genomic large insert libraries associated with QTL can be used to select individuals carrying desired traits for breeding programs and to identify causative genes. In this study, we prepared a BAC library and present the female and male linkage maps of yellowtail with microsatellite markers obtained from BAC-end sequences (BESs) and a yellowtail genomic library. The BAC library consists of 110,592 clones with an average insert size of 140.7 kb, representing a 16-fold coverage of the genome. From 5,324 BESs, 743 primer pairs were designed to amplify a mapping panel and 373 primer pairs were identified. A total of 464 microsatellite markers derived from a yellowtail genomic library were also mapped. These 837 markers were mapped on female and male maps in 24 linkage groups. The difference of recombination rates between the female and male maps was very small (Female:Male=0.99:1). All linkage groups in the maps indicated that the locations of the sex-specific recombination hot-spots were very different in males and females. Female and male linkage maps of yellowtail were constructed using BAC-clones information. After a genomic region has been shown to be linked to trait of economic importance, this high quality BAC library resource and mapped BAC clones information are crucial in the identification and functional characterization of the genetic variation.

Ozaki A, Yoshida K, Fuji K, Kubota S, Kai W, Suzuki J, Akita K, Koyama T, Aoki J, Kawabata Y, Nakagawa M, Hotta T, Tsuzaki T, Okamoto N, Araki K, Sakamoto T (2013). Q Quantitative Trait Loci (QTL) Associated with Resistance to a Monogenean Parasite (Benedenia seriolae) in Yellowtail (*Seriola quinqueradiata*) through Genome Wide Analysis. *PLOS ONE* 8: 6, e64987

Benedenia disease caused by the ectoparasite *Benedenia seriolae* is a serious parasitic disease in marine aquaculture finfish, leading to secondary viral or bacterial infections. Because fish rub their bodies against the fish cage to remove the parasite, the mortality is quite high especially in juveniles. Also the method to remove requires a lot of time, cost and effort. Benedenia disease is difficult to prevent in marine aquaculture systems. Genetic variation has been indicated to play a significant role to determining the susceptibility to this parasitic disease, however, the mechanisms involved in the differential response to infection remain poorly understood. To evaluate the genetic basis of Benedenia disease resistance in yellowtail (*Seriola quinqueradiata*), a genome-wide and chromosome-wide linkage analysis was initiated using F1 yellowtail families. Two major quantitative trait loci (QTL) regions on linkage groups Squ2 and Squ20 were identified and then confirmed in F1 families. These QTL regions explained 32.9– 35.5% of the phenotypic variance. On the other hand, the QTL related to growth was found in other linkage groups (Squ7). Therefore, Benedenia disease resistance QTL was not correlated with fish size. At the results, we have discovered the first genetic evidence that contributes to detailing the phenotypic resistance to Benedenia disease, and the results will help resolve the mechanism of resistance to this important disease of yellowtail.

Pre-Spawning Carotenoid Fortified Diets Improve Reproductive Traits of Channel Catfish, *Ictalurus punctatus* and Subsequent Performance of the Progeny

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Keywords: carotenoids, broodfish diets, channel catfish, egg quality, progeny performance

ABSTRACT

Availability of a consistent number of ovulatory competent channel catfish females is a pre-requisite for efficient production of channel catfish, *Ictalurus punctatus* ♀ x blue catfish, *I. furcatus* ♂ F$_1$ hybrid in hatcheries. Raising hybrid catfish in production ponds enables the catfish farmer to harness improved growth rates, survival, and feed conversion. Production of hybrid catfish embryo production involves hormone-induced spawning of channel catfish, fertilization of stripped eggs with blue catfish sperm, and hatching of fertilized eggs in troughs, similar to channel catfish. High quality broodstock maturation diets are an essential key for successful and sustainable production of hybrid catfish fry from hatcheries. Carotenoid fortification in brood fish diets has been suggested to improve maturation and egg quality in aquatic animals.

An 8-week pre-spawning broodfish nutrition study was conducted 2 months prior to spawning in twenty 1.5 m diameter, 760 L plastic tanks supplied with recirculated pond water and continuous aeration. Four carotenoid fortified diets were prepared by mixing the required quantity of carotenoid in water and sprayed on a 35% protein commercial catfish feed in a concrete mixer, followed by a coating of menhaden oil. The carotenoid treatments were: 1) 50 mg/kg astaxanthin; 2) 100 mg/kg astaxanthin; 3) 25 mg/kg lutein and 25 mg/kg zeaxanthin; 4) 50 mg/kg lutein and 50 mg/kg zeaxanthin and 5) control diet that was sprayed with the same quantity of water and oil. Four tanks were randomly allocated to each treatment. Brood fish were fed with respective feed three times a week to satiation.

Broodfish fed with 100 mg/kg astaxanthin fortified feed had a higher (P < 0.05) percentage of gravid females suitable for hormone injection, gonadosomatic index, and percent ovulation compared to other treatment groups. Gravid females from all 5 treatments were subjected to hormone-induced spawning procedures to produce hybrid catfish fry in two spawning trials. Relative fecundity, percent fertilization, percent hatch and fry produced per kg female body weight (BW) did not differ (P > 0.05) among treatments. Progeny derived from females fed a diet fortified with 100 mg/kg astaxanthin had higher (P < 0.05) resistance to *Edwardsiella ictaluri* (cause of enteric septicemia) disease challenge. These fish also demonstrated reduced stress response (cortisol levels) to low dissolved oxygen under controlled conditions. However, maternal feeding of carotenoids did not invoke any growth advantage to the progeny.

The results of the present study suggest a pre-spawning diet supplemented with 100 mg/kg astaxanthin improves maturation, gonadal mass, and enhances physiological response to induced spawning in channel catfish broodfish.

INTRODUCTION

In 2012, the U. N. Food and Agriculture Organization (FAO) reported that global production of fish, crustaceans, mollusks and other aquatic animals reached 157 million tons. While, wild fish harvest has remained around 90 million tons since 1991. Farm-raised or aquaculture production has continued to increase from 34.6 million tons in 2001 to 67 million tons in 2012. Presently, about 600 aquatic species are cultured worldwide in 190 countries with facilities of varying intensities and technological sophistication. The demand for quality and large quantities of fry and fingerlings produced for aquaculture production is high. Hence, complete control over the reproductive cycle of fish is critical for efficient and greater aquaculture production.

Channel catfish, *Ictalurus punctatus* – a leading US aquacultured species has been declining in production since 2003 (USDA NASS, 2012). This decline in production is attributed to high feed costs, competition from lower-priced imports, and economically attractive land-use alternatives. The

possibility of improved production traits resulting from hybridization of channel catfish with blue catfish has long been recognized (Dunham et al. 1990), but inefficiencies in hybrid fry production stemming from inconsistencies in egg quality and suboptimal hatching conditions still exist. Another limiting factor for consistent and large scale hatchery production of hybrid catfish is the availability of mature broodstock. Acceleration and synchronization of maturation in channel catfish is needed for higher hatchery production of hybrid catfish. Egg quality is influenced by broodfish nutrition, husbandry, and environmental factors. Supplementing nutrients to broodfish diet may provide a practical means of improving egg quality.

Maturation is a process of conditioning broodstock to stimulate gonad development to induce spawning, and facilitate stripped eggs to produce viable offspring (Juarez et al. 2003). Reproduction in fish, as in other vertebrates, is affected by environmental, social, and nutritional factors. The effects of food ration size and nutrient composition of the diets on reproduction have been investigated in several important aquaculture species (Bromage, 1995; Hardy, 1985). The biochemical composition of the ovary reflects the typical nutrient accumulation associated with maturation. The influence of nutrient availability on reproductive physiology and broodstock performance in fish has been reviewed previously (Hardy, 1985; Bromage, 1995; Izquierdo et al. 2001; Pavlov et al. 2004). These studies investigated the effects of nutrient supplements including polyunsaturated fatty acids, vitamins C and E, and the carotenoid pigments. Salze et al. (2005) suggested the differences in egg quality between wild and farmed broodstock are due to differences in carotenoid pigments in oocytes (Salze et al. 2005).

Past studies by Grung et al. (1993); Latscha (1991) and Whyte et al. (1998) have suggested carotenoids be included as an essential nutritional additive during gonadal maturation of fish. Aquatic animals, including shrimp are unable to produce astaxanthin (or any carotenoid) *de novo.* They obtain carotenoids from microalgae or microcrustaceans and take up the corresponding (3S, 3'S) and (3R, 3'R) isomers (Whyte et al. 1998). The majority of astaxanthin within the epidermal tissue is in a mono-esterified form. Carotenoid and protein complexes are combined to form carotenoproteins and are present in exoskeleton (Howell and Matthews, 1991). Carotenoids are synthesized through the isoprenoid pathway similar to the production of diverse compounds such as essential fatty acids, steroids, and vitamins A, D, E, and K.

Among the various classes of natural pigments, the carotenoids are the most widespread and structurally diverse pigmenting agents. In combination with proteins, they are responsible for brilliant yellow to red colors in plants, and the wide range of blue, green, purple, brown, and reddish colors of fish and crustaceans. The general distribution and metabolic pathways of carotenoids has been provided in Figure 1 (Latscha, 1991).

To optimize brood fish maturation procedures and achieve successful and sustainable hatchery production of channel x blue hybrid catfish fry, there is a need for high quality maturation diets. This study evaluates the effect of pre-spawning carotenoid fortified diets to improve maturity of channel catfish amenable for hormone induced spawning in the production of channel catfish female x blue catfish male F1 hybrid production in controlled hatchery conditions.
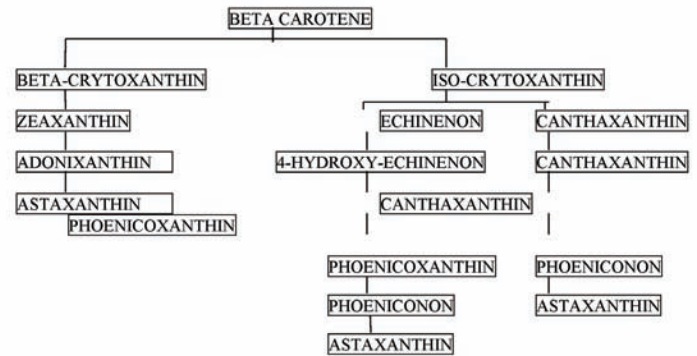
Figure 1. General distribution and metabolic pathways of carotenoids (Latscha, 1991)

MATERIALS AND METHODS

Fish and Facilities: Approximately 4-year old 200 mature female channel catfish of the 'Delta' strain were used in the study. These fish were hand selected based on superior secondary sexual characteristics and stocked in twenty 1.5 m diameter 760 L plastic tanks on February 18, 2011 at the Thad Cochran National Warmwater Aquaculture Center pond facility, Stoneville, Mississippi, USA. Ten fish were randomly stocked in a tank that was supplied with continuously recirculated pond water and diffused air from an air stone supplied with a regenerative blower (water temperature 21.8 to 24.7°C, pH 8.1 and dissolved oxygen > 6.2 mg/L). Brood fish were acclimated for two weeks in tanks and were fed with 35% protein commercial catfish feed three times weekly to satiation.

Diets: Five broodfish diets were prepared with three synthetic carotenoids: Carophyll Pink (10% active concentration of astaxanthin), luten (5%

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active concentration), and zeaxanthin (5% active concentration) (DSM Nutritional Products, Switzerland). Diets were prepared by spraying the required quantity of synthetic carotenoids on 35% protein commercial catfish feed (Delta Western, Indianola, MS, USA). The synthetic carotenoid was weighed accurately and mixed in 500 mL of distilled water for 30 minutes on a shaker and the resulting mixture was sprayed on 25 kg of feed in a mini electric concrete blender for 15 minutes, followed by a spraying of 500 mL of menhaden oil for 10 minutes. Four broodfish diets either contained 1) 50 mg/kg astaxanthin; 2) 100 mg/kg astaxanthin; 3) 25 mg/kg lutein and 25 mg/kg zeaxanthin; or 4) 50 mg/kg lutein and 50 mg/kg zeaxanthin. The fifth diet was a control diet sprayed with the same quantity of water with no carotenoids but oil.

Pre-spawning broodfish nutrition study: Four tanks were randomly allocated to a diet, with forty fish allocated per diet. Brood fish were fed with respective feed three times a week to satiation for 8 weeks. Individual broodfish were measured for total length and weight at the beginning and the end of the study. At the end of the feeding, two fish were randomly selected from each tank (8 fish per diet) and approximately 2 g oocytes were biopsied from a fish. The oocytes and the prepared feed were measured for carotenoid concentration by high performance liquid chromatography (Craft Technologies, Inc. Wilson, NC). The yellowness of the eggs was also measured by digital photography, using Adobe Photoshop software (Adobe Systems, Inc., San Jose, CA, USA).

Reproductive performance: Individual fish in each tank were examined for maturation; gravid females from each tank were identified at weekly intervals during the course of the feeding study. Gravid channel catfish females were placed in individual soft mesh bags and suspended in a 10,000 L concrete tank supplied with flow-through water and air (water temperature 26.1°C, pH 8.6 and dissolved oxygen > 6.0 mg/L). LHRHa (lutenizing hormone releasing hormone analog, Western Chemicals, Ferndale, Washington, USA) was administered in two doses, a priming intraperitoneal injection of 20 μg/Kg body weight (BW), followed by a resolving dose of 80 μg/Kg BW15h later, following the protocols described by Kristanto et al. (2009). Stripped eggs from individual females were weighed and enumerated based on a sample of eggs to derive relative fecundity (eggs/kg BW). Stripped eggs were fertilized with blue catfish sperm following the procedures outlined by Chatakondi et al. (2011). Percent fertilization was

expressed as a proportion of the total number of live eggs to the total number of eggs enumerated in a sample 24h post-fertilization. Disassociation of the chorion was an indicator of an unfertilized egg. Percent hatch was expressed as a proportion of the total number of live hybrid sac fry to the total number of fertilized eggs.

Progeny performance: Growth study: Hybrid catfish fingerlings were produced by female channel catfish fed with five pre-spawning carotenoid feeds. Twenty five aquaria were randomly assigned to five groups of hybrid catfish. A 10-week growth study was initiated in flow-through 80L aquaria; each aquarium was stocked with 25 randomly selected hybrid fingerlings produced by female channel catfish fed with one of the pre-spawning carotenoid feeds. Fingerlings were fed with 40% protein commercial floating catfish feed to satiation once daily. Individual fish in an aquarium were weighed to the nearest 0.1g and measured to the nearest 0.1 cm in length at the beginning and at the end of the study.

ESC (Enteric Septicemia of Channel catfish) disease challenge: At the end of the growth study, 80 fish were randomly selected from each of the five treatments and were stocked in four 80-L aquaria. Fingerlings from five treatments were stocked in 20 aquaria at our Unit's fish health facility. Fish were randomly assigned to one of the twenty aquaria represented the five treatment groups. Three additional aquaria were stocked with 20 fish per tank to serve as control. Fish stocked in 23 aquaria were subjected to immersion challenge of *Edwardseilla ictaluri* (confirmed by the MSU Fish Diagnostic Lab). Protocol to induce Enteric Septicemia of Catfish (ESC) with virulent E.*ictaluri* (2.0 X 10^{6} CFU mL^{-1}) final concentration to induce ESC disease, as described previously by Wolters and Johnson (1994) was followed. Fish were observed for 21 days and the associated mortality in individual tanks was recorded daily. Fish were fed with 35% protein commercial floating catfish feed (Delta Western, Indianola, MS) daily to satiation.

Low dissolved oxygen stress test: At the end of the growth trial, fingerlings were subjected to low dissolved oxygen followed by measurement of blood cortisol to determine stress response. Eighteen fish were randomly selected from each of the five treatments and were held in fifteen aquaria, three replicated aquaria per treatment (6 fish/aquaria) at 21.6 °C. Water flow in the 15 aquaria was stopped and nitrogen gas was bubbled into the aquaria, following methods described by Small (2004). Nitrogen

diffusion was stopped when DO levels reduced from 100 to 50 or 25% saturation. Two fish per tank were randomly sampled, anaesthetized with 6 mg/L Metomidate hydrochloride and bled from the caudal vasculature into labeled 1.5 mL centrifuge tubes (Small and Davis, 2003). Blood samples was centrifuged at 10,000 rpm for 7 minutes to separate plasma, and cortisol levels were measured by radioimmunoassay (RIA) using the Coat-A-Count Cortisol kit (Siemens Healthcare Diagnostics, Inc., Los Angeles, CA), using procedures outlined by Davis et al. 1993.

Statistical Analysis: Growth parameters of pre-spawning female catfish fed with carotenoid fortified diets were analyzed using one-way ANOVA. Variables evaluated and compared across treatments were: percent of mature females, percent ovulation of selected females, relative fecundity, and percent eggs hatched. A one-way ANOVA was conducted to compare progeny attributes among treatments: percent weight gain, mean survival to ESC disease challenge, and mean cortisol stress response to low dissolved oxygen. Data were arc-sine transformed to normalize data prior to analysis. If differences were significant, means were separated by Tukey's *ad hoc* test. In all statistical comparisons, traits were considered significant at P < 0.05.

RESULTS

Feeding carotenoid fortified feeds eight weeks prior to spawning did not improve growth traits of mature catfish. However, mean gonadosomatic index of female channel catfish fed 100 mg/kg of Astaxanthin (13.6%) was higher (P =0.01, F=13.38) than catfish fed control or other carotenoid fortified feeds. Feeding this diet also resulted in higher (P=0.004, F=10.88) maturation (82.3% gravid females) and mean (p=0.05, F=15.12) percent ovulation of females responding to hormone injection(77.5%). However, relative fecundity, percent fertilization, percent hatch and fry/kg of female BW did not differ among females fed carotenoid fortified feeds or the control feed (Table 1). Moreover, maternal feeding of carotenoids during pre-spawning did not influence progeny growth. However, progeny derived from channel catfish females fed a 100 mg/kg Astaxanthin had higher (P =0.05, F=9.97) survival (80%) to ESC disease challenge compared to progeny derived from mothers fed the control or other carotenoid diets. The mean stress response (plasma cortisol) of the progeny derived by channel catfish fed carotenoid diets at 100 and 50% dissolved oxygen saturation did not differ. However, the mean stress response of the progeny derived from channel catfish females fed 100 mg/kg of Astaxanthin had lower (P=0.02, F=10.56) cortisol levels at 25% dissolved oxygen saturation compared to progeny derived from mothers fed control or other carotenoid feeds.

DISCUSSION

There is an extensive evidence supporting the vital role of carotenoids in the physiology and overall health of aquatic animals, and suggests carotenoids are essential nutrients and should be included in aquatic diets (Grung et al. 1993). The present study addresses the potential of pre-spawning carotenoid supplemented feed to improve gonadal maturation of channel catfish females to enhance their responsiveness to hormone induced spawning in the production of channel x blue hybrid catfish.

The use of carotenoids as pigments in aquaculture species include 1) antioxidant and provitamin A, 2) enhanced immune response, 3) reproduction, 4) growth, 5) maturation, and 6) photoprotection (Howell and Matthews, 1991). The effect of dietary supplementation of carotenoids has been studied in three categories: 1) knowledge of the factors that influence pigmentation, 2) mechanisms of

Table 1. Reproductive performance (Mean + SE) of channel catfish females fed with pre-spawning carotenoid fortified diets and subsequent progeny performance.

Parameter	Control	50 mg/Kg Astaxanthin	100 mg/Kg Astaxanthin	25 mg/kg Luten + 25 mg /kgZeaxanthin	50 mg/kg Luten + 50 mg/kg Zeaxanthin
Reproduction					
Gonadosomatic index	9.7+1.8	9.8+2.1	14.6*+ 1.3	11.1+1.4	11.4+0.8
Gravid females (%)	55.2+2.9	65.8+4.6	82.3*+5.6	57+3.3	66.4+3.7
Ovulation (%)	55.5+2.9	60+4.6	77.7*+2.8	55+3.3	68.5+2.8
Fecundity (eggs/kg)	5600+277	5792+343	6100+414	5397+516	5606+388
Fertilization (%)	88.6+3.1	94.3+1.7	91.7+2.3	90.8+4.3	84.8+4.4
Hatch (%)	38.7+3.6	40.2+5.6	45.3+4.9	37.9+2.2	35.1+7.3
Fry/Kg	1125+337	1222+218	1204+408	1150+256	1080+363
Progeny performance					
Weight gain (g)	7.6+1.6	9+1.4	10.6+3.3	7.6+2.6	6.6+1.5
ESC Survival (%)	56+2.8	64+3.2	80*+4.4	55+3.6	60+4.0
Cortisol (ng/mL)	45.6+2.8	37.8+3.4	28.7*+2.2	46.1+3.4	43.5+4.6

*mean performance within a row differ from different treatments (P<0.05)

absorption, deposition, metabolism, and biological activity of pigments and 3) search for alternate sources of pigments in aquaculture diets to enhance marketability of aquaculture produce. Typically, Beta-carotene is converted into Beta-cyoptoxanthin or Iso-cryptoxanthin, both of these groups eventually degrade to 'astaxanthin'. Most carotenoids present in the wild are astaxanthin, astaxanthin esters, zeaxanthin (present in spirulina) will also be converted to astaxanthin (Latscha, 1991). It is also observed that in aquatic animals, total carotenoid content can change depending on species, tissue, seasons, feeding habits, and sex. These type of dramatic changes in total carotenoid content have been reported for fish (Leclercq et al. 2010), shrimp (Yanar et al. 2004), cephalopods (Van den Branden et al. 1980), and bivalves (Campbell, 1969).

Pre-spawning carotenoid diets did not favor growth of broodfish as fish tend to limit food intake prior to spawning. However, it was interesting to note that broodfish fed with 100 mg/kg astaxanthin improved maturation. This improved broodfish condition for artificial stimulatation of gonad development to facilitate spawning and enhanced the quality of strippable eggs, improved the production of viable offspring. Our results were similar to studies conducted by Watanabe and Kiron (1995) and Huang et al. (2008).

Carotenoid content in aquatic organisms can be markedly increased by feeding higher levels of natural carotenoids. However, natural feed ingredients rich in carotenoids are expensive and do not make the carotenoid available efficiently to the animal (Ponce-Palafax et al. 2006). The exclusive use of pelleted feeds over natural feeds to raise aquaculture species for uniformity, efficiency, and cost has been well studied (Zaripheh and Erdman, 2002). Hence, use of a specific refined source of cartoenoids is a desirable alternative for incorporating into pelleted diets to meet nutritional requirements of aquacultured species (Amar et al. 2012).

Administration of dietary astaxanthin has been demonstrated to enhance resistance in shrimp to oxygen depletion stress (Chien et al. 1999), salinity stress (Darachai et al. 1998), thermal stress (Chien et al. 2003), and ammonia stress (Pan et al. 2001). In this study, progeny derived from maternal feeding of a diet supplemented with 100 mg/kg astaxanthin had higher survival following *Edwardsiella ictaluri* disease challenge. Similar to this study, improved immunological and stress response was observed by supplementing carotenoid diets to shrimp (Estermann, 1994). Astaxanthin has also been suggested to have an important role in animal health as an antioxidant agent through inactivation of free radicals produced from normal cellular activity and stressors (Chien, 1999). Astaxanthin also possess 10 times greater anti-oxidative properties than β-carotene and 100 times greater than α-tocopherol (Shimidzu et al. 1996).

The results of the pre-spawning 100 mg/kg astaxanthin fortified diet in this study improved ovulatory competency of channel catfish, maturation, and hence made them more suitable for hormone-induced spawning. Progeny from this treatment showed reduced mortality to ESC disease challenge under controlled conditions apart from reduced tolerance to low dissolved oxygen stress conditions.

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Experimental Study on Broodstock Management of Barfin Flounder *(Verasper moseri)* Under the Concept of Minimum Kinship Selection

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ABSTRACT

In this study, we applied the concept of minimum kinship selection to broodstock management of Barfin flounder using three microsatellite DNA markers. The breeding plan was designed based on the kinship value, which was calculated among the broodstock individuals using microsatellite DNA markers. Moreover, individual identification and artificial insemination techniques were developed to support the breeding plan. The broodstock consisted of 37 wild and 41 hatchery-reared Barfin flounders. Each individual was identified using a PIT tag. The sex was recognized according to the gonad shape, as analyzed by ultrasound. Artificial fertilization was performed based on the factorial mating design, excluding closely related individuals. Furthermore, the numbers of larvae used for seedling production were equalized among the pairs to maximize the effective population size. Using this procedure, we produced seedlings with high genetic variation that was comparable to that of the broodstock's, showing that the concept of minimum kinship selection is useful for genetic conservation in Barfin flounder stock enhancement.

INTRODUCTION

The Barfin flounder (*Verasper moseri)*, is a large flatfish distributed from the southern Sea of Okhotsk to northern Japan. This fish is the highest-priced righteye flounder in Japan, but the capture fishery yields have been low since the 1970s due to severe depletion. This reduction has rendered the Barfin flounder a rare species. To correct this situation, a stock enhancement program with annual release of approximately 100,000 seedlings/year has been promoted since 1987. This program is inferred to be effective because released seedlings are recaptured every year. However, the risk of losing genetic diversity from the base population is ever-present: the proportion of hatchery born fish in the broodstock has been increasing because obtaining wild Barfin flounder is almost impossible. For that reason, optimal broodstock management is necessary to avoid inbreeding and loss of genetic variation in future generations.

The basic principles for Japanese marine stock enhancement programs released in February 2005 request responsible promotion of stock enhancement with consideration of the conservation of genetic diversity (MAFF 2005). Appropriate measures on the genetic level are necessary in artificial reproduction because the loss of genetic diversity might easily progress by inbreeding and genetic drift, particularly in species at a low population level. Akkeshi Station of the National Center for Stock Enhancement, FRA, has been developing broodstock management and artificial fertilization techniques in a rare species, Barfin flounder (*Verasper moseri*), for conservation of genetic diversity (Minami 1994). This paper introduces efforts undertaken for conservation of genetic diversity. Specifically in the selection of parents to be used, well-planned artificial fertilization, and the selection of hatched larvae for release. However, it is noteworthy that the development of techniques to actively produce genetically diverse seed has only just begun for Japanese fishery stock enhancement. Therefore, techniques introduced here have not been established yet and still include problems to be mitigated or resolved.

Avoidance of Inbreeding

Population genetic diversity exists in a group of individuals with much genetic variation. However, for the healthy maintenance of a population, the group must comprise genetically adapted individuals instead of a group of individuals with various genetic defects. Therefore, to conduct artificial reproduction methods for the conservation of genetic diversity, technologies to produce genetically adapted individuals are needed. Genetic factors to reduce individual fitness include increased homozygosity of harmful recessive genes due to inbreeding. To avoid this, modified breeding methods (such as circular group mating) and the utilization of registered lineage information have been promoted in livestock breeding (Sasaki 1994). Recently, genetic markers such as microsatellite DNA

have been developed for use in various organisms, making it possible to avoid inbreeding by using genetic information directly. Akkeshi Station, in collaboration with Tohoku University, developed an inbreeding avoidance plan utilizing hypersensitive DNA markers for Barfin flounder (Maria et al. 2003). Specific methods are described below. The genetic information of all parents in a stock are compiled in a database utilizing three sets of hypersensitive microsatellite DNA markers developed in naturally occurring Barfin flounder. The genetic relatedness of all parent pairs is obtained based on that information, and pairs with genetic relatedness of not more than 0 are judged as having a low genetic relation and are identified as combinations that can be bred. Inbreeding is avoided by planned breeding of the combinations by artificial fertilization. Genetic relatedness (R) is calculated using the following formula (Queller and Goodnight 1989).

$$R = \frac{\Sigma \{(Pxj - P^*) + (Pyi - P^*)\}}{\Sigma \{(Pxi - P^*) + (Pyj - P^*)\}}$$

Where, Pxi is the frequency of allele i in the first marker gene locus of individual x, Pyj represents i the frequency of allele j in the first marker gene locus of individual y, and P^* is the allele frequency for all the parents in the stock. Additionally, genetic relatedness (R) ranges from -1 to 1, and becomes larger with increases in the number of alleles in common.

In Akkeshi Station, there were 30 male and 48 female mature Barfin flounder in FY 2005. The mean number of alleles per marker gene locus in this parent population was 30.7, and the mean ratio of heterozygotes was 0.907. Genetic relatedness was calculated for all parent pairs. Results show

that 506 pairs of 1,440 pairs in the total number of combinations could be judged as having a high probability of inbreeding. Therefore the remaining 934 pairs were identified as combinations that were available for breeding (Table 1).

Selection of the Male Parent

To enhance the genetic diversity of seed, it is necessary to use a large number of parents. However, an increase in the number of parents does not engender the enhancement of genetic diversity if the parents are closely related, i.e., if they have much genetic information in common. At Akkeshi Station, the genetic relationship among male parents available in a production season was examined in detail before proceeding with artificial fertilization. Thereby, the genetic diversity of the seed produced throughout the season was enhanced by limiting the number of genetically close male parents to be used and by changing parents for every production lot.
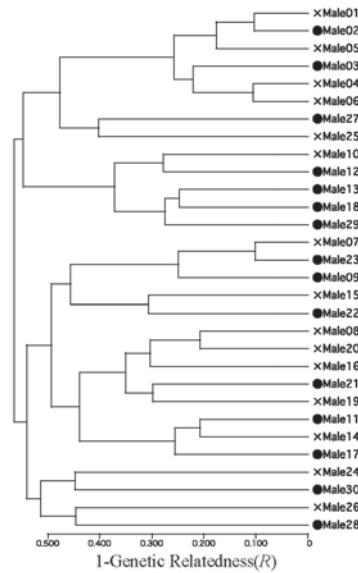
The genetic relation among 30 mature male parents available in FY 2005 was examined in detail using microsatellite DNA information (Fig. 1). Based on the results, 15 fish were selected to be used in the first round of artificial fertilization.

Figure 1. Genetic relationships among male parent Barfin flounder obtained by UPGMA based on the genetic distance (1-R) among individuals
 ●: Male parent used in the first artificial fertilization in FY 2005.
 ×: Male parent not used in the first artificial fertilization in FY 2005.

Table 1. Genetic relatedness between Barfin flounder pairs estimated based on microsatellite DNA information (Genetic Relatedness, R)

♀ \ ♂	Male02	Male03	Male09	Male11	Male12	Male13	Male17	Male18	Male21	Male22	Male23	Male27	Male28	Male29	Male30
Female02	0.60	0.48	−0.20	−0.18	−0.13	−0.15	−0.15	−0.15	−0.18	0.00	−0.20	0.01	−0.13	−0.17	−0.13
Female03	0.41	0.87	−0.18	−0.17	−0.13	−0.15	−0.15	−0.15	−0.17	−0.18	−0.03	0.11	−0.13	−0.16	−0.13
Female04	1.00	0.31	−0.21	−0.19	−0.13	0.18	−0.16	0.03	−0.19	−0.21	−0.21	0.01	−0.14	0.02	−0.14
Female05	0.66	0.70	−0.02	−0.17	−0.13	−0.15	−0.15	−0.15	−0.17	−0.20	−0.19	0.14	−0.13	−0.17	−0.13
Female07	0.55	0.74	−0.18	−0.17	−0.13	0.11	−0.15	−0.01	−0.17	−0.19	−0.04	0.11	−0.13	−0.02	−0.13
Female08	0.65	0.70	−0.21	−0.19	−0.15	0.13	−0.17	0.00	−0.19	−0.04	−0.21	0.13	−0.15	−0.01	−0.15
Female22	−0.02	−0.21	0.80	0.02	−0.15	−0.15	0.01	−0.15	0.02	0.20	0.60	0.01	0.06	−0.17	−0.13
Female29	−0.12	−0.13	−0.11	−0.09	−0.05	−0.08	−0.08	−0.07	−0.09	−0.12	−0.11	−0.08	0.12	−0.09	−0.05
Female32	−0.14	−0.15	0.06	−0.12	0.11	−0.10	−0.10	−0.09	−0.12	0.05	0.06	−0.10	0.11	−0.11	−0.07
Female38	0.03	0.17	−0.15	0.05	−0.09	−0.11	0.04	−0.11	0.05	−0.16	−0.15	0.04	−0.09	−0.13	−0.09
Female46	0.22	−0.01	0.03	−0.15	−0.10	−0.12	−0.13	−0.12	0.04	0.02	−0.17	0.03	−0.10	−0.14	−0.10
Female47	0.21	−0.12	−0.10	−0.09	−0.05	−0.08	−0.08	−0.07	−0.09	−0.11	−0.10	−0.08	−0.05	−0.08	−0.05
Female48	−0.12	0.03	−0.12	−0.10	0.12	−0.09	−0.09	−0.08	−0.10	−0.12	−0.12	−0.09	−0.06	−0.09	−0.06

□: Pairs judged as having low genetic relationship, ■: Pairs judged as having high genetic relationship

Avoidance of Genetic Drift Caused by Instability in the Quality of Gametes

When fertilized eggs are collected from artificial fertilization, it is difficult to obtain eggs and sperm of high quality from all parents. This is due to individual differences in the time of maturation and mismatch between the ovulation cycle and the egg collection plan. The qualitative evaluation of Barfin flounder gametes has not yet been put into practice. Therefore, adverse results from paired low-quality gametes are unavoidable in 1:1 pairings, even if the broodstock themselves are genetically of high quality. At Akkeshi Station this is circumvented by employing diallel mating. Gametes from each individual are crossed with all other individuals of the opposite sex that do no cause inbreeding, thereby avoiding gene loss from instability in the quality of gametes to the greatest extent possible.

Stripped sperm are diluted about 100-fold with artificial seminal plasma so that the fertilization procedure can be conducted smoothly (Mochida et al. 2000; Plate 1.). Next, eggs are stripped sequentially from the female parents. They are dispensed uniformly to male parents chosen to avoid inbreeding. Diluted sperm are added to the stripped eggs for fertilization. In the first artificial fertilization in FY 2005, an average of 107,000 eggs per individual were stripped from 21 female parents and dispensed into 6 to 14 aliquots (Plate 2). They were then fertilized with sperm from the 15 male parents described above. There were 211 combinations of artificial fertilization.

Plate 1. Sperm diluted with artificial seminal plasma.

Plate 2. Eggs stripped from one female parent and subdivided.

Egg Management

Differences exist in the developmental and hatching rate of fertilized eggs obtained by artificial fertilization. These differences are derived from instability in the quality of gametes and other factors. Thus, fertilized eggs are best managed in individual tanks by broodstock cross to ascertain separately if they are useful in seed production. However, it is not realistic to manage fertilized eggs obtained from diallel mating of hundreds of combinations simultaneously in individual tanks.

Previous research on the cryopreservation of Barfin flounder sperm (in collaboration with Fisheries Laboratory, Faculty of Agriculture, Kinki University, and Stock Enhancement Section, Hokkaido National Fisheries Research Institute, FRA) has shown that the fertilization rate for sperm with low motor activity is equal to that of highly active sperm when the amount of sperm added is increased. Based on these results, in our artificial fertilization procedures we use about ten times the amount of milt found experimentally to result in high fertilization rates even with sperm exhibiting low motor activity. Thus, fertilization performance in our procedure is unlikely to decrease significantly due to instability in the quality of sperm. The following discussion is made on the assumption that fertilization performance is unaffected by an individual male parent.

Artificially fertilized eggs are pooled by individual female parent in egg management tanks (200-ℓ Artemia hatching tank; Plate 3.). On the day following artificial fertilization, the developmental status of about 200 floating eggs is observed, and batches with a developmental rate of less than 60% is discarded. When hatching is completed in all tanks, the numbers of hatched larvae are estimated using the volumetric method. The developmental status of fertilized eggs obtained using the first artificial fertilization in FY 2005 showed a developmental rate as low as 25–50% in 7 of 21 female parents. These batches were discarded the day following artificial fertilization. The remaining fertilized eggs from 14 females were managed continuously in individual tanks. Hatching began 10 days after fertilization to yield 447,000 larvae.


Plate 3. Egg management tanks (managed until hatching separately by individual female parental origin).

Selection of female parent

Male parents were selected to avoid the repeated use of genetically close individuals. Similar processing was conducted for female parents with successful batches of fertilized eggs (i.e. hatch rate over 60%). The genetic relationships between these females were examined in detail. If a group of two or more females were found to be genetically very similar, only a single female's batch of larvae from that group would be retained for seed production.

The genetic relationships between the 14 females that produced a sufficient amount of hatched larvae in FY 2005 were examined in detail using microsatellite DNA. As a result, females IDNO01 and IDNO07, and females IDNO30 and IDNO32 were judged to be very closely related. Therefore, only one female from each group was used (larvae from two females were dropped). As a result, 420,000 hatched larvae from 12 females were used in seed production (Fig. 2).
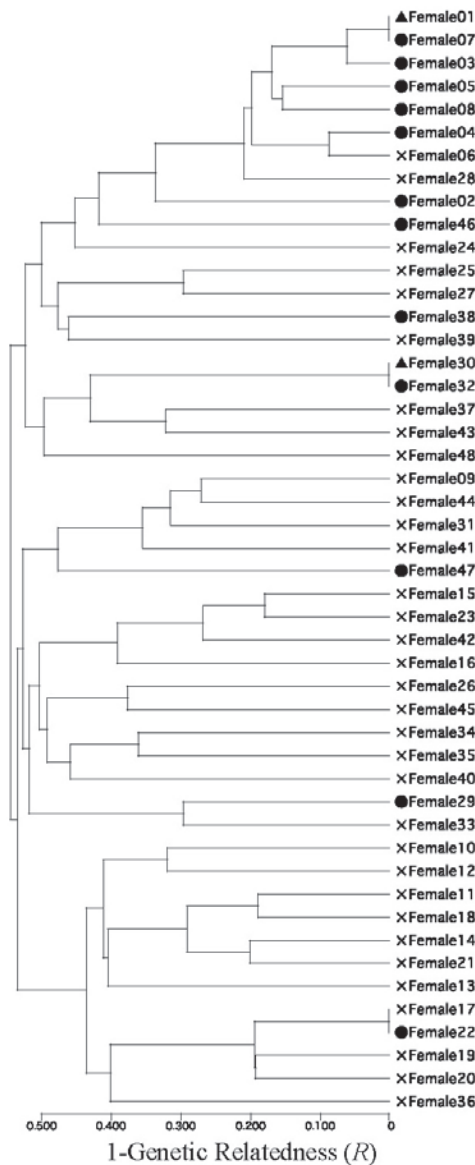
Hatched larvae culling (Equalization of family size) Fertilized eggs (hatched larvae) to be used in seed production are generally managed for quality, and are adjusted to a planned number around the time of transfer to the seed production tank. In that sense, hatched larvae are more or less subject to selection pressure. Empirical criteria such as the fertilization rate, hatching rate, malformation rate, and the size and floatability of larvae have been used as indicators for selection. However, this selection method promotes the uneven utilization of hatched larvae derived from specific parents, and is therefore ineffective for the conservation of genetic diversity. To conserve genetic diversity 'balanced thinning', by which all parents used in artificial fertilization produce the same number of offspring, is considered to be effective.

Hatched larvae were selected for seed production in such a way that the variance of the number of hatched larvae derived from each female-male cross (family size) was minimized. Solver in MS Excel (Microsoft, Redmond, WA) was employed to find values minimizing the variance of the number of larvae selected for seed production from each parental cross, given a total number of larvae needed for seed production. The total number of larvae needed for the first seed production in FY 2005 was 216,000. Thus, 216,000 of 420,000 available larvae were selected using the method described above (Table 2). The original range and coefficient of variation of the total number of hatched larvae from each cross before selection were 0.96–5.78 thousand and 0.394, respectively, which became 0.56–2.06 thousand and 0.251 after selection using the described methodology. Therefore, the total number of hatched larvae derived from individual female and male parental origin (family size) was homogenized.

Figure 2. Genetic relationships among female Barfin flounder obtained by UPGMA based on the genetic distance (1-R) among individuals
●: Females that supplied hatched larvae for seed production.
×: Females that produced an insufficient amount of hatched larvae.
▲: Females that produced a sufficient amount of hatched larvae were not used for seed production considering genetic relationships between females.

Table 2. Number of Barfin flounder hatched larvae derived from individual female and male parental origin obtained from diallel mating with consideration to inbreeding avoidance, and list of selection results with an aim to homogenize the total number of hatched larvae derived from individual female and male parental origin (family size)

Process	♀	Male02	Male03	Male09	Male11	Male12	Male13	Male17	Male18	Male21	Male22	Male23	Male27	Male28	Male29	Male30	Total
Before culling	Female02			4,233	4,233	4,233	4,233	4,233	4,233	4,233	4,233	4,233		4,233	4,233	4,233	50,796
	Female03			2,400	2,400	2,400	2,400	2,400	2,400	2,400	2,400	2,400		2,400	2,400	2,400	28,800
	Female04			3,740	3,740	3,740		3,740		3,740	3,740	3,740	3,740	3,740		3,740	37,400
	Female05			1,409	1,409	1,409	1,409	1,409	1,409	1,409	1,409	1,409		1,409	1,409	1,409	16,908
	Female07			1,973	1,973	1,973		1,973	1,973	1,973	1,973	1,973		1,973	1,973	1,973	21,703
	Female08			5,255	5,255	5,255		5,255	5,255	5,255	5,255	5,255		5,255	5,255	5,255	57,805
	Female22	5,000	5,000			5,000	5,000		5,000						5,000	5,000	35,000
	Female29	862	862	862	862	862	862	862	862	862	862	862	862		862	862	12,068
	Female32	4,580	4,580		4,580		4,580	4,580	4,580	4,580			4,580		4,580	4,580	45,800
	Female38			5,044		5,044	5,044		5,044		5,044	5,044		5,044	5,044	5,044	45,396
	Female46		3,507		3,507	3,507	3,507	3,507	3,507			3,507		3,507	3,507	3,507	35,070
	Female47		2,050	2,050	2,050	2,050	2,050	2,050	2,050	2,050	2,050	2,050	2,050	2,050	2,050	2,050	28,700
	合計	10,442	15,999	26,966	30,009	35,473	29,085	30,009	36,313	26,502	26,966	30,473	11,232	29,611	36,313	40,053	415,446
After culling	Female02			1,520	1,520	1,520	1,520	1,520	1,520	1,520	1,520	1,520		1,520	1,520	1,520	18,240
	Female03			1,520	1,520	1,520	1,520	1,520	1,520	1,520	1,520	1,520		1,520	1,520	1,520	18,240
	Female04			1,958	1,958	1,958		1,958		1,958	1,958	1,958	1,958	1,958		1,958	19,580
	Female05			1,409	1,409	1,409	1,409	1,409	1,409	1,409	1,409	1,409		1,409	1,409	1,409	16,908
	Female07			1,650	1,650	1,650		1,650	1,650	1,650	1,650	1,650		1,650	1,650	1,650	18,150
	Female08			1,650	1,650	1,650		1,650	1,650	1,650	1,650	1,650		1,650	1,650	1,650	18,150
	Female22	2,767	2,767			2,767	2,767		2,767						2,767	2,767	19,369
	Female29	799	799	799	799	799	799	799	799	799	799	799	799		799	799	11,186
	Female32	2,056	2,056		2,056		2,056	2,056	2,056	2,056			2,056		2,056	2,056	20,560
	Female38			1,986		1,986	1,986		1,986		1,986	1,986		1,986	1,986	1,986	17,874
	Female46		1,827		1,827	1,827	1,827	1,827	1,827			1,827		1,827	1,827	1,827	18,270
	Female47		1,391	1,391	1,391	1,391	1,391	1,391	1,391	1,391	1,391	1,391	1,391	1,391	1,391	1,391	19,474
	Total	5,622	8,840	13,883	15,780	18,477	15,275	15,780	18,575	13,953	13,883	15,710	6,204	14,911	18,575	20,533	216,001

* It was assumed that the fertilization performance is not different by individual male parent.

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Marine Fish Hatchery Technology and Research at the Thad Cochran Marine Aquaculture Center

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ABSTRACT

The Thad Cochran Marine Aquaculture Center is composed of 8 new laboratory buildings dedicated to the development of Gulf of Mexico marine fish aquaculture; 4 other buildings have been used by the joint Gulf Coast Research Laboratory/USDA Marine Shrimp Program. The buildings include live feed production systems, hatchery, maturation, juvenile and adult production systems, as well as wet and dry laboratories for genetic, disease, reproductive physiology, nutritional, and behavioral research. In addition, several buildings are used for teaching and public educational purposes. The primary objectives of the Center are to enhance Mississippi Gulf waters with commercially and recreationally important species and develop aquaculture technology for commercial transfer. The immediate goals of the Center are to develop the methods to capture, maintain, and spawn broodstock of marine fish in order to facilitate production, rearing, tagging, releasing, and assessing of juveniles in a stocking program. The facility employs cutting-edge technology, peer-reviewed research, and hands-on testing to grow fish in an environmentally sustainable and economically feasible manner. Research is funded through partnerships with state and federal agencies, as well as private companies. The facility is unique in that all hatchery and production tanks are closed, recirculating seawater systems filled with artificial seawater, insuring biosecurity between tanks and the environment. The research developed at the center can be divided into five programs: Diseases, Genetics, Microbiology, Reproductive Physiology, and Nutrition. These programs are implemented in the context of 6 main projects: Spotted Seatrout Aquaculture and Stock Enhancement, Red Snapper Aquaculture, Live Feed Culture, Recirculating Seawater Technology, Marine Shrimp Farming, and New Species Development. Over the last few years, more than a half-million Spotted Seatrout (*Cynoscion nebulosus*) have been stocked into Mississippi waters. Seatrout have been tagged and released and an assessment program is being developed in collaboration with the Mississippi Department of Marine Resources. The second major project focuses on Red Snapper (*Lutjanus campechanus*); several thousand have also been released in recent years. The Red Snapper Program has been significantly expanded during the last 2 years with the construction of the new Red Snapper Larval Rearing and Grow-out Buildings. Improvements in live feed production have been key to the success of these expanding hatchery production projects. Additional species have been the focus of the New Species Development research, including Cobia (*Rachycentron canadum*), Tripletail (*Lobotes surinamensis*) and Atlantic Croaker (*Micropogonias undulatus*). The Center has led an effort to establish a Gulf of Mexico Marine Fish Hatchery Consortium that would link a number of research and private scientific institutions on the Gulf coast, improving communication and coordinating joint research on Gulf fish species.

INTRODUCTION

The Thad Cochran Marine Aquaculture Center (CMAC) is the centerpiece of the marine aquaculture program of The University of Southern Mississippi (USM) Gulf Coast Research Laboratory (GCRL) in Ocean Springs, MS. The Board of Trustees of the State Institutions of Higher Learning approved the name of the new center on October 19, 2006. The CMAC houses GCRL's marine aquaculture research and graduate education programs. The University is investing heavily in marine aquaculture at the CMAC's 224-acre Cedar Point site (CMAC 2013). Funding for the CMAC came from the university, Mississippi Department of Marine Resources (MDMR), National Oceanic and Atmospheric Administration (NOAA) and United States Department of Agriculture (USDA).

Two-thirds of harvested marine species for which data exist are either at or below the levels required for sustainability. As human populations increase, greater pressure is placed on marine fisheries resources through habitat destruction and demand for food and recreation. It is estimated that aquaculture of Gulf fish species would double the seafood output of the Gulf of Mexico ($818 million in 2011; NMFS 2012). Additionally the recreational fishing industry ($9.8 billion in 2012) would also realize expanded employment and business opportunities as natural populations are restocked with hatchery produced fingerlings (NMFS 2011).

The CMAC is proud to be one of leaders in marine aquaculture and stock enhancement research, implementation, and technology transfer for the northern GOM. The CMAC has been a leader in the establishment of a Gulf of Mexico Hatchery and Fisheries Restoration Consortium composed of six institutions from the five Gulf States and Maryland: Gulf Coast Research Laboratory/University of Southern Mississippi (GCRL; lead institution), University of Texas Marine Science Institute (UTMSI), Louisiana University Marine Consortium (LUMCON), Auburn University (AU), Mote Marine Laboratory (MML), and University of Maryland-Baltimore (UMB). The consortium is built on established relationships and will employ the highest quality science and economic approaches to implement and transfer technology with the goal of raising significant numbers of fish for fishery restoration and stimulating private sector small business development. The work of CMAC and the consortium will result in advanced technologies for use by Gulf States fishery agencies and private industry.

Responsible Approach

The CMAC is committed to the design of research programs and production systems that adhere to a responsible and sustainable approach to the production of marine organisms for enhancement and commercial aquaculture purposes (Blaylock and Whelan 2004; Gold et al. 2004; Blaylock et al. 2000). Marine resource management agencies are seeking tools such as scientifically robust stock enhancement procedures that would expand the repertoire of management tools for heavily fished species.

This requires that all the programs optimize operations based on the following five principles:

- Genetics
  - Understanding and maintaining natural population structure
  - Assess and mitigate effects of stock enhancement on genetic diversity and fitness of supplemented populations

- Nutrition
  - Optimizing survival and development in the hatchery
  - Producing live feeds and sustainable artificial feeds

- Disease
  - Insuring health of wild and cultured fish
  - Establishing biosecurity protocols

- Assessment of effectiveness of stock enhancement
  - Assessing survival of released fish and contribution to the fishery
  - Tracking changes in wild populations

- Closed, recirculating systems
  - Increasing sustainability by reducing environmental inputs and outputs
  - Environmentally isolated systems decrease risk of disease introduction or transmission

Facilities

The Thad Cochran Marine Aquaculture Center (CMAC) is composed of eight new buildings that are used for marine fish culture and enhancement and four older buildings that are used for marine shrimp culture (Figure 1).

Figue 1. Aerial view of the Thad Cochran Marine Aquaculture Center site at Cedar Point. (1) Red Snapper Broodstock and Larval Rearing Building, (2) Aquaculture Visitors' Pavilion, (3) Aquaculture Growout Building, (4) Research Building, (5) Aquaculture Demonstration Laboratory, (6) Red Snapper Growout Building, (7) Shrimp Production Greenhouse, (8) Shrimp Maturation and Hatchery Buildings, (9) Fish Health Building, (10) Wet Laboratory Building and (11) Multi-Trophic Reclamation System.

- Aquaculture Visitors' Pavilion serves as a complete research and education center for the Cedar Point site. The building is 11,000 ft² and combines the complete spectrum of production systems needed to culture marine fish. There is an algae culture room, rotifer production room, artemia hatching room, dry and frozen feed preparation room, water quality analysis laboratory, egg incubation room, larval rearing room with 20- 1,500 L cone-bottomed hatchery tanks, maturation/broodstock room with 6-25,000 L photoperiod and temperature controlled

tanks, staff offices and visitors' atrium with educational displays and large screen video monitor connected to the GCRL website and cameras mounted in broodstock tanks. All of the spotted seatrout go through larval rearing in this building. Broodstock of several different species have been maintained in the maturation room.

- Red Snapper Broodstock and Larval Rearing Building (7,650 ft²) is dedicated to the production of juvenile red snapper for enhancement. The building includes live feed preparation rooms for both rotifers and *Artemia*, an egg incubation room, a large-scale copepod production room, larval rearing room for 12-1,500 L cone bottom hatchery tanks, and two maturation/broodstock rooms with 3 m deep, 39,000 L broodstock tanks with photoperiod and temperature control.

- Aquaculture Growout Building (7,200 ft²) is used to produce juvenile fish for later release and adults for broodstock. There are a variety of nursery and production tanks that range from 7,000 L to 48,000 L.

- Red Snapper Growout Building (7,200 ft²) is similar in design to the Aquaculture Growout Building but is dedicated to red snapper. The tank systems in this building are currently under construction.

- Aquaculture Demonstration Laboratory (5,512 ft²) is used to develop new species for production aquaculture. This building contains areas for egg incubation (120 L), larval rearing (1,000 L), nursery (1,000 L), adult growout and broodstock/maturation (>45,000 L). There is also a dry laboratory for water quality testing and dry feed storage.

- Wet Laboratory Building (6,300 ft²) is designed to accommodate replicated studies in support of any of the Center's activities with three rooms containing tanks ranging from 20 L aquaria in racks to 1,000 L cone-bottomed tanks. Currently, one of the rooms is being used for large-scale production of copepods.

- Fish Health Building (7,100 ft²) serves as the quarantine and strip spawning facility for wild fishes so as to minimize the risk of introducing pathogens into the facility. There are four separate tank areas and a fish health laboratory.

- Research Building (27,000 ft²) houses a variety of laboratories, offices, conference rooms and classrooms used for graduate classes and public education. Most project faculty are housed in this building.

- Shrimp Production Greenhouse is composed of 12- 80 metric ton (80,000 L) raceways for the commercial demonstration of marine shrimp production in biofloc systems. This is a greenhouse style facility with supplemental gas-powered heating and liquid oxygen funded by the USDA (Ogle et al. 2006).

- Shrimp Maturation/Hatchery Buildings (three buildings) were built in collaboration with the USDA's Marine Shrimp Farming Program. They contain areas for algae production, rotifer and artemia, shrimp hatchery tanks, maturation/broodstock tanks, and small production raceways.

PROJECTS

Most of the research and educational activities currently conducted on the CMAC campus can be divided into six projects: (1) Spotted Seatrout Aquaculture and Stock Enhancement; (2) Red Snapper Aquaculture; (3) Live Feed Culture; (4) Recirculating Seawater Technology; (5) Marine Shrimp Culture; and (6) New Species Development. These projects are a collaborative effort between the CMAC and the Mississippi Department of Marine Resources (MDMR) and Coastal Conservation Association (CCA).

Spotted Seatrout Project

The goal of the project is to develop the methods to acquire, maintain and spawn broodstock of spotted seatrout (*Cynoscion nebulosus*) to facilitate production, rearing, tagging, releasing and assessing of juveniles in a stocking program. Currently, the fish are used primarily for wild-stock enhancement, although their use for commercial aquaculture is being explored with a commercial collaborator.

The spotted seatrout, sometimes referred to as speckled trout or speck, is the most popular recreational fish species in the Gulf of Mexico. Naturally predatory and occasionally cannibalistic, their natural tendencies make cultivating them in tanks challenging. The GCRL scientists began developing the seatrout program in 2004 with Tidelands Trust Funds administered by the Mississippi Department of Marine Resources.

Captive wild adult spotted seatrout spawn in the CMAC broodstock tanks: 6-25,000 L covered circular tanks with photoperiod and temperature control (Figure 2). Scientists regularly skim the eggs from these broodstock tanks; the eggs are incubated in 120 L cone-bottomed incubators and the hatched larvae are transferred to 1,500-L cone-bottomed larval rearing tanks (Figure 3). The larvae are fed enriched rotifers and artemia for the first 20-30 days post-hatch. Later, they are weaned to artificial pellets and grown to fingerling size (>100 mm) before being tagged and released in Mississippi waters.

Figure 2. Aquaculture Visitors' Pavilion Broodstock Tanks. The broodstock tanks all have temperature and photoperiod control as well as closed, recirculating filter systems.
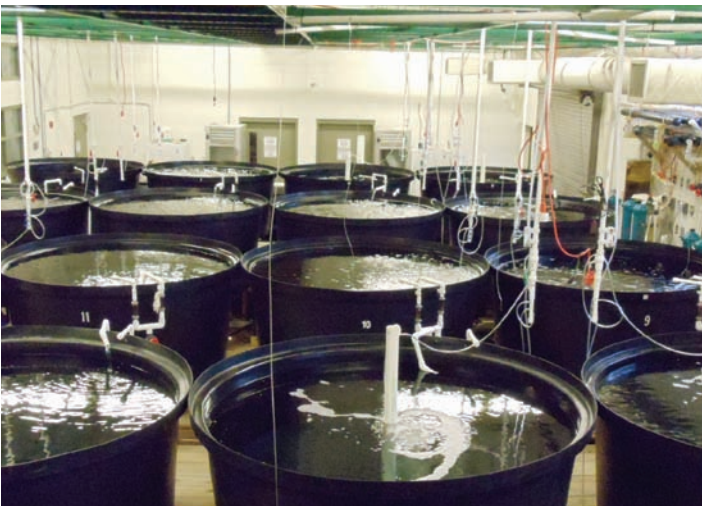

Figure 3. Larval rearing tanks located in the Aquaculture Visitors' Pavilion. These tanks can be used for a variety of marine species.

Spotted seatrout benefit from CMAC's recirculating, closed-water systems due to better control of environmental conditions in the tank. They reduce environmental impacts as well because very little discharge occurs as a result of the environmentally-friendly technology. Broodstock, larval rearing

tanks, and production tanks all have particle filters, biological filters, protein skimmers, and sterilization, i.e., ultraviolet light or ozone injection. All systems also have access to pure oxygen injection but this is usually only required in higher density production tanks; it is available for emergencies. In fact, CMAC is the only facility in the world growing spotted seatrout at this size using closed, recirculating systems.

Scientists tag the fish before releasing them in order to monitor them in the wild (Figure 4). These released fish are tagged with coded wire tags and fin clips are taken for genetic analyses. A genetic monitoring program was initiated in 2010 and currently focuses on (i) understanding population structure of spotted seatrout in the region to assist in the definition of management units for the program, and (ii) assessing potential effects of the program on genetic diversity in order to define appropriate targets for broodstock effective size and stocking levels (Saillant 2011). We maintain two populations of brood fish and the juvenile fishes are stocked back into the two bay systems, Davis Bayou and Bay St. Louis. The ultimate goal is to calculate the cost effectiveness of stock enhancement, including determining the optimal size, locations, and time of year for releasing reared spotted trout. Impacts on natural populations are also monitored using genetic analyses. The CMAC has built strong collaborations with recreational anglers and conservation groups like CCA, and actively participates in several local fishing tournaments.

Figure 4. Tagging and releasing spotted seatrout.

Red Snapper Project

The red snapper, *Lutjanus campechanus*, supports major commercial and recreational fisheries in the Gulf of Mexico. The species observed a significant decline between the 1970s and 1990s and is subject to close management and severe harvest restrictions in US waters. NOAA's National Marine Fisheries Service has designated red snapper as an overfished species. The red snapper is considered a primary candidate for US marine finfish aquaculture for both stock enhancement and commercial production.

GCRL began developing red snapper aquaculture in the early 2000s and is currently the only facility that

has the capacity to grow red snapper larvae (Ogle and Lotz 2006). Red snapper larvae are especially challenging to culture because they require very small nauplii of copepods as an initial live feed. The production of these copepod food items is itself difficult and still performed at an experimental scale.

Red snapper eggs are currently obtained by hormonal induction of ovulation and spermiation in pre-mature females and males caught in the wild (Figure 5). Eggs and sperm are collected by hand stripping and the eggs are fertilized *in-vitro* with the sperm to produce embryos. Fish acclimated to captivity engage in gametogenesis but inhibitions of gamete maturation have been observed and spontaneous spawning of captive broodstocks is inconsistent and often leads to unfertile spawns (Bardon et al. *in press*). Larvae are fed invertebrate live prey (copepods, rotifers and artemia) for 30-40 days before being weaned on dry feed and grown to tagging size. Research on the nutritional requirements of larval red snapper is in progress (Saillant et al. *in press*).

Figure 5. Hormone injection and stripping of red snapper broodstock.

Juveniles produced for stock enhancement are tagged using coded-wire tags or identified using genetic tags, and released on offshore reefs monitored by MDMR to evaluate their survival and recruitment to the fishery. Grow-out of red snapper to market size is also being evaluated in intensive closed, recirculating systems used by CMAC.

Current program objectives include increasing the production capacity of juveniles for stocking on Mississippi offshore reefs, developing captive spawning technologies, and improving husbandry methods for the production of juvenile fish for stock enhancement as well as market size red snapper.

Live Feed Culture Project
The culture of many marine fish species requires the concurrent culture of multiple species of live feed, e.g., algae and micro-crustaceans (Benetti et al. 2008). The easiest marine fish species to culture require only artemia nauplii, usually enriched, while other species require both enriched artemia and enriched rotifers. Several different "strains" of rotifers have been used. The most difficult marine fish species to culture require copepods either due to the smaller size required by the fish's small mouth (gape) or the superior nutritional quality of copepods compared to S- or SS- rotifers.

Algae
The CMAC dedicates several areas to marine microalgae culture and uses the same basic protocol. The algae, *Isochrysis galbana* (T. iso) is grown in 18 L clear bags suspended in front of two rows of fluorescent grow lights. Nutrients and carbon dioxide are added as the cultures grow and each bag is harvested on day 10. The Red Snapper Larval Rearing Algae Room is capable of producing 150 bags (Figure 6); the Visitor's Pavilion Algae Room can produce another 40 bags. The algae are used almost exclusively to feed copepods.
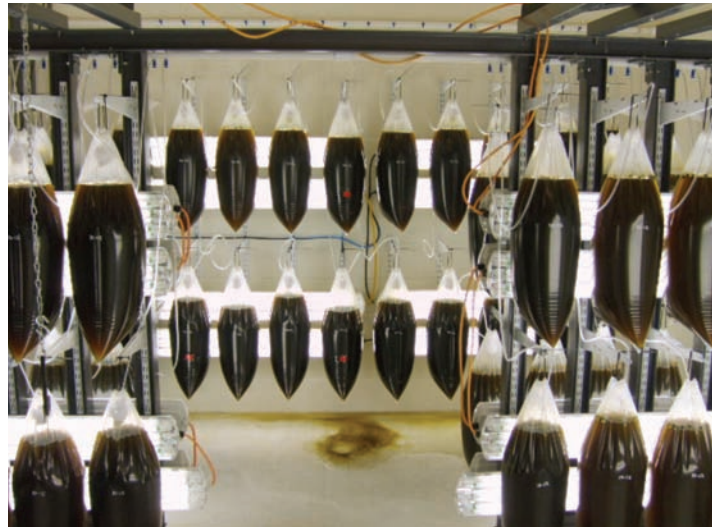

Figure 6. Algae Culture Room. *Isochrysis galbana* (T. iso) is grown in 18 L bags with continuous light.

Rotifers and Artemia
Rotifers are cultured in 100 or 200 L cone-bottom tanks. Cultures are maintained at densities ranging from 2,500 to 3,000 rotifers per ml. The rotifers are fed continuously with algal paste (Rotigrow, Reed Manufacturing). They are harvested daily and enriched before being fed to the marine fish larvae. The CMAC has a capacity to produce approximately 750 million rotifers per day. *Artemia* are hatched in 500 L cone-bottomed tanks (6 total). They are hatched as needed and enriched before feeding to the marine fish larvae.

Copepods
The CMAC copepod systems evolved from a wild harvest system based in several outdoor 60 metric ton tanks (Ogle et al. 2005) to a two-phase, batch

tank system in a greenhouse to the current biosecure, indoor batch/continuous system described here. All current production is located indoor in biosecure laboratories, ensuring that no pathogens are introduced with the copepods. This type of biosecure system is unique to CMAC.

The CMAC Large-scale Copepod Production System (Figure 7) first became fully operational in the Wet Laboratory Building in 2012 (Sarkisian et al. 2013). This system increased our production capacity in 2012 by 100% and saved labor needed to operate by more than 50%. We produced approximately 25 million copepod eggs/d and provide 6-12 million nauplii/d for feeding to the red snapper larvae. We significantly increased production and simultaneously optimized labor required to produce copepods. A similar but larger system is currently operational in the Red Snapper Broodstock and Larval Rearing Building. The combined Wet Laboratory and Red Snapper Production Systems will give us the capacity to produce over 30 million eggs/d, 10-15 million nauplii/d, and 5-10 million older stage copepods/d.

Figure 7. Large–scale copepod production in the Wet Laboratory Building. Upper tanks are Growout tanks stocked with eggs. Lower tanks are Egg Production tanks that are stocked with adults from Growout tanks. Small tank at bottom is the Collection tank where eggs are collected in a submerged plankton net.

Recirculating Seawater Technology Project
A critical component of our responsible approach to marine aquaculture is the application of modern filtration technology to develop closed, recirculating seawater systems for hatchery, nursery production, and broodstock tank systems. Biosecurity is another critical component of our responsible approach and goes hand in hand with closed seawater systems. This means that all seawater systems no matter how small or large are filled with water constituted with artificial sea salts and include particle filters, biological filters,

protein skimmers, and some type of sterilizer. Small systems usually include ultraviolet sterilizers and larger production systems include ozone injection. Most systems are temperature controlled with heat pumps and all buildings have oxygen piped from a central liquid oxygen tank for high production or emergency situations.

The only net seawater losses are due to filter backwashing. Due to the scale of the CMAC, a significant amount of water is lost in backwashing. For this reason, the CMAC has constructed an engineered, multi-trophic reclamation system. The reclamation system (Figure 8) is centrally located on the CMAC site. Backwash water from any tank system can be transported to the reclamation system and the processed water can then be stored in a reservoir and transported back. The reclamation system is composed of geotextile bags to separate liquid and solid effluent. The solid effluent retained in the geobag is dried and used as a substitute for commercial fertilizer in the production of salt marsh plants for wetlands remediation. A propeller-washed, floating bead filter, protein skimmer, and ozone further process the liquid fraction of the effluent before circulating it through an aquaponics greenhouse where salt tolerant plants reduce the dissolved nutrient level.

Marine Shrimp Farming Project
The CMAC and GCRL support acceleration of United States and Mississippi marine shrimp farming industries. The GCRL was a charter member of the U.S. Marine Shrimp Farming Program that began in 1985 with the support of the US Departments of Commerce and Agriculture. Under this program, GCRL developed a national and international reputation as a center in (1) closed-system water-reuse

Figure 8. Multitrophic Reclamation System used to reclaim high nitrogen, high sediment wastewater. Upper left is the bead filter for initial particle removal followed by geobag in upper right for fine particle entrapment. The water then flows through seagrasses in the greenhouse for nitrogen removal (lower picture).

shrimp culture, (2) disease detection and prevention and (3) reproduction research. As a result, GCRL has transferred aspects of these technologies to commercial seed production facilities world-wide. In 2004, a commercial-scale, next-generation greenhouse culture facility consisting of 12- 80 metric ton raceways was constructed and operated (Ogle et al 2006). The Marine Shrimp Farming Project became a project managed by the CMAC recently as the center expanded; it attracts graduate and postgraduate students for advanced study and provides technical support for the national marine shrimp farming industry.

New Species Development Project

The Aquaculture Demonstration Building will be the principal site for new species development, but the Aquaculture Visitor's Pavilion has been used for this purpose. This is the last of the programs to be implemented since the construction of buildings and tank systems and the operation of the Seatrout and Red Snapper programs have dominated CMAC's attention for the last few years. Currently, CMAC is developing methods for Atlantic croaker and tripletail. The CMAC has been successful culturing the former (Sarkisian et al. 2010; Leclercq et al. *in press*) but spawning has been an obstacle for the latter. Future species that CMAC has targeted include amberjack, grouper, and tuna species.

Project Summary

- Spotted Seatrout
  - Captive broodstock established
  - High survival of larval fish and decreased cannibalism
  - Successful releases at four Mississippi sites, >500,000
  - Genetic monitoring of wild populations initiated
- Red Snapper
  - Successful spawning of wild fish with hormone injection
  - Initial success in larval rearing with copepods as first food
  - Successful release on offshore reefs
- Live Feed Culture
  - Established dependable protocols for rotifer and artemia production
  - Significantly expanded algae and biosecure copepod production
- Recirculating Seawater Technology Development
  - Improved water quality for larval rearing
  - New closed, recirculating production systems
  - Established biosecurity
  - Implementation of Engineered, Multi-Trophic Reclamation System
- Marine Shrimp Farming
  - Designed and constructed closed-system, water-reuse shrimp culture systems
  - Improved disease detection and prevention
  - Reproduction of local and exotic white shrimp through the life cycle
- New Species Development
  - Tripletail, Croaker, Amberjack, Tuna

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Feeding Hatchery-Produced Larvae of the Giant Grouper *Epinephelus lanceolatus*

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ABSTRACT

Research on larval rearing of giant grouper *Epinephelus lanceolatus* was carried out at the Pacific Aquaculture and Coastal Resources Center, University of Hawaii at Hilo. Fertilized eggs from captive broodstock were stocked in an intensive production system in three 1 m³ round tanks with controlled aeration, temperature of 27.6 ± 0.2 °C, and salinity of 29.5 ± 0.3 ppt. Grouper eggs hatched 30 hours post-fertilization under these conditions, at day post hatch (DPH) 2 yolk sac larvae had a mean total length of 2.4 ± 0.2 mm . Two feeding strategies were tested: in Tank 1, trochophore larvae of the Pacific oyster (*Crassostrea gigas*) were fed to the fish in DPH 2 and 3, then feeding shifted to enriched S-type rotifers (*Brachionus rotundiformis*) in DPH 4. In Tanks 2 and 3 first food consisted of a mix of calanoid copepods (*Parvocalanus crassirostris*) and enriched S-type rotifers, both were fed to the fish twice every day starting in DPH 3. At DPH 6 massive fish mortality occurred in the trochophore-rotifer treatment (Tank 1) as apparently the fish were not able to ingest the rotifers, in DPH 8 mortality was total with this treatment. Fish in the mixed feeding regime in Tanks 2 and 3 presented better growth and survival. At DPH 10 a co-feeding treatment was started in Tank 2, the same was done in Tank 3 at DPH 17 with a microdiet of 200-300 *µ*m particle size. In DPH 12 and 13 high fish mortality occurred in Tanks 2 and 3 due to fish being trapped in the water surface, sprinklers were used to improve fish survival and prevent this problem. Starting at DPH 14, instar I nauplii of *Artemia* was supplied to the fish tanks followed by a mix of instar I nauplii and enriched metanauplii two days later. *Artemia* was fed two times per day. At DPH 20 the supply of rotifers and copepods to the tanks was terminated. Grouper larvae presented high growth rates during the *Artemia* feeding stage. Metamorphosis started at 25 DPH and at 35 DPH it was not yet completed with larvae remaining pelagic with a mean total length of 27.3 ± 2.5 mm. Aggressive behavior and cannibalism was observed starting at DPH 30, but it was not extensive. As larvae started to settle in the bottom, fish shelters were provided for protection. Weaning was initiated at 35 DPH and ended ten days later. Survival from hatched to pre-weaned larvae was estimated at 2.1%.

INTRODUCTION

The giant grouper *Epinephelus lanceolatus* is one of the largest teleost fish inhabiting coral reefs. This species is also found in estuaries and harbors, and can grow to over 2 meters in length and 400 kg in weight. It is the most widely distributed grouper species in the world, occurring in the Indo-Pacific region from the east coast of Africa to the Hawaiian Islands, and from southern Japan to Australia (Heemstra and Randall, 1993). *E. lanceolatus* is one of the two grouper species native to Hawaii, but it is extremely rare in Hawaiian waters. It is an important species in subsistence fisheries in the Pacific Islands and is highly appreciated in the live reef food fish trade in Southeast Asia, which supplies the Honk Kong market. Due to the decline of giant grouper wild populations, the species has been classified as vulnerable and is currently a protected fish species in several countries (Shuk Man and Ng Wai, 2006).

Aquaculture production of giant grouper was achieved in Taiwan in 1995 (Chang, 2009), and the species is currently the subject of aquaculture research in Australia, Vietnam, Philippines and the United States. However, limited information exists on *E. lanceolatus* production by aquaculture. As occurs in the culture of many other marine fish, the most challenging aspect in the larval rearing of giant grouper is the high mortality occurring when larval fish are not supplied with live food of adequate size and nutritional quality at the start of exogenous feeding. One strategy to deal with this problem is the provision of a wide diversity of live food organisms by fertilization of pond water (Tew et al., 2013). The larviculture methods for giant grouper applied in Taiwan include the use of microalgae, oyster eggs, mussel trochophore larvae, rotifers, copepods and *Artemia* (Pierre et al., 2007).

Aquaculture provides opportunities for the conservation and restocking of depleted populations of giant grouper, the reduction of fishing pressure on wild populations, and diversification of fish production. Nonetheless, there are major constraints in the expansion of grouper culture which include the unreliable supply of juveniles and difficulties of spawning in captivity. Of all groupers consumed in China and SE Asia, only 15–20% originates from fish farms (Tupper and Sheriff, 2008). The importance of the production of grouper larvae and juveniles through aquaculture has been highlighted in the International Standard for the Trade in Live Reef Food Fish (WWF, 2004), in which preference is given to the use of hatchery-reared fingerlings for live reef fish aquaculture. Therefore, the aim of the present study was to contribute to the development of hatchery technologies for the culture of giant grouper by testing a larval rearing protocol using calanoid copepods, oyster trochophore larvae and small size rotifers at the start of exogenous feeding.

METHODOLOGY AND RESULTS
Eggs Incubation and Hatching

Research on the larval rearing of giant grouper was carried out in the fish hatchery at the Pacific Aquaculture and Coastal Resources Center (PACRC), University of Hawaii at Hilo. Fertilized eggs from captive broodstock were obtained from Kampachi Farms, Kailua-Kona, Hawaii. Approximately 36,000 viable eggs were transported, acclimatized to local in-door conditions and stocked (12,000 eggs per tank) in three round tanks with central drain and conical bottom filled with one cubic meter of filtered seawater. Soft aeration in each tank was provided by a centrally-placed air stone, temperature was constantly maintained at 27.6 ± 0.2°C by means of heaters, and salinity was 29.5 ± 0.3 ppt during the incubation period and larval rearing. Constant illumination over the tanks provided a photoperiod of 24L:0D from day post-hatch (DPH) 1 to 20, which was then changed to natural photoperiod of 12L:12D. The daily water exchange in the tanks was progressively increased from 10% in DPH 1 to 300% at DPH 24. Tanks were cleaned every day by siphoning out waste from the bottom and scooping out debris from the surface. The fertilized giant grouper eggs had a mean diameter of 0.89 ± 0.01 mm and hatched 30 hours post-fertilization. Fish growth (total length) was determined with a calibrated slide under a stereoscopic microscope throughout the larval development. Fish larvae survival was also monitored.

Feeding with Live Food Organisms and Co-Feeding

At DPH 2, the yolk sac larvae had a mean total length of 2.4 ± 0.2 mm, the mouth and anus were closed, and half of the yolk was still present, by DPH 3 the mouth opened (Figure 1). The microalgae *Isochrysis galbana* was supplied every day to all tanks from DPH 1 to 20 at a density of 30,000 to 45,000/ml. Two feeding strategies were tested to deal with the first exogenous feeding. In Tank 1, hatchery-produced trochophore larvae of the Pacific oyster (*Crassostrea gigas*) were fed in DPH 2 and 3, then at DPH 4 the feeding was shifted to enriched S-type rotifers (*Brachionus rotundiformis*) at a density of 20/ml. The size range of the rotifers was 90-130 μm. In Tanks 2 and 3, the fish food consisted of a mix of calanoid copepods (*Parvocalanus crassirostris*) (0.5 to 3.0/ml) and enriched S-type rotifers (10/ml), both were fed to the fish twice every day starting at DPH 3. After each feeding the water flow was stopped for one hour. Size range of copepods fed to the fish was 60-800 μm, meaning that females carrying eggs were present in the larval tanks releasing nauplii and copepodites into the water. Rotifers were cultivated in 180 L cylindrical tanks and fed Roti-Grow Plus (Reed Mariculture, USA) at a rotifer density of 400-500/ml. Enrichment of rotifers was done in separated tanks with Ori-Green (Skretting, Canada) for a minimum of 3 hrs. Copepods were cultivated in multiple 200 L tanks and fed mixed microalgae, copepod density in the tank was 20-30/ml and they were harvested every day.

Figure 1. Larvae of giant grouper *Epinephelus lanceolatus* in day-post hatch (DPH) 3 with a total length of 2.9 mm. At this stage the mouth was open. Each side of the squares in the background of the larval fish pictures corresponds to 1 mm.
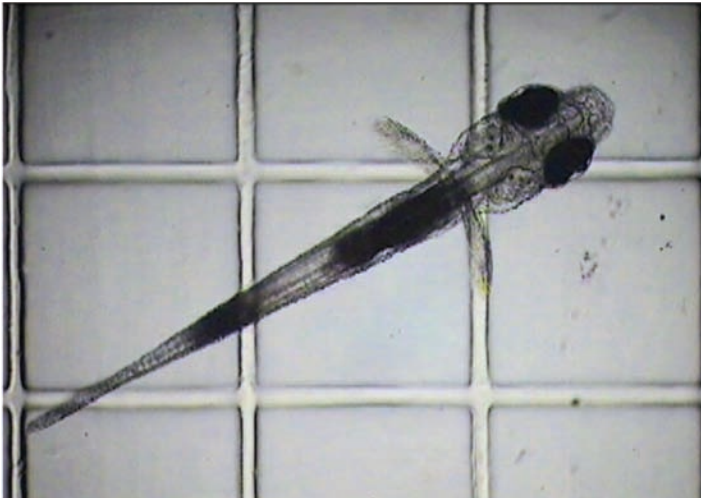
Figure 2. Giant grouper larvae with the yolk sac completely resorbed at DPH 4. Some food was present in the digestive tract. Total length in this stage was 3.0 mm.

Figure 4. At DPH 9 the grouper larvae started to develop the two ventral or pelvic spines. Mean total length was approximately 3.5 mm.

At DPH 4 the yolk sac in larvae measuring 3.0 ± 0.0 mm was completely resorbed (Figure 2), however, food was already present in the digestive tract of fish in the mixed copepod/rotifer feeding. In DPH 6 massive larval fish mortality occurred in Tank 1 where rotifer were the only food source as the fish were not able to ingest the rotifers, by DPH 8 mortality was total in this feeding treatment. In contrast, fish in the mixed feeding regime (Tanks 2 and 3) were actively feeding with full guts, which was confirmed by microscopic observations as the larvae had a transparent body (Figure 3). At DPH 9 the larvae continued eating live foods and were evenly distributed in the water column. At this age that the two ventral or pelvic spines started to develop in fish larvae (Figure 4). At DPH 10 and a length of 3.7 ± 0.2 mm, a co-feeding treatment was initiated in Tank 2 with a commercial fish microdiet (O-range, INVE Aquaculture, USA) of 200-300 μm particle size. The microdiet was offered in excess to the fish four times every day.
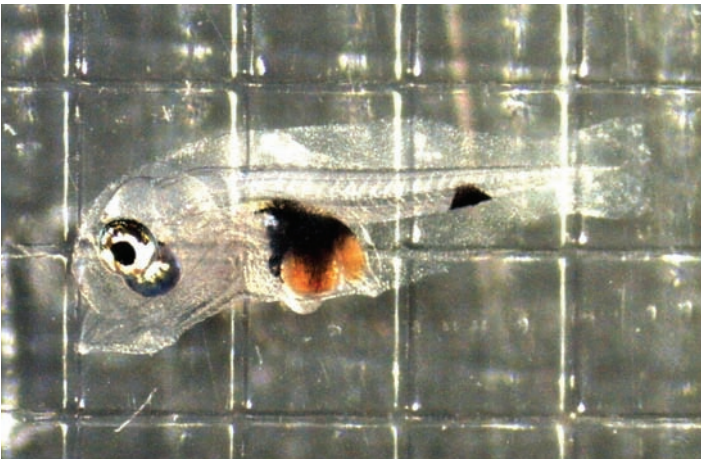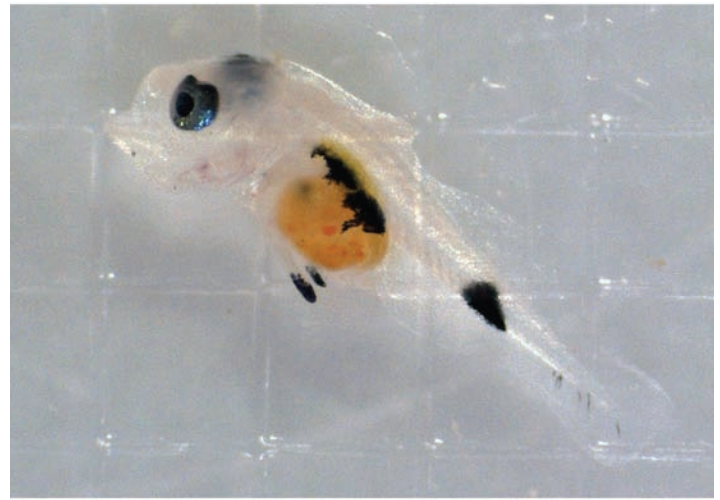
Between DPH 12 and 13 at an approximate body length of 4.0 mm, significant fish mortality occurred in Tanks 2 and 3. During this period the fish larvae were swimming actively around the tank, with some fish grazing for food in the tank wall. However, some fish larvae tended to swim towards the water surface probably to inflate the swim bladder. However, some fish were not able to break the water tension due to long dorsal and ventral spines which significantly increased their length at this stage. This caused the fish larvae to lean and get trapped on the water surface, fish were not able to swim downwards and died. A method using rotating water sprinklers prevented the fish larvae from getting trapped on the surface, these devices proved essential to improve fish survival at this stage. In each tank one sprinkler was placed at the center 20 centimeters above the water surface and was constantly spraying most of the surface (Figure 5).

Figure 3. Digestive tract full of food in giant grouper larvae was evident at DPH 8.

Figure 5. In each larval tank one small rotating sprinkler was placed at the center of the tank to prevent mortality of grouper larvae on the water surface. Grouper larvae survival was significantly improved with this method.

In DPH 14 when the fish larvae length reached 5.1 ± 0.3 mm (Figure 6), the microdiet fed to the larvae was increased in particle size to 300-500 μm. Despite being fed the same mixed rotifer/copepod ration, growth of fish larvae in Tank 2 (co-feeding treatment) was higher than in Tank 3 (only live food, no co-feeding). Therefore, at DPH 17 co-feeding was also applied in Tank 3. The supply of instar I nauplii of *Artemia* (5/ml) was initiated on DPH 14 in Tank 2, two days later a mix of instar I nauplii and enriched metanauplii (1-3/ml) was fed to the fish. Feeding instar I *Artemia* was done until DPH 35, and feeding enriched *Artemia* was done until the end of weaning. The same *Artemia* feeding protocol was applied in Tank 3 starting at DPH 17 (Figure 7). Decapsulated *Artemia* cysts (GSL) were incubated in brine shrimp hatchers (Aquatic Ecosystems, USA) and enriched with DHA Selco (INVE Aquaculture) for 24 h. Non-enriched instar I and enriched *Artemia* were fed two times per day. At DPH 20 the supply of rotifers and copepods to the tanks was terminated and the only food provided to the fish was enriched *Artemia*. In general, grouper larvae presented high growth rates during the *Artemia* feeding stage (Figure 8). At DPH 27 the larvae had a mean length of 16.5 ± 0.8 mm (Figure 9) and distributed all around in the lower half of the tank swimming in a clockwise direction.

Figure 8. Growth curve (total length) of giant grouper *E. lanceolatus*, and the applied feeding protocol during the larval stages. In general, live food was provided twice a day and the microdiets four times per day (see text for details).

Figure 9. A few days before DPH 27 grouper larvae started the pigmentation of the head region. The dorsal and ventral rays reached their maximum size at this age. Mean total length was 16.5 mm.
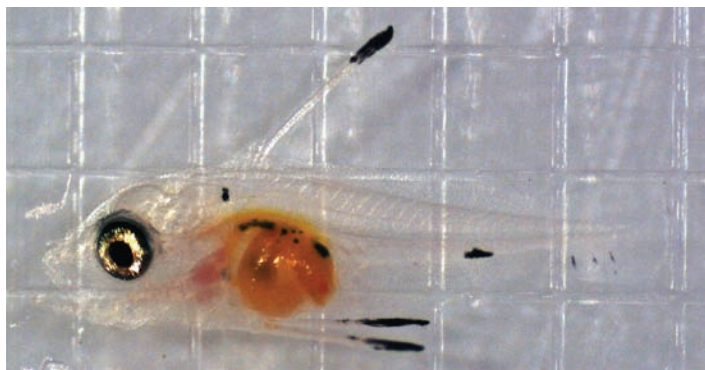

Figure 6. Larvae of giant grouper at DPH 14 reached a mean total length of 5.1 mm. At this stage the dorsal and ventral spine were well developed and growing.
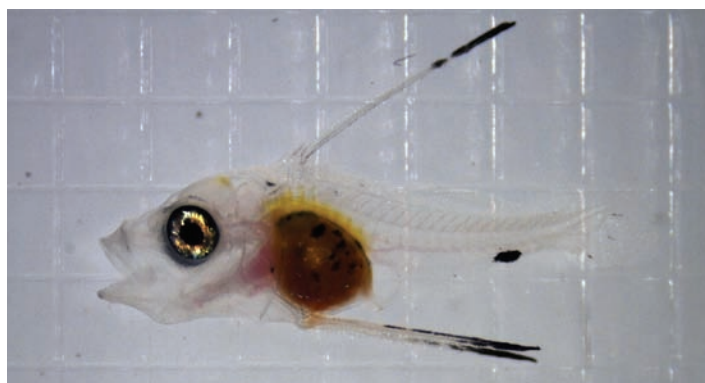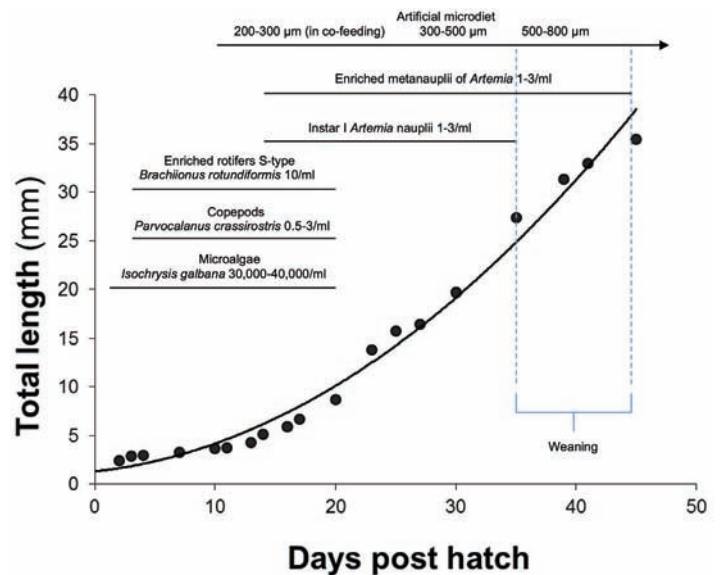

Figure 7. The grouper larvae at DPH 17 was feeding on instar I *Artemia* nauplii and enriched metanauplii. Larval body at this stage was not pigmented. Mean total length was 6.7 mm.

Aggressive Behavior/Cannibalism

Aggressive behavior in grouper larvae was first observed around DPH 30, when larger fish started to chase around smaller fish, biting their caudal tail but not swallowing them. In this period, most fish tended to settle in the bottom. Fish cannibalism or big fish swimming with a smaller fish in their mouth, was first observed at DPH 36. However, it was not common and only a few larger fish were observed with this behavior. If the aggressor fish was tipped with the finger or a hard object, it released the larvae in their mouth. It was interesting to observe that aggressive fish did not attack fish of a similar size. Fish sorting by size, or removing larger fish from the tank helped to

reduce the aggressive behavior, which was observed until DPH 41. Another applied measure to prevent fish mortality by aggressive behavior was the setting of fish shelters in the tank. This was done by placing "fish condos" in the tank, i.e. groups of seven pieces of one inch PVC pipes of approximately 15 centimeters in length where fish could hide from aggressive fish (Figure 10). The diameter of the PVC pipes was increased according to the size of the fish. Survival from hatched fish to pre-weaned larvae under the described conditions was estimated at 2.1%.
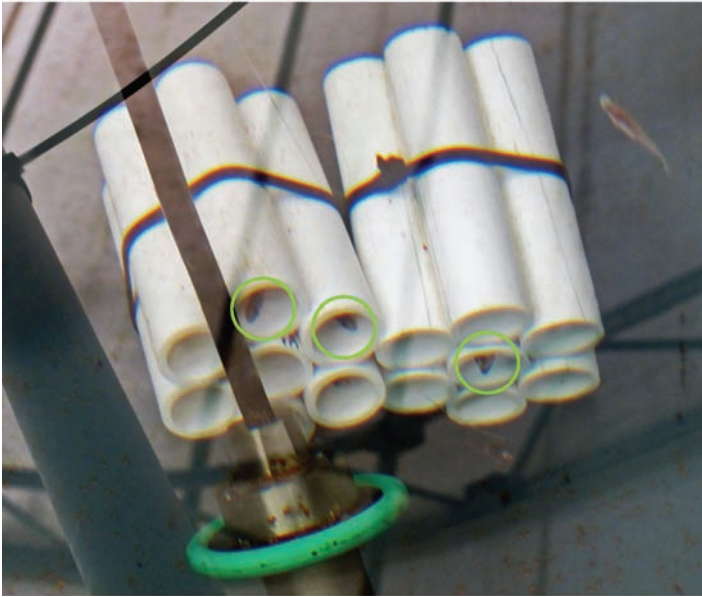

Figure 10. At DPH 30, aggressive beahavior and cannibalisms was observed in some of the larger groupers in the tank. Fish "condos" made of PVC pipes were placed near the bottom of the tank and next to the wall to provide shelter for smaller fish. The circles in the picture indicate fish occupying the shelters.

Fish Metamorphosis and Weaning

Metamorphosis started at DPH 25 when the head region started pigmentation, ten days later metamorphosis was not yet completed as the fish larvae remained pelagic. The body of fish remained partially transparent until approximately DPH 35, in DPH 38 at approximately 30 mm of length, fish body was observed to be completely pigmented with at least two bands of pigments across the posterior half of the body (Figure 11). At around DPH 41, the majority of fish exhibited benthic orientation. Fish seemed to look for protection from other fishes in the bottom of the tank or in the PVC pipes, and only moved along the water column when they were searching for feed particles. In some species of carnivorous marine fish, the end of metamorphosis indicates the time when fish are able to be fed exclusively on inert microdiets. For the giant grouper in this study, weaning was initiated at DPH 35 and lasted 10 days, within the period when they completed metamorphosis. A mix of a commercial microdiet with particle sizes 300-500

and 500-800 μm was used in the first days of weaning. During the weaning period, a daily reduction of 10% of the *Artemia* ration was applied, and the ration of the microdiet was increased as the larvae adapted to it. In the first feeding of the day, the microdiet was manually supplied to the fish followed half hour later by the enriched *Artemia*. During weaning the inert microdiet was supplied four times per day and the *Artemia* twice a day. It is important to note that grouper larvae were not attracted to floating feed particles, highlighting the importance of using submersible or sinking diets for weaning and post-weaned giant grouper. At DPH 45 and a mean total length of 35.4 ± 1.1 mm, the live food was completely removed from the feeding protocol and fish were completely weaned. Survival after weaning was approximately 70% of the pre-weaned fish. A feed particle size of 800-1200 μm was added to the microdiet mix as the bigger fish were ingesting it actively. At this stage, grouper juveniles were completely metamorphosed (Figure 12).

Figure 11. Around DPH 38 and 30 mm of total length, fish body was completely pigmented with at least two bands of pigments across the posterior half of the body. A few days later most of the grouper larvae exhibited benthonic behavior.
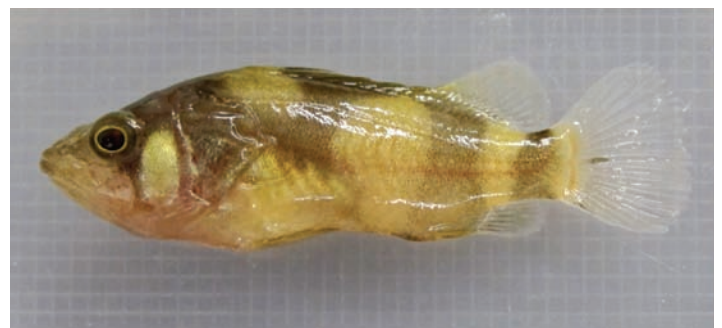

Figure 12. At DPH 45 giant grouper larvae were completely weaned. At this stage, grouper juveniles were completely metamorphosed at 35.4 mm of total length.

DISCUSSION

At present, no significant aquaculture production of giant grouper occurs outside Taiwan. This work describes the first larviculture of giant grouper at PACRC where research to produce local species of marine fish for aquaculture is currently in progress. The results of this study contribute to knowledge of grouper aquaculture in the United States, which has previously focused on the culture of Nassau grouper *E. striatus* (Powell and Tucker, 1992; Tucker and Woodward, 1996; Watanabe et al., 1996). Development of *E. lanceolatus* larvae was similar to that reported for the red grouper *E. morio* larvae (Colin et al., 1997). The larval rearing methods applied in giant grouper culture are similar to those applied for other groupers of smaller size, e.g. Malabar grouper *E. malabaricus* (Tookwinas, 1989), orange-spotted rockcod *E. suillus* (Duray et al., 1997), red-spotted grouper *E. coioides* (Doi et al., 1997; Meei Su et al., 1997), longtooth grouper *E. bruneus* (Sawada et al., 1999), and a hybrid of *E. coioides* and *E. lanceolatus* (Kiriyakit et al., 2011). Larval feeds for these species generally consist of small size live food organisms such as oyster trochophore larvae, nauplii of copepods, and small strain rotifers at first exogenous feeding. Copepods have been used with success in the culture of grouper larvae (Rimmer et al., 2011) and larvae of other marine fish species, where they constitute the most important nutritional factor in improving fish growth and survival during the early post-yolk sac larval stages. Another critical period during larval rearing of giant grouper in the hatchery is the time of swim-bladder inflation, when fish swim to the water surface and can become trapped due to the surface tension. This phenomenon has also been described in other grouper species as "surface aggregation mortality" (Sugama et al., 2012). In our study this problem was satisfactorily solved by the use of water sprinklers, which also helped in the water aeration and water exchange in the larval tank. Fish survival during weaning could be improved by postponing at least one week the start of weaning (García-Ortega, 2009), which in the present study was initiated at DPH 35. Although delaying the time of weaning increases production costs because live food is used for a longer period, increased fish survival may compensate for those higher costs. This is the case with the giant grouper, which can attain retail prices of about US$ 169/kg in the Honk Kong market (Shuk Man and Ng Wai, 2006).

Due to the environmental conditions and current restrictions in the use of coastal areas in the United States, the production of marine fish species in intensive recirculating production systems seems to be the most promising alternative for grouper culture (Tucker, 1999). The vulnerable status of many species of groupers around the world encourages their production by aquaculture, for conservation purposes or to supply fish markets. In this study the feasibility of giant grouper fry production in Hawaii was demonstrated. Further work is needed to refine protocols for the production of the species under controlled conditions in land-based hatcheries.

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The authors of this paper review multiple spawning, larval rearing and growout trials for the Nassau grouper (*Epinephelus striatus*). They were able to successfully spawn the grouper by multiple methods including combinations of induced (HCG) and natural ovulation along with artificial and natural fertilization. During multiple larval rearing trials using different size tanks, feeding regimes, and other parameters the best survival recorded was 5% from fertilization to 98 dph. During growout grouper reached a mean weight of 1.5 kg at 23 months with a respective food conversion ratio of 0.9-1.3. At 28 months the mean weight was 2.0 kg with a food conversion ratio of up to 1.8.

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This paper represents one of the first successful spawning and larval rearing trials for the red grouper (*Epinephelus morio*). This paper offers an in-depth description of larval development of this grouper from egg to juvenile focusing on morphometrics, pigmentation and behavior. Furthermore, this paper describes how salinity and temperature affects growth and development. Eggs averaged 0.95mm diameter and hatched in ~30 hours at 24°C with metamorphosis occurred starting 35 days post hatch at a size of 20 mm standard length.

Kiriyakit A., W.G. Gallardo, and A.N. Bart. 2011. Successful hybridization of groupers (*Epinephelus coioides* x *Epinephelus lanceolatus*) using cryopreserved sperm. Aquaculture 320: 106-112.

In this recent paper the larval rearing of a grouper hybrid from *E. coioides* eggs and *E. lanceolatus* sperm is described. Feeding of hybrid larvae was progressively done using oyster trocophore larvae, rotifers and *Artemia*. This is an important paper because little if any information exists in the larval rearing and development of the giant grouper. In addition, this paper reports rotifers were used without copepods in the successful larval rearing of this hybrid, which was not achieved in our study with *E. lanceolatus*. Additional information is provided on the larval development and metamorphosis of the hybrid fish that in some aspects is similar to the developmental stages we observed during in the larval rearing of *E. lanceolatus*.

Conditioning Technologies for Flatfish Stock Enhancement: Global Progress and Pitfalls

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ABSTRACT

Hatchery-reared flatfish (e.g., flounders, halibuts, soles) often exhibit irregular swimming, feeding, and cryptic (burying and color change) behavioral patterns compared with wild conspecifics. These behavioral "deficits" are assumed to lead to increased predation risk once fish are released. Conditioning flatfish before release may offer fish an opportunity to refine these behaviors, which may increase survival and subsequent recruitment to the fishery. Examples of conditioning strategies that have been applied in the hatchery include providing sediments in rearing tanks, live feeds, or predator cues. Providing winter flounder, *Pseudopleuronectes americanus*, with live white worms, *Enchytraeus albidus*, during rearing conformed post-release feeding performance closer to that of wild fish. Strategies also can be applied at the release site, such as short-term release into acclimation cages before full release. Danish scientists have been cage-conditioning reared Atlantic turbot, *Psetta maxima*, before release since 2004, and this practice has resulted in lower post-release mortality. Stocking facilities in Japan have found that cage-conditioned Japanese flounder, *Paralichthys olivaceus*, have significantly higher fishery recapture rates and enhanced burying and feeding skills. In both Denmark and Japan, studies indicate that intensive recollection efforts near release sites may disproportionately sample weaker fish. In the U.S., evidence suggests that cages themselves attract predators, so cage designs have been modified in recent years. Successful conditioning of stocked fish before release can increase post-release recapture. However, choosing a location that can be monitored adequately may be just as important if the success of a stocking effort will influence future efforts.

INTRODUCTION

A successful stocking program requires survival of released fish; and to achieve this, released, hatchery-reared fish must be able to adjust to their new environment, feed successfully, and avoid predation (Howell, 1994). However, all reared fish are not equal. The end products of fish reared for aquaculture and those reared for stock enhancement vary greatly. For aquaculture, the ideal end product is a large-sized individual that was grown most economically (in both a financial and temporal sense) with preferably a pleasant flavor. For stock enhancement, the ideal end product is an individual that can survive in the wild until recruitment (e.g., to the fishery; to the spawning stock). Conditioning fish for stock enhancement can increase survival and recapture rates of released fish.

In the last 20 years, great advancements have been made in formulated feed production, including the design of feeds that take longer to break down in water or contain more non-fishmeal based ingredients, as well as feeds accepted by younger and smaller stages of fish. These are great feats for aquaculture. However, how do these advances in formulated feeds affect fish reared for stock enhancement - other than increasing the temporal gap between the last time reared fish may have seen live prey in the hatchery (probably in the larval or early juvenile stages) and encountering them as released fish in the wild? The degree to which fish reared for stock enhancement may need conditioning before release will depend on the type of fish reared and its particular ecological niche and associated behaviors.

Global Progress

Japan is the only country that has been successful in exhibiting high recapture rates of stocked flatfish (up to 30%; Fujita, 1996), economic profitability of stocking efforts (Kitada et al., 1992; Kitada, 1999), stabilization of fisheries catch (Yamashita and Aritaki, 2010), and evidence of biological contribution to wild spawning stocks (Kitada and Kishino, 2006). Indeed, the governing structure of Japan (localized prefectures) has contributed to such successes. Japanese stocking efforts are funded primarily by taxes from citizens and sales income of fishermen (Yamashita and Aritaki, 2010). Although Japanese scientists suggest that feeding behavior of hatchery-reared Japanese flounder can be made more natural by conditioning fish with live or more realistic feeds and by providing fish with sandy substratum before release (Tanaka et al., 1998), few hatcheries engage in conditioning strategies for flatfish stock enhancement. Exceptions include Obama Laboratory, Japan Sea National Fisheries

Research Institute, Fisheries Research Agency, which has been conducting pre-release, experimental cage conditioning for Japanese flounder since 2008 (Walsh et al., 2012; 2013), and Hyogo Prefectural Center for Stock Enhancement, which provides hatchery-reared juvenile marbled flounder with a diet of frozen mysids before release (T. Minamiura, Hyogo Prefectural Center for Stock Enhancement, pers. comm.).

Experimental releases of Atlantic turbot, mostly financed by the European Union, national governments, and the fishing industry, have been conducted in Belgium, Norway, Spain, and the United Kingdom totaling approximately 36,000 fish (Ellis and Nash, 1998; Danancher and Garcia-Vazquez, 2007; Støttrup and Sparrevohn and 2010). Ellis and Nash (1998) showed that releases significantly increased abundance of the local population. Releases of Black Sea turbot, *Psetta maeoticus,* by Russia and Turkey in the 1990s consisted of over 165,000 fish, and evidence suggests that these releases contributed to increased fish abundance in subsequent years (Maslova, 2002; Støttrup and Sparrevohn, 2010). In Denmark, stocking of Atlantic turbot, European plaice, *Pleuronectes platessa*, and flounder, *Platichthys flesus,* has been conducted since 1988, totaling over 3 million released flatfish to date (Støttrup 2004; Støttrup et al., 2002; Støttrup and Sparrevohn, 2010). Danish stocking efforts are funded via fees charged for recreational fishing licenses through the National Coastal Fisheries Management Program (Støttrup and Sparrevohn, 2010). Since 2004, Danish scientists have been cage conditioning reared Atlantic turbot before release, and this practice has resulted in a much lower post-release mortality than when fish were not conditioned (Sparrevohn and Støttrup, 2008; Støttrup and Sparrevohn, 2010).

Currently in the United States, flatfish stocking consists of only smaller-scale and mostly experimental efforts. The only official, ongoing program, established by the Texas Parks and Wildlife Department in 2006, exists for southern flounder, *Paralichthys lethostigma*. Details regarding the success of that program, however, are unavailable, although approximately 20,000 fish have been released to date (Sikes, 2011; Tompkins, 2010). An experimental, small-scale release of summer flounder, *Paralichthys dentatus,* (N = 1500) was conducted in North Carolina in 1999, and released fish were conditioned to predators by exposure to caged adult blue crabs, *Callinectes sapidus,* prior to release (Kellison et al., 2003). Since 1999 experimental releases of winter flounder, *Pseudopleuronectes americanus,* have been conducted by the University

of New Hampshire (UNH; approximately 27,000 fish released in total to date), and in September 2012 approximately 5,000 fish were released into salt ponds surrounding Martha's Vineyard, Massachusetts, with local community involvement (Zeiber, 2011). UNH protocols have included cage conditioning of flounder before release since 2004; however, evidence arose that the cages themselves attracted predators, mostly crabs (Fairchild et al., 2008), so cage design has been modified in recent years.

TECHNOLOGIES
Marking Methods
In Japan, hatchery-reared Japanese flounder exhibit a high incidence of malpigmentation on their abocular side, which forms a natural marker of a stocked fish. These marks are found to some degree in over 95% of hatchery-reared fish, while they are rarely seen in wild fish (Tominaga and Watanabe 1998). Before stocking, fish with malpigmentation on the ocular side must be removed, as these visually evident individuals may draw predators to the release site and put normally pigmented fish at increased risk.

In Japan and Denmark, fish for stocking have been marked en masse by submerging fish in an alizarin compexone (ALC) dye bath to stain the otoliths. However, no external mark is evident on the fish and otoliths must be removed to detect the stain. Coded-wire tags have also been used to identify winter flounder juveniles (E. Fairchild, University of New Hampshire, pers. comm.). However the equipment for tagging and detecting tags is quite expensive (between $5000-$75,000 depending on the unit plus the cost of tags), and again, with these tags, no external mark is evident.

In Japan, when fish history such as size, time, or place of release needs to be tracked for recaptured fish, Japanese flounder are marked by fin cuts, chemical or fluorescent dyes, anchor tags, or genetic tags (Yamashita and Aritaki, 2010). Many stock enhancement facilities in Japan currently burn or brand individuals for release, and different patterns or locations of marks can be used to identify different groups of fish (Walsh et al., 2012; 2013). Survival rate of branded individuals is high if fish are over 7 cm and scar tissue can be evident years after release (Okouchi et al., 2004). In the U.S., young winter flounder are marked by neon colored visible implant elastomer tags, which allows distinction of different treatments of fish via the application of different colors, combinations of colors, or locations on fish. Similar marking of Japanese flounder with latex tags

was practiced in Miyako Bay, Japan, until 1988, when concerns regarding the consumption of tag residuals in captured fish led to the abandonment of this technique (Okouchi et al., 2004). In Denmark, when 1-year old turbot are released, fish are large enough to receive inexpensive anchor tags with individual identification numbers.

Conditioning Strategies

Conditioning can be implemented in the hatchery or in the wild (Table 1). In the hatchery, fish can be exposed to rearing tanks with sediment, predator exposure, or be provided with live or life-like diets for all or a portion of feed. In the wild, fish can undergo operant behavioral training, where fish can learn to gather at a sound or light for feeding. Fish also can be initially released into acclimation cages before true release in the wild (Sparrevohn and Støttrup, 2007; Fairchild et. al, 2008; Walsh et al., 2012; 2013).

Table 1. Conditioning strategies for stocking flatfish.

Location	Strategy	References
Hatchery	tanks with sediments	Tanda (1990); Ellis et al. (1997); Miyazaki et al. (1997); Tanaka et al. (1998); Kellison et al. (2000); Fairchild (2002); Fairchild and Howell (2004)
	live (or life-like) diets	Furuta (1996); Tanaka et al. (1998); Walsh et al. (2009)
	predator exposure	Kellison et al. (2000); Fairchild (2002); Hossain et al. (2002); Arai et al. (2007)
Release site	acclimation cages	Sparrevohn and Strottup (2007); Fairchild et al. (2008); Walsh et al. (2012; 2013)
	operant behavior conditioning	Anraku et al. (1998)

In the hatchery, providing rearing tanks with sediment allows flatfish to begin camouflaging pigment change and develop burying skills. In addition, the frequency of off-bottom swimming behavior in juvenile Japanese flounder was lower in tanks with sand than in bare tanks (Miyazaki et al., 1997).

In the U.S., summer flounder survival was significantly higher for fish reared with an isolated blue crab present in the tank than for those fish reared without the predator conditioning (Kellison et al., 2000). However, the increased survival of conditioned fish was not as high as that for wild fish. In Japan, observational learning improved predator avoidance for hatchery-reared Japanese flounder that witnessed predation of conspecifics (Arai et al., 2007).

At the University of New Hampshire in the U.S., investigation of live diets for flatfish stock enhancement has been explored (Walsh et al., 2009). Sub-adult brine shrimp (*Artemia* sp.), white worms (*Enchytraeus albidus*), and common burrower amphipods (*Leptocheirus plumulosus*) have been

cultured as feeds. The most promising feed is white worms, which have been reared in Russia since the 1940s for hatchery-reared sturgeon (Ivelva, 1973). In the field, hatchery-reared winter flounder grown on white worms exhibited similar survival, diet profiles, and RNA/DNA composition as wild fish (Walsh et al., 2009). In Japan, marbled flounder at Hyogo Prefectural Center for Stock Enhancement are fed a mixture of minced frozen mysids with additional formulated feed to boost nutritional content (T. Minamiura, Hyogo Prefectural Center for Stock Enhancement, pers. comm.). However, formulated feed is stopped 2 weeks before release to focus fish on more natural feed types.

Operant behavioral training may be especially useful in sea ranching efforts. Work conducted in Japan revealed Japanese flounder gathering when a sound cue was provided even when food was not available (Anraku et al. 1998).

Finally, there is acclimation cage conditioning, which allows hatchery fish to experience substrates and sediments, wild (live) food sources and a predator-free existence before actual release. Researchers in Denmark began cage conditioning turbot in bottomless cages to expose fish directly to sediments. Fish are conditioned for 3-6 days in areas with a small (< 30 cm) tidal range (Støttrup et al., 2002; Sparrevohn and Strøttrup, 2007). However, when this technology was transferred to Japan, a large problem was that predators easily entered the cage through the open bottom. To address this, Japanese cages contain mesh bottoms and sand is put inside. Japanese cages also zipper closed so that fish can be stocked and released easily from the cage. Takahama Bay, Japan, has 30 cm tides, and fish are conditioned for 6-7 days (pers. obs).

When this technology was transferred to the U.S., there were two problems. First, crab predators were even more abundant, and ripped holes in the soft nylon cage mesh with their claws. Since crabs gather on the bottom, floating cages were deployed just below the surface. However, survival in these cages was highly variable, and fish did not gain the benefit of experiencing sediments before release; therefore, a new U.S. bottom cage design was constructed of more durable polypropylene mesh. This material was too rigid to close with zippers, so cable ties were used. However, the second problem is that the tidal range in the New Hampshire is between 1 to 5 m, so when tides rose, fish swam to the surface of the cage and

wedged themselves between the cable ties to escape. The newest design has a self-adjusting height that rises with the tide; the cage opening is never submerged. This design was first used in September 2012 with high fish survival and retention (E. Fairchild, University of New Hampshire, pers. comm.).

Success of Cage Conditioning

To examine the benefit on burying ability, Obama Laboratory in Japan placed 20 juvenile Japanese flounder in covered, sand-bottom tanks for 5 minutes before and after the cage-conditioning period (Walsh et al., 2013). Fish that underwent one week of acclimation cage conditioning had significantly better burying abilities than those that did not.

To examine the benefit on feeding ability, experimental trials were conducted using natural live prey – mysids in 2008 and 2009 (Walsh et al., 2013). Each individual flounder was offered 5 mysids. Conditioned fish performance fell between that of wild and non-conditioned fish. Wild fish performed significantly better than non-conditioned fish in both years, but conditioned fish performance was not significantly different from either wild or non-conditioned fish.

A large-scale release of approximately 13,000 Japanese flounder by Obama Laboratory in 2010 revealed significantly higher recapture rates of conditioned fish by fishermen (Walsh et al. in press). This cooperative effort involved set net fishermen, fish traps, shrimp and sea cucumber trawlers, and recreational fishermen. Danish researchers found that mortality of cage-conditioned turbot was half that of non-conditioned fish (Sparrevohn and Strøttrup, 2007).

Pitfalls

Ensuring that released fish are morphologically, ecologically, genetically, and behaviorally similar to wild conspecifics is necessary for an effective release program (see Le Vay et al., 2007, for review). Development and testing of conditioning strategies that are easy to implement, economically feasible, and effective is ongoing. Unfortunately, the number of studies that have been able to monitor and track the fate of released conditioned fish is few.

Walsh et al. (2012; 2013) observed that non-conditioned fish, mostly non-feeding individuals, were caught more often than conditioned fish by < 0.5 m/s boat beam trawl when researcher-initiated recapture efforts were applied. Similarly, Sparrevohn and Støttrup (2007) found that the catchability of non-conditioned turbot caught by beam trawl was 10% higher than that of cage-conditioned fish. This may

indicate that intensive researcher recollection efforts at, or near, the release site disproportionately sample weak fish that are not feeding or moving. Efforts and money for recapture may be better spent on involving more local fishermen, especially since cooperative efforts generate more interest and publicity in the stocking. Involving more fishermen also may promote the reporting of recaptured hatchery-reared catch by those not directly involved in the project, and thus, amplify the level of monitoring conducted.

Even without implementing a conditioning strategy, one of the greatest difficulties of a stocking exercise is the level of post-release monitoring. In many cases, 1% recapture rates are the norm. Choosing a location that can be monitored adequately may be just as important as choosing a location where stocking is predicted to succeed, which is essential if the success of a stocking effort will influence future efforts (i.e., funding, resources, support). Stocking agencies have an obligation to conduct post-release monitoring in an attempt to assess stocking effectiveness, especially if the stocking effort is funded with taxation of citizens or fees from fishermen (Yamashita and Aritaki, 2010). In addition, there is a biological and ecological responsibility to evaluate what, if any, effect the stocking has on local fish populations and their habitats.

SUMMARY

The most prominent countries conditioning flatfish for stock enhancement are Japan, Denmark, and the U.S. Among the three countries there is great communication and collaboration – with U.S. and Danish researchers going to Japan and Japanese researchers going to the U.S. to investigate technology transfer. Acclimation cages are the most implemented strategy for flatfish worldwide and are short-term, inexpensive, and effective, but require site-specific adjustment. Live or life-like diets show great potential to be implemented in some capacity for rearing stocked flatfish. Frozen mysids are already being provided in Japan, and the potential to mass culture white worms shows promise for the future.

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Walsh, M. L., E. A. Fairchild, N. Rennels, S. C. Farina, W. H. Howell, R. Mercaldo-Allen, and C. Kuropat. 2009. Rearing Diets for Winter Flounder Optimize Weaning Success in Hatchery, Wild. Global Aquaculture Advocate. May/June: 48-50.

The objectives of this work were to quantify feeding-related performance of reared-then-released winter flounder juveniles that were reared on different feeds (both live and formulated) in the hatchery. The authors evaluated how feeding history translated to wild feeding performance once individuals were released into nature (caged in-situ) by examining survival, growth, feeding onset and incidence, stomach fullness, diet composition, and nucleic acid-based condition. Fish raised on live diets exhibited higher survival and growth than those reared on formulated, pellet feeds.

Støttrup, J. and C. R. Sparrevohn. 2010. Stock enhancement Europe: Turbot *Psetta maxima*, pp. 219–236., in: Practical Flatfish Culture and Stock Enhancement (H. V. Daniels and W. O. Watanabe, Eds.) Ames, IA, USA: Wiley-Blackwell Publishing.

This chapter provides a thorough yet concise description of Atlantic turbot releases throughout Europe. The authors discuss the aims and rationale of turbot stocking and include information regarding marking and tagging techniques, choice of release site, release strategies, and conditioning.

Walsh, M. L., H. Fujimoto, T. Yamamoto, T. Yamada, Y. Takahashi, and Y. Yamashita. 2013. Post-release performance and assessment of cage conditioned Japanese flounder, *Paralichthys olivaceus*, in Wakasa Bay, Japan. *Rev. Fish. Sci.* 21(3).

The authors examined both the immediate (burying, feeding) and longer-term (movements, recapture rates) benefits of cage conditioning juvenile Japanese flounder before stocking. Recapture rates of cage-conditioned fish caught by local fishermen were greater than those of non-conditioned fish (i.e., those released directly from hatchery tanks). In addition, burying ability was assessed by releasing recently conditioned and non-conditioned fish into tanks and quantifying the number of fish that buried. Likewise, feeding ability was assessed by providing tanks of conditioned and non-conditioned fish with prey and quantifying how many prey remained after a period. These laboratory experiments revealed that conditioned fish exhibited enhanced burying and feeding performance compared to non-conditioned fish. This work was the first to examine flatfish conditioning strategies using market data and to evaluate experimentally the performance of hatchery-reared flatfish that have been cage-conditioned.

Fairchild, E. A., N. Rennels, and W. H. Howell. 2008. Predators are attracted to acclimation cages used for winter flounder stock enhancement. Rev. Fish. Sci. 16: 262–168.

This paper addresses a negative consequence of using acclimation cages for stocking fish: that the cages in themselves may attract predators. Crab densities significantly increased in the vicinity of acclimation cages and continued to increase each day, even when cages were empty (i.e., contained no fish). Cages containing fish attracted an even higher abundance of crabs.

Study on High Incidence of Death Due to Collision of Hatchery-Reared Pacific Bluefin Tuna *Thunnus Orientalis* Juveniles in Net Cages

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ABSTRACT

Bluefin tuna have been cultured in many countries and regions including the Mediterranean Sea, Mexico, Australia, and Japan because of its high commercial value. The recent controversial issues of tuna capture-based aquaculture due to the decline of wild stocks catalyzed the need for a stable supply of artificially-reared tuna juveniles for aquaculture purposes. The reasons for this are two-fold: to reduce the negative impacts on wild-stocks, and to promote the aquaculture industry. In 2002, Kinki University succeeded in reproducing Pacific bluefin tuna *Thunnus orientalis* (PBT) in captivity. Various studies to apply artificially-reared juveniles to a mass-culture system have been carried out actively since then, however a high mortality of hatchery-reared juveniles is frequently observed after transfer from indoor tanks to net cages at around 30 days post hatch. The mortality is likely due to collisions with the cage nets at night/dawn. Techniques are being developed to prevent these collisions as the survival rate is much lower than that of other teleosts. In order to reduce the mortality in net cages, it is important to elucidate the developmental stages of reared PBT when the collisions occur in net cages.

Bone injuries can be considered as an index for obvious collisions due to the damage caused by physical impact. In the present study, bone injuries of dead juveniles were investigated in order to examine the prevalence of net collisions. Juvenile PBT were reared in 3 net cages (20 m in diameter) for 90 days after transfer from indoor tanks at Amami Station, Seikai National Fisheries Research Institute, Fisheries Research Agency, Japan. Dead fish were sampled and examined by the clearing and staining method or by dissection to detect injury to the vertebral column and parasphenoid. Primary injuries observed were dislocation and fracture of the vertebral column which usually occurred between the 1st and 15th vertebrae. The prevalence of injuries of the vertebral column and parasphenoid up to day 30 was 0.0-12.0%. Thereafter, the injuries drastically increased to 17.8-78.0% after transfer to nets from day 31 onward at a size of more than about 20 cm in TL. These results suggest that the mortalities of juvenile PBT larger than 20 cm in TL in cages were caused by net collision. In the future, further studies are required to reduce collision deaths for a consistent and stable supply of the seedlings for aquaculture.

INTRODUCTION

Bluefin tuna are of high commercial value and they are widely cultured in many countries around the Mediterranean Sea and in Mexico, Australia, and Japan. Most aquaculture industries are dependent on wild captured juvenile tuna and negative impacts on wild tuna stocks by capturing juveniles for aquaculture have been reported (Miyake et al. 2003; Masuma et al. 2011). To sustain the tuna farming industries and preserve wild tuna stocks, a stable supply of artificially-reared juvenile tuna for aquaculture must be established. Recently, Pacific bluefin tuna *Thunnus orientalis* (PBT) have been reared under aquaculture conditions through their complete life cycle (Sawada et al. 2005), and rearing techniques for mass-culture of PBT in indoor tanks until about 30 days post-hatching (DPH) are being developed (Tanaka et al. 2010; Masuma et al. 2011).

However, high mortality is observed after transferring the juvenile PBT from indoor tanks to sea cages (Miyashita et al. 2000). The high mortality is likely due to collisions with the cage nets because of visual disorientation at night/dawn and the high swimming speed of juvenile PBT. The relatively low scotopic vision sensitivity of the juvenile PBT compared to other marine juvenile fish such as grouper *Epinephelus septemfaciatus*, greater (also known as purplish) amberjack *Seriola dumerili*, ocellate puffer *Takifugu rubripes*, and red sea bream *Pagrus major* have been examined (Ishibashi et al. 2009). It has been observed in indoor tanks fitted with polyethylene netting on the walls that when juvenile PBT come into contact with the net wall they subsequently exhibit abnormal

behavior and subsequently collide with the wall at night (Ishibashi et al. 2009). Also, juvenile PBT are visually disoriented due to incompatibility of the retinomotor response from scotopic to photopic vision with the change in ambient light intensity at dawn (Masuma et al. 2001). On the other hand, the swimming ability of PBT rapidly increases in the juvenile stage because of morphological and functional development of the muscle, caudal fin, and caudal keel. These developments are linked to an increase in swimming speed, but the steering and braking ability from pectoral and pelvic fins is still immature in the juvenile stage (Miyashita 2002). Therefore, it is possible that the visually disoriented juveniles cannot control their high power swimming and thus collide with the net walls at night/dawn.

Various studies to prevent juvenile PBT from collisions have been conducted so far. Ishibashi (2006) reported that the daily mortality rate of juveniles reared in 30 m diameter circular sea cages had a tendency to be lower than that of juveniles reared in 6 m diameter circular sea cages. Also, to make up for visual disorientation at night/dawn and prevent juvenile PBT from touching and/or colliding with the net wall, Ishibashi et al. (2009) conducted trials in sea net cages illuminated by overhead lights providing 200-3000 lx intensity at the water surface. As a result, collisions were reduced and survival rate was drastically improved.

However, despite the success of subsequent rearing trials, the survival rate still fluctuates and is less than 50% one month after transfer from indoor tanks to sea cages. This figure is considerably lower than for other common teleosts raised for aquaculture such as grouper, red seabream, and greater amberjack with survival rates of over 80% for the initial month in sea cages. Therefore, further technical developments are needed before a stable supply system of artificially-reared tuna for aquaculture can be achieved on a commercial scale.

In order to be able to reduce the mortality of juvenile PBT reared in sea cages, it is important to elucidate the prevalence of collisions with the walls of sea cages. Because collisions cause physical impact to juvenile PBT, they lead to injuries of the vertebral column and/

or parasphenoid (Miyashita et al. 2000). As bone injuries of the vertebral column or parasphenoid were previously observed only in dead fish (not surviving fish; Higuchi et al. 2012), it is thought that these bone injuries cause mortality in juvenile PBT. Although collisions don't always lead to bone injuries, bone injuries can be considered an index of obvious collisions. In the present study, the injuries of the vertebral column and parasphenoid observed in dead fish were investigated in order to examine the developmental stage of reared PBT at which collisions (termed here as 'collision death') occur in sea cages.

MATERIALS AND METHODS
Experimental Fish
Experimental PBT juveniles were reared from fertilized eggs at Amami Station, Seikai National Fisheries Research Institute, Fisheries Research Agency, Japan. We transferred 1,024 (group 1), 3,617 (group 2) and 14,404 (group 3) PBT juveniles each into one of three circular sea cages (20 m in diameter) at Amami Station on 15 July, 30 August, and 17 September 2010, respectively (Table 1). The ages of groups 1, 2, and 3 at transfer were 36, 34 and 30 DPH, respectively (Table 1). The mean total lengths (TL) for groups 1, 2, and 3 were 5.94 ± 1.05, 4.44 ± 0.64, and 3.20 ± 0.42 cm, respectively (means $\pm$ s.d.; Table 1). The fish were initially reared in a nylon net (7 m in depth, 6 mm opening in mesh) attached to the sea cages, and transferred to a different nylon net (12 m in depth, 25 mm opening in mesh) with growth. The fish were initially fed minced sand lance *Ammodytes personatus* to satiation, followed by whole sand lance with growth. The sea cages were illuminated by artificial lighting (MDWL-12X; Marinetech Co., Fukuoka, Japan) placed above the centre of the sea cage from dusk to dawn in order to reduce the frequency of collisions with the net wall (Ishibashi et al. 2009). The light intensity just above the water's surface in the illuminated sea cages was approximately 500 lx during the night. Dead fish were daily sampled from day 0

Table 1. Summary statistics of PBT stocked in sea cages and survival rate at day 30, 60 and 90.

| Group | Summary statistics of PBT stocked in sea cages | | | Survival rate (%) | | |
	Number of fish	Age (dph)	Total length (cm)	day 30	day 60	day 90
1	1,024	36	5.94 ± 1.05*	27.2	19.9	19.0
2	3,617	34	4.44 ± 0.64*	8.0	6.9	6.2
3	14,404	30	3.20 ± 0.42*	4.1	3.3	2.6

*Mean ± s.d.

to 90 by a SCUBA diver. The TL and wet body weight (BW) of the sampled fish were measured. The survival rates at day 30, 60, and 90 after transfer to sea cages were calculated by counting the number of surviving fish in photographs that a SCUBA diver took from the bottom of the sea cage using a camera (PSS100; Canon Inc., Tokyo, Japan) mounted with a fish-eye lens (UWL-100 28AD; INON Inc., Tokyo, Japan). The water temperature was measured at 5 m in depth around the sea cages from July to December.

Growth Estimation of PBT Reared in Sea Cages

To estimate the growth of the PBT reared in sea cages, the von Bertalanffy growth function (VBGF) was fitted to the relationship between TL / BW and number of days post-transfer. The VBGF is:

$$L_t = L_\infty (1 - \exp(-K(t - t_0)))$$

where L_t = the TL (unit: cm) at age t (day); L_∞ = the asymptotic TL (cm); K = the growth coefficient; and t_0 = the hypothetical age (days) when the TL would be zero.

$$W_t = W_\infty (1 - \exp(-K(t - t_0)))^3$$

where W_t = the BW (unit: g) at age t (day); W_∞ = the asymptotic BW (g); K = the growth coefficient; and t_0 = the hypothetical age (days) when the BW would be zero.

The parameters of VBGF were estimated by the least-squares method using Solver in MS Excel (Microsoft, Redmond, WA). The BW of group 2 was not estimated by VBGF because of the low number of measured samples.

Examination of Bone Injuries of Dead PBT

Dead fish were sampled on day 5, 10, and 16-20 (pooled in this period) and fixed in 10% formalin. Thereafter, they were cleared and stained with alizarin red S by Potthoff's method (Potthoff 1984) to examine the injuries of the vertebral column (Fig. 3a-A) and parasphenoid (Fig. 3a-B). For the dead fish sampled on day 21-30, 31-60, and 61-90 (pooled in these periods), the injuries of the vertebral column were directly observed after removing the muscle of the specimens. The prevalence of the bone injuries of the vertebral column and parasphenoid was calculated as follows:

Prevalence of bone injuries (%) = (number of dead PBT having bone injuries on each day or period / number of dead PBT on each day or period) x 100

RESULTS

Survival Rate of the PBT and Water Temperature in Sea Cage

The survival rates of group 1, 2, and 3 were 27.2%, 8.0%, and 4.1% on day 30, 19.9%, 6.9%, and 3.3% on day 60, and 19.0%, 6.2%, and 2.6% on day 90, respectively (Table 1). High mortality was observed until day 60 in all the groups. Thereafter until day 90, dead fish were only infrequently observed.

The water temperature increased until early August, and sharply decreased from late October to early December at 5 m depth around the sea cages. During the experimental periods (day 0 to 90), each rearing water temperature of groups 1, 2, and 3 ranged from 25.8 to 28.5 °C (27.0 °C average), from 23.7 to 27.6 °C (26.1 °C average) and from 22.4 to 27.6 °C (25.3 °C average), respectively (Fig. 1).
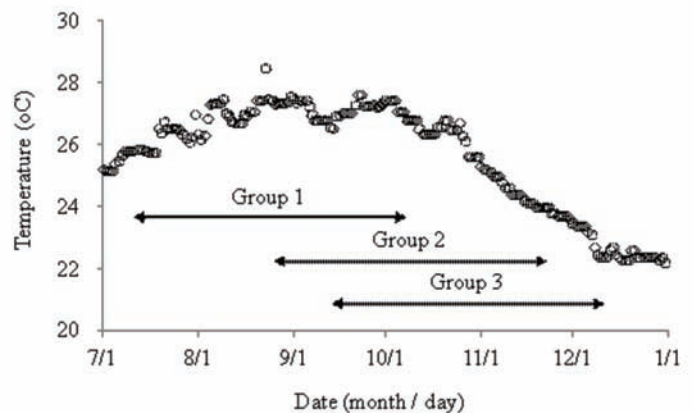

Fig. 1 The water temperature measured at 5 m depth around the sea cages from July to December 2010. The arrows indicate the 90-day experimental periods for groups 1, 2, and 3.

Growth of PBT Reared in Sea Cage

The VBGF parameters for TL of PBT reared in sea cages were L_∞ = 176.7, K = 0.0027, t_0 = -9.7 for group 1, L_∞ = 607.8, K = 0.0007, t_0 = -8.8 for group 2, and L_∞ = 607.9, K = 0.0006, t_0 = -5.8 for group 3 (Fig. 2). The VBGF parameters for BW of PBT reared in sea cages were W_∞ = 879283.7, K = 0.0011, t_0 = -14.3 for group 1 and W_∞ = 879260.3, K = 0.0009, t_0 = -5.2 for group 3 (Fig. 2). The growth coefficients of the VBGF for the PBT reared in sea cages, in descending order, were group 1, 2, and 3.
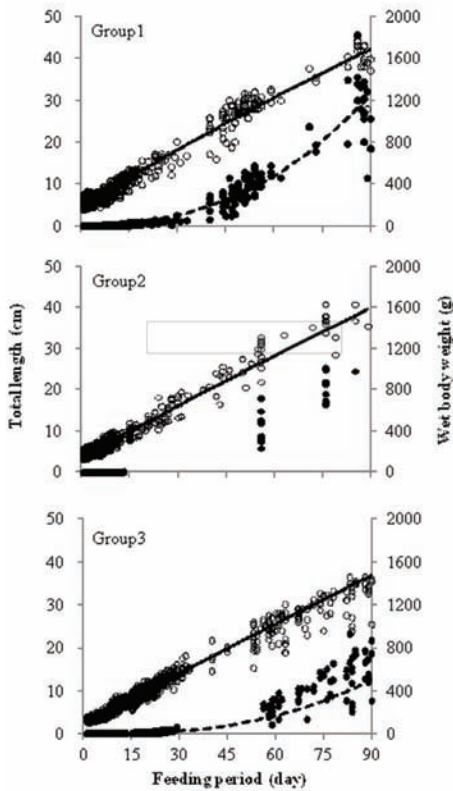
Fig. 2 Growth of the PBT juveniles reared in sea cages estimated by the von Bertalanffy growth function. White circle: total length of dead PBT juveniles; black circle: wet body weight of dead PBT juveniles; solid line: von Bertalanffy growth curve of total length; dashed line: von Bertalanffy growth curve of wet body weight.
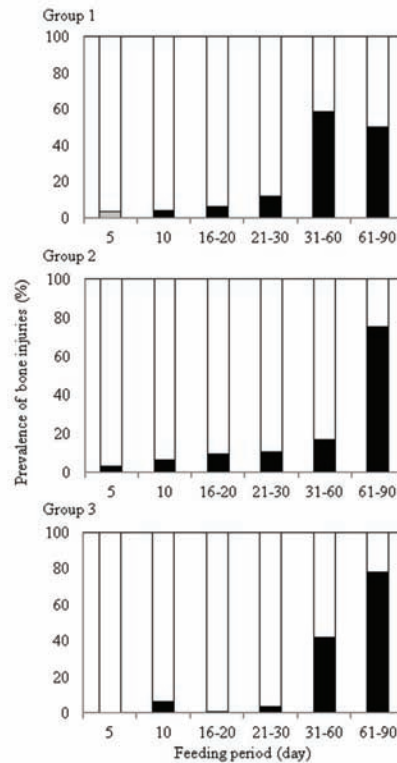

Fig. 4 Prevalence of bone injuries of the vertebral column or parasphenoid found in dead PBT up to day 90 after transfer. Black columns: injuries to the vertebral column; grey column: injuries to the parasphenoid; white columns: no bone injury.

Examination of Bone Injuries of Dead PBT

Injuries of the vertebral column and parasphenoid of the dead PBT were observed (Fig. 3). Dislocation and fracture usually occurred between the first to the 15th vertebral column (Fig. 3c). The prevalence of injuries both to the vertebral column and parasphenoid was 0.0-12.0% until day 30, and drastically increased to 17.8- 78.0% from day 31 onward (Fig. 4).
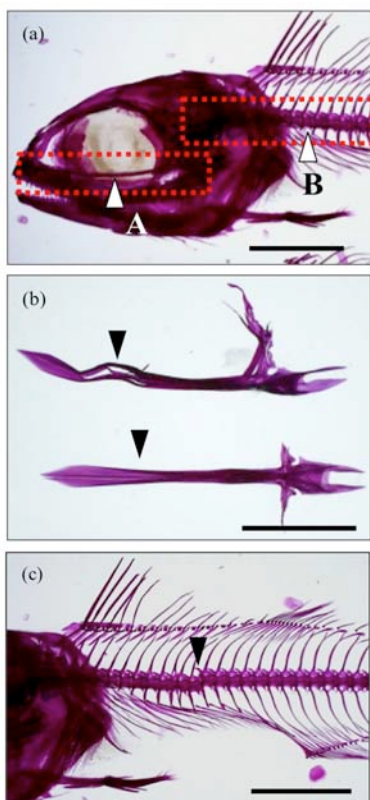

Fig. 3 The injuries of the vertebral column or parasphenoid of juvenile PBT. (a) A PBT cleared and stained with Alizarin Red S. Arrowheads indicate A) the parasphenoid and B) the vertebral column. (b) Parasphenoids excised from PBT showing fracture (upper) or non-fracture (lower) at day 5 after being transferred. Arrowheads indicate the fracture positions. (c) The dislocation of the vertebral column (arrowhead) of a dead PBT at day 10. Scale bars: 0.5 cm.

DISCUSSION

The growth of PBT was in descending order, group 1, 2, and 3. In particular, the growth of group 3 was much less than that of groups 1 and 2. The estimated BW of group 3 by VBGF was half that of group 1 on day 90 after transfer. Environment may be a factor, as the water temperature dramatically decreased and from late October the average rearing temperature for group 3 was approximately 2 °C lower than that of group 1. Therefore, the reduced growth for group 3 could be related to a decrease in rearing water temperature.

The prevalence of bone injuries drastically increased in dead fish greater than 20 cm TL from a month onward after transfer, and accounted for more than 50% of the mortality in all three groups. These results suggest that collision death increased and was the main cause of mortality in sea cages after fish reached a size of approximately 20 cm TL. It is known that muscle development, including the number and cross-sectional area of red and white fibers is linked to increased swimming speed in fish (Matsuoka et al. 1984). In PBT, number and cross-sectional area of red and white fibers drastically increases in juveniles of about 20 cm TL, and the cross-sectional area of red and white fibers in fish of about 25 cm TL approximates the same level as that of 2-year old fish (Miyashita 2002). Also, Tamura et al. (2009), using computational fluid dynamics analysis reported that for PBT the pectoral fins generated lifting force and the ratio of

the lifting force to downward gravitational force increased and was equal in fish of TL greater than 20 cm. Further, at about 25 cm TL in PBT the caudal fin and caudal keel, which develop in association with increasing swimming speed and assist stabilization of the swimming orbit as a hydrofoil wing, respectively, are morphologically and functionally equal to that of the adult PBT (Miyashita 2002). Therefore, the morphological function of PBT develops to enhance swimming ability as they grow into juveniles of 25 cm TL. In the present study, the PBT grew to exceed 20 cm TL in all trials 31-60 days after transfer, when the prevalence of bone injuries observed in the dead fish increased. Thus, the high frequency of collision death in juveniles of longer than about 20 cm TL from one month after transfer from indoor tanks to sea cages could be due to the drastic increase of swimming speed in PBT.

Low prevalence of bone injuries in dead fish was observed at the size of about 5 to 15 cm TL during day 5 to 30 after transfer from the indoor tanks to the sea cages. In contrast, the prevalence of bone injuries in dead PBT increased when the fish grew to 5 cm body length (BL) in an indoor tank rearing experiment and reached about 80% at the size of 10 to 16 cm BL (Miyashita et al. 2000). That suggests the collision death frequently occurs in juvenile PBT of about 5 to 16 cm BL in indoor tanks. Moreover, Miyashita et al. (2000) reported that the high frequency of collision deaths was caused by an imbalance in the rapidly developing swimming ability versus the steering and braking ability by fins and caudal keels of fish of 5 to 16 cm BL. In contrast, Ishibashi et al. (2009) reported that artificial lighting of sea cages during the night led to reduced collision with the net wall by juvenile PBT and improved survival rate to 73% up to day 23 after transfer compared to 12% survival in a non-lighted control sea cage. This study indicates that artificial lighting might reduce juvenile PBT collision with the net wall up to the initial month after transfer, but beyond one month after transfer the juvenile PBT might develop too much swimming speed to avoid collision even with the artificial nighttime lighting. Further, although the juvenile PBT during the initial month after transfer could escape bone injuries caused by smashing into the net wall due to their immature swimming ability and the artificial lighting, the juvenile PBT might suffer skin injuries from contact and/or collision with the net wall. In fact, it was reported that skin injuries were observed in most of the dead PBT during the initial month after transfer (Ishibashi et al. 2009). Because juvenile PBT have very poor tolerance to physical stress due to immature skin and scale formation (Ishibashi 2011), skin injuries may cause fatal damage in the initial month after transfer. In the future, an accurate estimation of the frequency of collisions in juvenile PBT of about 5 to 20 cm TL in sea cages should be conducted by ethological analysis using a video camera.

In conclusion, we suggest that the primary cause of mortality in juvenile PBT greater than about 20 cm TL is collision with the net wall. Although mortality during day 30 to 90 after transfer seemed to be lower than during day 0 to 30, about 20 to 40% of the surviving juveniles at day 30 died by day 90. Therefore, it is very important that the survival rate from one month onward after transfer is improved by preventing collision death with the net wall. Honryo et al. (2012) and Ishibashi (2006) reported that artificial lighting with adequate light intensity from dusk to dawn and a lattice pattern attached to the net wall leads to reduced collisions with the net wall. Further study is needed to support these findings and to reduce collision deaths for a consistent and stable supply of PBT fingerlings for aquaculture.

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Collisions with the walls of tanks or nets caused high mortality of northern bluefin tuna during the juvenile stage. In this study, juveniles were reared in indoor tanks from 30 to 120 day post-hatching (DPH) and in a sea net cage from 42 to 150 DPH, and dead fish were examined to detect injury of the bones using x-ray. As a result, more than 98% fish were dead at the end of both experiments. The proportion of dead fish with bone injuries, especially to the vertebral column and the parasphenoid, increased after fish reached 50 mm in BL (35 DPH), and exceeded 60% in fish with BL 85 mm (40 DPH) or greater in the indoor tanks. Also, the injuries of the vertebral column and the parasphenoid were observed in most of dead fish in the net cage. These results show that the loss of juvenile bluefin tuna was caused by collision with the tank or net wall that fatally damaged the bones of the vertebral column and the parasphenoid.

Masuma, S., Kawamura, G., Tezuka, N., Koiso, M. and Namba, K. 2001. Retinomotor responses of juvenile bluefin tuna *Thunnus thynnus*. Fisheries Science, Vol 67. Pp 228-231.

In bluefin tuna culture, a high mortality of juveniles is caused by collision with the tank and net cage walls at dawn. This collision can possibly be attributed to visually disoriented behavior of the fish. To examine this possibility, the authors carried out retinomotor response experiments with juvenile bluefin tuna and measured ambient light intensity at the culture site at dawn. The light intensity at which the transition from scotopic to photopic vision takes place was 7.52 lx and the time taken by the transition was 15 min. At dawn, the ambient light intensity rapidly increased from scotopic light intensity level and attained photopic light intensity level in 10 min. This incompatibility of the retinal adaptation with the change in the ambient light intensity could cause the visual disorientation of the fish. It is therefore possible that the visually disoriented juveniles cannot control their high power swimming and thus collide with the walls at dawn.

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In a lab-based study using infrared camera, the death of juvenile Pacific bluefin tuna (PBT) was associated with touching and/or colliding with the net wall attached to an indoor tank followed by abnormal swimming behavior, and finally becoming moribund during the night and dawn. Also, the scotopic visual threshold of juvenile PBT was at least 40 fold inferior to that of juvenile red sea bream, ocellate puffer, purplish amberjack, and grouper. To make up for low scotopic vision sensitivity of juvenile PBT and to prevent juvenile PBT from touching and/or colliding with the net wall, the authors illuminated sea net cages by overhead lights providing 200-3000 lx intensity at the water surface. As a result, survival rate of juvenile PBT in the sea net cages significantly improved to 73% up to 23 days post-transfer compared to 12% survival in the non-light control net cage.

The Cooperative Culture of Seaweed in New England—How Research, Industry, and Extension Are Cultivating a New Field in Aquaculture

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ABSTRACT

A partnership of research, industry, and extension in the Northeast U.S. has resulted in successful commercial culture of native seaweed species in Maine and Long Island Sound. Development of nursery culture and grow-out technologies for sugar kelp (*Saccharina latissima*) in the Seaweed Biotechnology Laboratory at the University of Connecticut and the lab and farm of Ocean Approved, LLC, of Portland, ME has allowed for the establishment of the first commercial kelp farm in the United States and an educational and research farm in Long Island Sound.

The technology was transferred to Maine in the form of a multi-species research seaweed nursery, as well as an extension-led partnership with shellfish farmers to integrate kelp lines on six different shellfish farms along the coast. The first regional seaweed aquaculture workshop was held as a result of this work, bringing together members of the seaweed industry, potential new farmers, researchers, extension, students, and entrepreneurs. This emerging new field continues to grow in the Northeast with the sharing of ideas, technologies and information through the open and collaborative relationships of extension, research, and industry.

INTRODUCTION

As the world's population continues to grow, so will the need for production of high quality food. Aquaculture is the fastest growing animal food and aquatic plant-producing sector worldwide, and already provides nearly 50% of the world's food fish and approximately 96% of the world's aquatic plants (Fao, 2010). Asian countries are leaders in aquaculture production, producing the majority of all cultured organisms including finfish, shellfish, and marine macroalgae (seaweeds). The United States has a small aquaculture sector, consisting mainly of inland freshwater fish production with a smaller marine aquaculture sector that produces Atlantic salmon and shellfish. Per capita seafood consumption is rising in the U.S., and is predicted to increase along with the seafood trade deficit, which was $10 billion in 2008 (FAO, 2013). As wild fishery catches decline or remain stable, the culture of aquatic species for food will become more important as we undergo a transition from wild harvesting to farming on our coasts. This development of aquaculture in our marine waters is akin to the development of agriculture, and its development and success will require similar approaches.

History of Extension in US

The United States government established the land grant university system through the Morrill Act in 1862, with the intention to "promote the liberal and practical education of the industrial classes in the several pursuits and professions in life," and expanded this system by establishing agricultural experiment stations and research centers in 1887. The agricultural extension service was added to the national land grant college system by the Smith-Lever Act in 1914, "in order to aid in diffusing among the people of the United States useful and practical information on subjects relating to agriculture…" These three segments—education, research, and extension—form the total mission of our national public universities. This system has been a crucial part of the development and transformation of the agricultural industry in the past century.

Traditional Modes of Extension

The traditional extension model has generally been described as a linear model, a system of knowledge transfer from the university to the farmer, and a needs transfer, from the farmer to the university, with the extension agent acting as the instrument of this communication. In this model, the researcher approaches the problem or question as a scientist, as a neutral, removed, objective observer of the problem in order to pursue scientific answers or solutions. This has been called research and development of the 'first order,' where the research component tends to be detached from the system with limited success in the ultimate utilization of the research outcomes by the majority (Ison & Russel, 2000). While the traditional model has been successful in many ways, including bringing about the agricultural revolutions around the world, the linear research-to-extension-to-farmer

model is no longer effective in a world of multiple actors and considerations, and requires a more inclusive system of collaboration and partnerships (Rahman, 2002).

New Variables in the Modern Age

With the burgeoning population and associated environmental problems, increased stress on natural resources, and rapid development of arable land are all complicated issues that involve many stakeholders. Besides environmental considerations, the motivations behind research and development are often market driven and are affected by management policies, non-government organizations (NGO's), and public perceptions. Increased competition between producers in a global economy requires research and extension that is market driven, that increases and diversifies production, and effectively links to processing and marketing systems (Sharma, 2002). The relationships between stakeholders are also changing, with increased connectedness, more access to information and technologies, and more intricate networks.

New Models of Extension

A new approach to research and extension has been suggested, a 'second order' of research and development, one that builds upon and improves the first order approach. This second order seeks to understand the traditions that our conceptions, management, and science of our environment are based on. It includes the study of relationships and takes into account the absolute linkages of humans and their environment (Ison & Russel, 2000). This approach is based on the idea that people create the world they live in and are responsible for their actions. It places the researcher inside the system that he is observing and places the responsibility of the outcome on all participants of a system. It is a collaborative approach, where the collaboration is based on a shared enthusiasm for action, and where this enthusiasm provides emotional energy and meaning for participants.

Research, extension, and farming are linked together as part of an integrated knowledge and technology system that includes the public, private, and organizational sectors. The integration of new technologies, producers and consumers, and the issues arising from this integration requires education, outreach, and communication —all of which are important components of effective extension (Warner, 2008). The linkages among different stakeholders can be strengthened through modern communication and information technologies, forming more extensive networks (Sharma, 2002). These technologies can also be utilized to provide a dynamic approach to keep technology at pace with new technology development, allowing businesses to compete in a global, rapidly changing marketplace (Morrissey & Almonacid, 2005).

Aquaculture in America

The development of marine aquaculture in the U.S. has emerged as a model for the new, 'second order' approach for research and extension (NOAA, 2011). While agricultural developments occur on privately owned property, marine aquaculture takes place in the commons—publicly held waters managed by state and federal policies. The coastal environment is also an open, dynamic system, which is influenced and affected by upstream activities and inputs. The very nature of this publicly held trust that is influenced by other human and industrial activities requires a multi-disciplinary approach to new aquaculture development, involving researchers, coastal managers, policy makers, industry, coastal communities, other coastal users, and the general public.

Development of marine finfish aquaculture in the U.S. began in the 1970s, and the industry has undergone several changes as it has evolved from an industry involving a number of small entrepreneurs with lower tech operations, to consolidation and vast improvements in culture technology of large-scale production. The salmon industry has suffered from major opposition and widespread negative public perception, preventing industry expansion (FAO, 2011). While wild fishery stocks continue to be classified as overfished and the value of cultured fish remains high, potential for industry expansion remains severely limited due to public concerns over environmental impacts. The shellfish industry, however, has been expanding and is now operating in almost all U.S. coastal states (FAO, 2011). These aquaculture industries have traditionally been single species farms; especially as the production of new culture technologies require a concentrated amount of effort. Recently, there has been a shift in thinking about the approach to marine farming, from single-species, production-focused systems to an ecological approach that includes cultivating two or more species at different trophic levels in order to mitigate the environmental impacts of a single-species farm (Barrington et al, 2009). This approach, called integrated multi-trophic aquaculture, or IMTA, can reduce environmental impacts of a farm, improve public perception of aquaculture, and increase revenue opportunity for the farmer through diversification (Neori, 2007).

In order for an integrated sea farm to provide an inorganic extractive component, it must include marine algae (Chopin et al, 2001). Seaweeds are marine macroalgae that have rapid growth rates, can produce large amounts of biomass, and can effectively take up dissolved inorganic nitrogen from land and sea based sources, including from animal (fish, shellfish) aquaculture (Ahn et al, 1998). Seaweed aquaculture is a large industry in Asia, where millions of tons are produced annually for food and extracts, but has remained only in the realm of research in the U.S. Seaweeds are valuable as food—representing over $5 billion annually worldwide, and the potential U.S. market is large, as the trend for consumption of more healthy and nutritious foods becomes more widespread (Buchholz et al, 2012). Some coastal states, like Maine and California, have traditional wild harvesters that currently sell dried sea vegetables, mainly through health food stores. The development of seaweed aquaculture requires all of these actors—researchers, farmers, processors, marketers, consumers, and public perception—to come together for a successful industry, and this is what extension can help do.

A New Industry for the Northeast

The challenge of building a new industry is multi-dimensional, as all parts of the production-to-consumption chain have to be established and effectively linked. This includes nursery, husbandry, harvesting, processing, and marketing infrastructure and experience. The seaweed industry in the Northeast must build upon prior research work in nursery and cultivation techniques, and work together with existing sea vegetable companies to establish markets for their crops. This type of industry must focus on enhancing existing markets and creating new markets for new types of cultured products. A large part of creating demand among American consumers is through education and exposure since sea vegetables are, for the most part, a foreign food to the average consumer. In order to supply markets, the products must be consistent and reliable with industry-wide quality. Aquaculture can provide this through optimal husbandry and culture conditions. As a new type of marine agronomy in the U.S., the development of husbandry techniques for seaweeds will need to be developed through partnerships with experienced mariners (sea farmers, fishermen). Results in the marine environment are largely site-specific and require numerous trials and adaptations in order to develop desired results. This new research and development will require extensive and effective funding support. Finally, the multi-dimensional quality of the farm-to-market supply chain for a

new product will require solid cooperation and communication among all of the players, and this will require effective extension.

Previous attempts to cultivate seaweeds in Maine (Yarish et al, 1998, Levine & Cheney, 1998) and Long Island Sound (Brinkhuis et al, 1987) have been short-lived or experimental. The research and experience gains failed to launch any viable commercial seaweed culture industry, though there was potential for cultivation for both food and energy production. Though knowledge was gained through these projects, the diffusion or "spill-over" effects did not reach any commercial end-user and the results of the projects were limited to several published papers and meeting proceedings. Individual projects working alone have limited success, since it takes collaboration with a shared enthusiasm for action for further development and success of a new technology or industry.

The development of a new seaweed aquaculture effort in the Northeast has been built around a strong center of collaboration between research, industry, and extension. This has resulted in the development of native seaweed nursery and grow-out technologies in Maine and Long Island Sound. This emerging industry continues to expand with the addition of new species, products, funding, infrastructure, and partners.

This project started with a product, an idea, and a desire to act. Ocean Approved, a Maine company that developed an innovative, fresh-frozen kelp product for the American consumer (Parkhurst, 2011), had a new approach to selling sea vegetables that also included a desire to ultimately culture the kelp that their business relied on. They sought out aquaculture research and development assistance from Dr. Charles Yarish at the University of Connecticut's Marine Biotechnology Laboratory in Stamford, and a partnership was formed. Dr. Yarish is one of the leading seaweed culture research scientists in the U.S., and has access to the experience and research of other culture operations around the world. There were two key steps to developing culture technologies for our native seaweeds—1) information and technology transfer and 2) adaptation to local species and needs. Four years of funding was supplied through NOAA and Connecticut Sea Grant programs resulting in the establishment of the first commercial kelp farm in the U.S., and the expansion of the technology throughout the Northeast.

This technology has expanded to support further work in Long Island Sound on bioremediation of

urban eutrophic waters (Kim et al, 2012), a research and educational kelp farm in Connecticut (Kim et al, 2012), and the diversification of kelp on an existing oyster farm (Lemoult, 2012). Key to the success of all of these spillover projects in Long Island Sound is the strong element of outreach, partnership, and education. The marine biotechnology lab at University of Connecticut (UConn) has a solid policy of research through collaboration, and education through experience. They consistently partner with students from a variety of institutions, including the Bridgeport Regional Aquaculture Science and Technology High School in Bridgeport, Connecticut, Rocking the Boat after school program in New York City, and Purchase College in New York, providing educational and research opportunities for a diverse student population. The UConn lab works closely with other research professionals from the public and private sectors and with industry partners to develop usable and applicable technology. They also utilize Sea Grant extension agents, from Connecticut to Maine, for education, networking, research, and extension.

The nursery technology co-developed by UConn and Ocean Approved was established at Ocean Approved's kelp nursery in Portland, Maine and successfully produced three seasons of juvenile kelp for cultivation. This partnership and technology transfer enabled the company to improve and adapt culture methods to increase efficiency and reduce cost. The first year of at-sea cultivation also resulted in the creation of a new, exclusively cultured product—a small-sized whole kelp for the gourmet market. The company is currently in its third year of cultivation and is growing as demand increases and technologies improve. Ocean Approved has been a successful collaborator and partner with interested research institutions, students, entrepreneurs, and the media to share the new technology as well as the enthusiasm for the potential of this new industry.

Through the Maine Sea Grant extension program, kelp cultivation technology has been transferred and further developed on existing shellfish farms along the coast. This was collaboration seaweed farmers (Ocean Approved), shellfish farmers, existing seaweed companies, and Maine Sea Grant marine extension agents to integrate *Saccharina latissima* onto mussel and oyster farms in Maine. Nursery-produced seeded lines were placed out underneath, between, and amongst existing shellfish gear to determine optimal physical and biological integration of sugar kelp. Environmental variables were observed along with growth and quality throughout the trial (December to June), and kelp was harvested and analyzed by

partner companies. The experience gained from the first year of cultivation was applied to the second year, with adjusted planting times and separate long line installations to improve growth and quality of the product.

Kelp harvested from the first trial was dried and given to various interested students and researchers. These spin-off projects were an unforeseen benefit to the kelp/shellfish integration trial, and the preliminary results are creating other research opportunities. Examples of these projects include using kelp for sea urchin (Carrier et al, 2012) and abalone feed, investigations into biofuel production (University of Maine), and analysis of kelp for fertilizer (UConn). It is expected that there will be further seaweed-related research projects throughout New England as the industry develops.

The extension-led research and development project has allowed for a greater number of participants and extensive dissemination of information. This outreach has been accomplished through various media outlets, presentations and demonstrations, and at a dedicated meeting. This meeting, entitled "The Seaweed Scene 2012," brought together members of the seaweed industry, potential new farmers, researchers, extension, students, and entrepreneurs to learn about seaweed aquaculture, the projects going on in the Northeast, and to network and share ideas. An online network was set up in the form of a listserv, and a Facebook page was established to share ideas and seek out collaborations. These IT network tools have enabled members of the seaweed industry, research, and the general public to interact in ways not otherwise possible.

Other seaweed cultivation trials have been initiated in other parts of New England, including projects in Maine, New Hampshire, Massachusetts, Rhode Island, Connecticut, and New York. New England already has an extensive shellfish aquaculture industry, and the development of seaweed culture technology is a natural fit with existing farms, enabling sea farmers to diversify ecologically and economically. Seaweed aquaculture could also be an opportunity for commercial fishermen to diversify into aquaculture. Besides food, seaweed aquaculture can be useful for water bioremediation, habitat restoration, and biomass production, and there are now plans underway throughout New England to further develop these potentials. In order to enable these projects to develop, a source of seed plants are required. This will require the establishment of regional seaweed nursery

facilities to support research and development as well as to provide seed to the emerging industry.

The nursery technologies developed at the seaweed research lab in Stamford, Connecticut, were transferred to Ocean Approved, LLC, in Portland, Maine to establish their commercial seaweed nursery , and to a research nursery at the Center for Cooperative Aquaculture Research (CCAR) in Franklin, Maine. This seaweed research nursery is crucial to the Northeast seaweed industry, and builds upon previous research on *Porphyra umbilicalis* culture in Maine (Blouin et al, 2007). Through a Maine Aquaculture Innovation grant, *P. umbilicalis* seed stock has been established at CCAR from a unialgal culture from the Maine coast. Mature, spore-producing plants were produced from juveniles within six weeks, and maintained in a tumble culture in the nursery. Nets were successfully seeded with this seed stock, and storage of seeded nets in deep freeze produced viable fronds after 2 months. Seed stock continues to be maintained for further research and production of seeded nets for culture at sea.

The research nursery in Maine is currently building culture capacity for native kelps (*Saccharina latissima*, *Laminaria digitata*, and *Alaria esculenta*), laver, (*Porphyra umbilicalis)*, and dulse (*Palmaria palmata)* (Figure 1). The development of seeding technology for native species will be coupled with partnerships and collaborations with new and existing sea farmers in order to develop successful husbandry protocols for the industry. Further collaboration with the existing seaweed industry will guide the development, harvesting, and processing of the new cultured products, in order to establish market outlets and build the farm-to-consumer chain.

This emerging new field continues to grow in the Northeast with the sharing of ideas, technologies, and information through the open and collaborative relationships of extension, research, and industry. Instead of separate linear research and development projects, all must work together in order for the seaweed culture industry to move forward. At the center of all of these developments is extension. Extension, instead of acting as a linear line of communication from researcher to industry, is at the center of a spoked wheel. All partners play a role in extension themselves by reaching out and collaborating with other industry, research, educational, governmental, and NGO partners, and are also part of a network that extension agents can work within. By acting as the center connecting point, extension is able to bring together normally disparate parts to enhance existing research, encourage new collaborations, and allow for needs and ideas to become successful realities.

Figure 1. (Left) *Saccharina latissima*, cultured on a mussel farm in Maine. (Center) *Porphyra umbilicalis* seedstock, cultured in seaweed nursery in Maine (Right) *Palmaria palmata*, experimental culture line on mussel farm in Maine.

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This Connecticut newspaper article summarizes the research work on native New England seaweed species for farms in Maine and Long Island Sound. A collaborative research project involving the University of Connecticut Marine Biotechnology Research Laboratory and commercial partners Ocean Approved, Llc in Maine resulted in the first successful kelp culture crops being cultivated both in Maine and Long Island Sound in the winter of 2011, with plans to further develop farming operations in the future.

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A Pilot Project to Stimulate Seaweed Production on Mussel Farms in Maine [Press Release] Retrieved from http://www.seagrant.umaine.edu/extension/kelp-mussels

This online press release from Maine Sea Grant summarizes the collaborative research work conducted by shellfish growers, seaweed farmers, and marine extension agents in Maine. The project was the first of its kind in Maine, integrating Saccharina latissima on six different shellfish farms (mussel and oyster) in Maine.

Morrissey, MT & S Almonacid. 2005. Rethinking technology transfer. *Journal of Food Engineering*, Volume 67, Issues 1–2, pp. 135-145. (http://www.sciencedirect.com/science/article/pii/S0260877404003346)

This article uses several new seafood processing innovation examples to highlight the importance of a new approach to technology transfer. In order to improve and compete in a global marketplace, businesses need more real-time, adaptable approaches to technology and research being developed in labs and Universities. In order to keep the speed of transfer up with the speed of new technology development, the authors suggest a more multi-disciplinary and dynamic approach.

Warner, KD. 2008. Agroecology as Participatory Science: Emerging Alternatives to Technology Transfer Extension Practice. *Science, Technology, & Human Values*, Vol. 33, No. 6, pp. 754-777.

Agricultural extension plays an important role of linking the research community with the business community, transferring new technologies from scientists to farmers, and problems of farmers as research questions back to scientists. Besides just technology transfer, extension plays a wider role as educators of the general public, integrating new technologies, producers, consumers, and issues arising from these. This article reviews these interactions by looking at environmental pollution issues and agroecological partnerships between growers, extension, and scientists. This type of participatory science uses collaboration, multidisciplinary approaches, and ecological approaches to improve the direction of agricultural developments.

Parkhurst, Emily. 2011, September 26. Kelp wanted: Seaweed farms expand in Maine's Casco Bay. The Forecaster. Retrieved from http://www.theforecaster.net/content/pnm-kelp-farm-near-chebeague-092811

This newspaper article reports on the expansion of the first commercial kelp farm in the United States, Ocean Approved, Llc, in Maine. They are a new company with two years of cultivation experience, with plans to expand their product lines and further develop seaweed aquaculture in Maine. Part of their plan involves sale of their native "seed" plants to other growers in Maine.

In situ Swimming and Settlement Behavior of Cultured Serranid Larvae, *Plectropomus Leopardus* and *Epinephelus Malabaricus*

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Keywords: Swimming and settlement behavior, cultured larvae, *Plectropomus leopardus, Epinephelus malabaricus*

ABSTRACT

Cultured serranid larvae of *Plectropomus leopardus* and *Epinephelus malabaricus* were released during daytime in June - July of 1997 and 1998 at various locations along a fringing reef at Ishigaki Island, southern Japan, and their behavior was observed by divers. At offshore (1 km) deeper sites (30 m), released larvae swam at an average depth of 10.2±5.1 m and swimming speed of 4.9±1.2 cm/s for *P. leopardus* and 7.5±4.5 m and 6.1±5.7 cm/s for *E. malabaricus*. When released at shallower sites (7 m) close to the reef edge (3 m), both species of larvae swam directionally to open water most likely to avoid predators. At sites 400 m offshore and 18 m deep released *P. leopardus* and *E. malabaricus* larvae settled on the slope at average depths of 16.4±2.7 m and 15.4±2.5 m, respectively. No significant differences were found between the two species in settlement depth, time to settlement, swimming speed, or swimming distance. When released at sites 400 m offshore and 18 m deep 3 m from the slope and 3m above the rubble bottom, 75.9 % of *P. leopardus* larvae settled underneath prominent coral on the slope, and 24.1 % settled underneath rocks.

INTRODUCTION

The serranid fishes *Plectropomus leopardus* and *Epinephelus malabaricus* are the most important fishery resources on coral reefs in Japan. The Research Center for Subtropical Fisheries, SNFRI, FRA, and Okinawa Prefectural Fisheries and Ocean Research Center, Ishigaki Station have been culturing these two serranids for aquaculture by fishermen and for stock enhancement by releasing juveniles into coral reef environments (sea ranching). In aquaculture and sea ranching, the quality of larvae and information about their life cycle, where they settle, and about early post-settlement habitat, is needed to optimize release techniques. Therefore, we conducted underwater observation studies at Ishigaki Island to address the following questions: 1) At what depth, speed and direction do larvae of these two serranids swim near coral reefs? 2) How settlement occurs and what habitat do they settle on?

MATERIALS AND METHODS
Study site

The study was carried out at three study sites; 1) a shallow site (250 m from the reef edge, 8 m depth), 2) a deeper offshore site (1 km from the reef edge, 35 m depth), and 3) a (shallower) offshore site (400 m from the reef edge, 18 m depth) in Urasoko Bay (24°29′ N, 124°13′ E), Ishigaki Island, southern Ryukyu Islands (Fig.1). Direction to the nearest reef edge at the shallower site was 65 degrees and that of the offshore deeper site was 170 degrees.
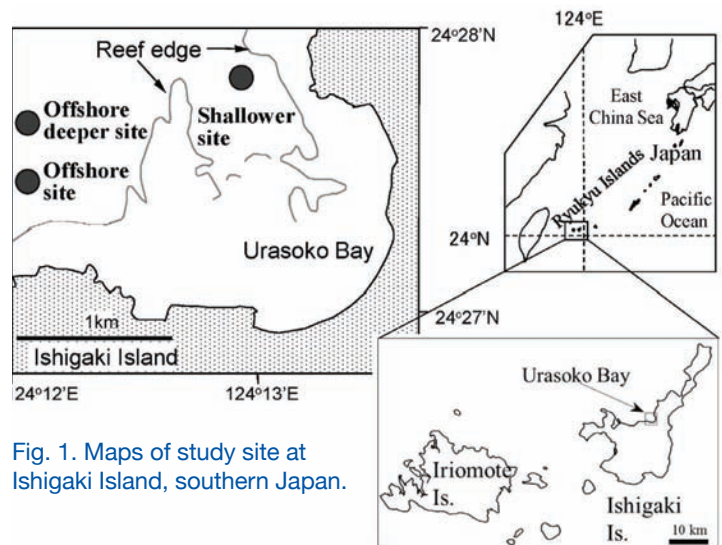

Fig. 1. Maps of study site at Ishigaki Island, southern Japan.

Cultured larvae

We used cultured pelagic phase and settlement stage larvae of both *P. leopardus* and *E. malabaricus* in experimental releases (Fig. 2). Pelagic phase larvae (ca. 30 days after hatching) have elongated second dorsal and pelvic fin spines. Settlement stage larvae (ca. 50 days after hatching), have proportionally decreased second dorsal and pelvic spine lengths and more pronounced body pigmentation (Masuma et al. 1993).
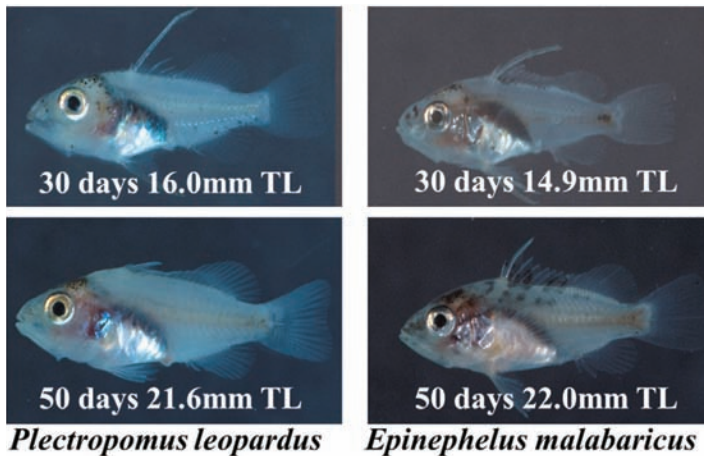

Fig. 2. Photographs of cultured serranid larvae, *Plectropomus leopardus* and *Epinephelus malabaricus*. Upper photographs of larvae are pelagic phase, and lower are settlement stage.

Underwater observations

Swimming behavior of larvae of the two serranids was observed during daytime (14:00-17:00) in June - July of 1997 and 1998, using the methods described by Leis & Carson-Ewart (1999). Two divers faced each other 2 to 3m apart, and an observer-diver released a larva into the water column from a bottle. The observer-diver followed the larva, and a recorder-diver followed the observer and recorded the depth, compass direction every 30 sec and flow measurement (General Oceanics: Digital Flow meter Model 2030R2 with a low speed rotor) at the end of the observation for distance travelled. Before release, a photo was taken of each larva in a plastic case with 1 mm slits (for scale), and the size of the released larvae were measured from the photographs. We followed each larva for 10 minutes unless the larva swam deeper than 20 m, in which case observation was discontinued to prevent decompression sickness. When the larva settled during the observation time, the settlement site was recorded. We pooled all observation data which were recorded every 30 sec, and analyzed the data statistically.

In addition, to determine whether larvae select settlement sites, we released *P. leopardus* settlement-stage larvae (n=29, size range 20-24 mm TL) individually at 3 m from the slope and 3 m above the rubble bottom at the offshore site, and recorded where they settled.

RESULTS AND DISCUSSION

Swimming Behavior of Pelagic Phase Larvae

At the offshore deeper site, we released 11 individuals of *P. leopardus* and collected 193 observations; we also released 11 individuals of *E. malabaricus* and collected 204 observations. Released larvae swam at an average depth and swimming speed of 10.2±5.1 m and 4.9±1.2 cm/s for *P. leopardus* and 7.5±4.5 m and 6.1±5.7 cm/s for *E. malabaricus* (Table 1). At shallower sites close to the reef edge, we released 15 individuals of *P. leopardus* and collected 130 observations; and we released 14 individuals of *E. malabaricus* and collected 111 observations. Released larvae swam at an average depth and swimming speed of 3.7±2.4 m and 3.8±3.3 cm/s for *P. leopardus* and 3.2±1.6 m and 4.8±4.0 cm/s for *E. malabaricus*.

Table 1. Swimming behavior of the pelagic phase larvae of *Plectropomus leopardus* and *Epinephelus malabaricus*.

| Releasing site		Size (mmTL)				Depth (m)	Speed (cm/sec)	
		Mean±SD	Size range	No.of individuals	No.of observations	Mean± SD	Mean±SD	Mini-Max.
Plectropomus leopardus	Offshore deeper site	17.4±2.0	14-21	11	193	10.2±5.1	4.9±1.2	0.6-13.3
	Shallower site	17.1±2.0	15-21	15	130	3.7±2.4	3.8±3.3	0.4-9.8
Epinephelus malabaricus	Offshore deeper site	16.7±4.1	12-23	11	204	7.5±4.5	6.1±5.7	1.2-19.5
	Shallower site	16.4±2.0	12-20	14	111	3.2±1.6	4.8±4.0	0.3-12.0
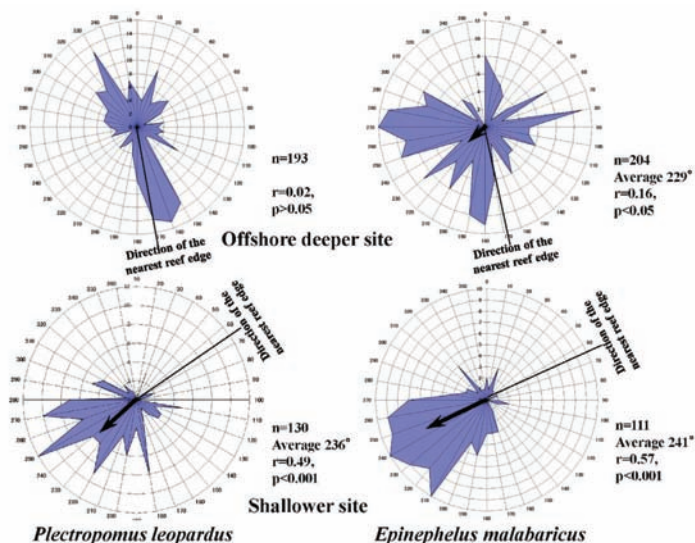
Fig. 3. Swimming direction of pelagic phase larvae released at offshore deeper site and shallower site near the reef edge.

66

The swimming direction was not significant, but generally either towards the reef or offshore, for *P. leopardus* (r=0.02, p>0.05, Rayleigh's test) and roughly towards the reef edge for *E. malabaricus* (average 229°, r=0.16, p<0.005, Rayleigh's test; Fig. 3). When released at shallower sites close to the reef edge, both species of larvae swam directionally to open water (*P. leopardus*: average 236°, r=0.49, p<0.001; *E. malabaricus*: average 241°, r=0.57, p<0.001, Rayleigh's test)

When we released larvae at the shallower sites, three of *P. leopardus* larvae and five of *E. malabaricus* were eaten by *Thalassoma hardwickii* (Labridae), *Pomacentrus philippinus*, *Neoglyphidodon nigroris* (Pomacentridae), or *Lutjanus bohar* (Lutjanidae) near the reef edge. Our data showed pelagic-phase cultured larvae released at Ishigaki Island swam deeper and more slowly compared to reports for natural larvae released in Australia (Leis and Carson-Ewart 1999). These differences were probably size related. Pelagic-stage larvae from the Australian study (size range 20.2-22.1 mm TL) were larger than larvae used in this Ishigaki Island study.

Plectropomus leopardus larvae are far from passive, being able to swim at speeds greater than the local ambient currents (Leis and Carson-Ewart, 1999). Wright et al. (2008) described auditory and olfactory abilities of *P. leopardus* as well developed at the settlement stage, and apparently sufficient to detect auditory and olfactory cues from the reef. It was found that the larvae of the two serranids swam towards reef or offshore after detecting the reef, and when they came close to the fringing reef, they swam to open water most likely to avoid predators.

At sites 400 m offshore and 18 m deep, we released 18 individuals of *P. leopardus* and 21 individuals of *E. malabaricus* at 5 m deep. Released *P. leopardus* and *E. malabaricus* settlement-stage larvae settled on the slope at average depths of 16.4±2.7 m and 15.4±2.5 m, respectively (Table 2). There were no significant differences between the two species in settlement depth, time to settlement, swimming speed and swimming distance. However the settlement behavior between the two serranids was completely different. *Plectropomus leopardus* larvae slowly approached the slope and swam rapidly the last 1-2 m to settle underneath prominent live or dead coral, presumably to avoid attacks by predators. In contrast, *E. malabaricus* larvae swam rapidly towards the slope at first and then approached slowly the last 1-2 m to settle. Cultured larvae of *P. leopardus* settled in deeper sites than natural ones in Australia (Leis and Carson-Ewart 1999).

Settlement Behavior of Settlement-Stage Larvae
When released 3 m from the slope and 3m above the rubble bottom at sites 400 m offshore and 18 m deep, *P. leopardus* larvae swam toward the slope or bottom at an average swimming speed of 9.1 cm/s. 75.9 % of *P. leopardus* larvae settled underneath prominent live or dead coral on the slope, and 24.1 % settled underneath rocks on the bottom within an average of 62.1 sec. (Table 3). On Green Reef, Great Barrier Reef, Australia, Light and Jones (1997) indicated that *P. leopardus* recruited to level patches of rubble substrata. However, our results showed that almost all larvae settled on the slope. They preferentially settled underneath prominent live or dead coral on the slope rather than underneath rocks on the bottom (Chi-square goodness-of-fit test, p<0.05).

Table 3. Settlement sites of *Plectropomus leopardus* released at 3 m from the slop and 3 m above the rubble bottom.

	Settlement on the bottom	Settlement on the slope	Chi-square goodness-of-fit test
No. of individuals	7	22	p<0.05
	24.1%	75.9%	

Plectropomus leopardus (23-24 cm TL), previously shown to be fish of age 1+ (Ebisawa 2000), distributed in the dead coral rubble area underneath the reef slope at Ishigaki Island (Shibuno et al. 2008). This is probably caused by ontogenetic shifts in habitat associations from the initial settlement sites to dead coral rubble area underneath the reef slope (Light and Jones 1997; Nakai 2002). *E. malabaricus* distributes in rocky shallow water reefs in the bay (Nakabo, 2002). Another study about their migration after settlement will be needed.

	Plectropomus leopardus n=18			*Epinephelus malabaricus* n=21			U-test
	Mean	±	SD	Mean	±	SD	
Size (mmTL)	21.2	±	0.9	20.3	±	2.5	ns
Size range	(20	–	23)	(16	–	26)	
Depth (m)	16.4	±	2.7	15.4	±	2.5	ns
Distance from the bottom (m)	2.8	±	1.4	5.2	±	2.5	p<0.01
Time to settlement (sec)	170.8	±	112.2	140.7	±	107.2	ns
Swimming distance (m)	20.1	±	15.1	22.0	±	18.2	ns
Swimming speed (cm/sec)	13.7	±	12.1	20.3	±	11.0	ns

Table 2. Settlement behavior of *Plectropomus leopardus* and *Epinephelus malabaricus*.

ACKNOWLEDGMENTS

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At the Japan Sea Farming Association, Yaeyama Station, coral trout broodstock have provided fertilized eggs by spontaneous spawning since May 1988. The authors described the embryonic and morphologica1 development, behavior, and growth of the coral trout from the egg to juvenile stages.

Leis, J. M. and B. M. Carson-Ewart. 1999. In situ swimming and settlement behavior of larvae of an Indo-Pacific coral-reef fish, the coral trout *Plectropomus leopardus* (Pisces: Serranidae). Marine Bio1ogy 134: 51-64.

Late larvae of the serranid coral trout *Plectropomus leopardus* (Lacepède), captured in light traps, were released during the day both in open water and adjacent to two reefs, and their behavior was observed by divers at Lizard 1sland, northern Great Barrier Reef.

Nakai, T. 2002. Management of fishery resources for groupers (Serranidae) in Okinawa, southern Japan. Fisheries Science, 68(Supplement): 431-432.

The authors show data on the distribution of groupers across the coral reef area at Iriomote Island, and a summary on the seed production of several species of grouper in Okinawa. Their data on the distribution of groupers provide additional information on the management strategy for groupers.

Shibuno, T., Y. Nakamura, M. Horinouchi and M.Sano. 2008. Comparison of reef fish community structures from mangrove estuary to coral reef slope, at Ishigaki Island, southern Japan. Ichthyological Research. 55(3):218-237.

To clarify seascape-scale habitat use patterns of fishes in the Ryukyu Islands (southern Japan), visual censuses were conducted in the mangrove estuary, sand area, seagrass bed, coral rubble area, branching coral area on the reef flat, and tabular coral area on the outer reef slope.

Larval Rearing Advancements for Yellowtail Amberjack in *(Seriola lalandi)* Southern California

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Keywords: *Seriola lalandi*, aquaculture, live feeds management, bacteria control

ABSTRACT

Hubbs-SeaWorld Research Institute (HSWRI) has been culturing yellowtail amberjack (*Seriola lalandi*) experimentally at its laboratory in San Diego, CA since 2003. In 2007, we began to intensively rear juveniles, and between 2007 and 2011, we achieved survival rates as high as 5.0% from egg to approximately 50 days post hatch (dph). In 2012, we continued to conduct manipulative larval rearing studies to improve overall larval performance. These studies targeted optimizing the live prey feeding regime and reducing bacterial loading in larval culture tanks.

First, we addressed our larval live prey feeding procedures. The traditional HSWRI feeding regime for yellowtail amberjack began with offering rotifers (*Brachionus plicatilis*) at 2 dph through 7 dph, 1st instar *Artemia* (*Artemia franciscana*) from 6 - 8 dph, and 2nd instar *Artemia* from 7 - 35 dph. In the first trial, larvae were co-fed rotifers, 1st instar *Artemia*, and 2nd instar *Artemia* from 3 - 20 dph in order to gain a better understanding of appropriate transition times, as well as ingestion levels of each feed type over time. Results showed that the larvae selected 1st instar *Artemia* as early as 3 dph (4.5 mm notochord length) and 2nd instar *Artemia* as early as 5 dph (4.6 mm notochord length). The second trial attempted to remove 1st instar *Artemia* from the current feeding regime because they do not have a functional feeding apparatus and therefore cannot be enriched. This trial consisted of three treatments: 1) rotifers to 2nd instar offered at 5 dph, 2) rotifers to 2nd instar offered at 7 dph, and 3) rotifers to 1st instar to 2nd instar (i.e. traditional HSWRI protocol). Results showed no significant differences in growth or survival among any of the treatments, indicating that larvae can be weaned from rotifers directly onto 2nd instar *Artemia* starting at 5 dph.

Second, we wanted to reduce bacteria levels in larval culture tanks. Bacterial load in the larval rearing environment is heavily influenced by inputs into rearing tanks (e.g. algae cells and live prey), as well as other factors like water treatment and temperature. We conducted two trials to evaluate methods to minimize the bacterial load in the water while maintaining good larval performance. Plating on thiosulfate-citrate-bile salts-sucrose (TCBS) agar was used to determine the presence and abundance of *Vibrio* species as the measurement variable. The first trial examined the use of bentonite clay as a turbidity agent to replace algae in a greenwater-type environment. This study consisted of three treatments: 1) clay with continuous feeding, 2) clay with batch feedings, and 3) algae paste with batch feedings. The results showed that both clay treatments had significantly fewer *Vibrio* colonies in the water column (377 ± 120 CFU) than the algae paste treatment ($5,692 \pm 2,396$ CFU) after 14 days of culture. Larval growth was not significantly different among the treatments, but survival was significantly higher in the clay treatment with continuous feeding ($14.1 \pm 2.6\%$) than either the clay with batch feeding ($2.3 \pm 0.5\%$) or algae paste with batch feeding treatments ($2.8 \pm 1.5\%$).

The second study attempted to limit bacterial loading in the larval rearing culture tank by siphoning the larvae into adjacent, clean tanks at 1, 5, and 9 dph. The results showed significantly fewer *Vibrio* colonies after each transfer event in the passive larval transfer tank treatment. Also, survival was significantly higher at 14 dph for transferred larvae ($43.9 \pm 13.5\%$) than larvae in the control tanks ($23.1 \pm 6.3\%$).

INTRODUCTION

The yellowtail amberjack (*Seriola lalandi*) is a member of the family Carangidae, found in temperate waters of the Pacific and Indian oceans (Baxter 1960, MacCall et al. 1976, Sumida et al. 1985, Kolkovski and Sakakura 2007). In the Eastern Pacific, adult and juvenile *S. lalandi* range from southern Washington State to Chile, including the Gulf of California, and are seasonally abundant in southern California, where they are called California yellowtail (CYT). Larval distribution is limited to warmer waters from as far north as Point Conception in California, throughout the Southern California Bight and south into Baja California (Suminda et al. 1985). CYT larvae have been found seasonally from April through October (Sumida et al. 1985).

Because of its status as a high value food fish, especially as sushi, the CYT along with several other species in the

genus *Seriola*, are a focus of the growing worldwide aquaculture industry. Multiple *Seriola* spp. are cultured commercially in several countries around the Pacific (Benetti 1997, Nakada 2002, Fowler et al. 2003, Benetti et al. 2005, Verner-Jeffreys et al. 2006). These species include yellowtail *S. quinqueradiata* (Japan and Taiwan), amberjack *S. dumerili* (Japan), and almaco jack *S. rivoliana* (Hawaii). *S. lalandi* is being commercially cultured in Japan, South Australia, and New Zealand. Japanese farmers rely primarily on wild caught juveniles (Nakada 2002), and Australia and New Zealand produce juveniles from domestic broodstock (Fowler et al. 2003). Although commercial culture of *Seriola* spp. in the United States occurs only in Hawaii (Verner-Jeffreys et al. 2006), CYT is considered a top candidate for culture in southern California and Baja California, Mexico. To capitalize on the potential of this species, Hubbs-SeaWorld Research Institute (HSWRI) began an experimental CYT breeding program in San Diego, CA in 2003 with locally caught wild adults. Since the breeding program remains experimental in nature, larval rearing protocols are still being refined.

MATERIALS AND METHODS

All larval rearing trials were conducted in the same experimental system, which maintained consistently good water quality operating in either a flow-through or recirculating mode. The experimental system was comprised of twenty-four 320L black conical bottom tanks connected to a fluidized bed sand biofilter, a three cubic meter bead filter, 150 watt UV sterilizer, and two 50 μm bags to filter the water before returning to the culture tanks. Dissolved oxygen (DO), pH, and temperature were monitored daily using a portable meter (model HQ40d, Hach Company, Loveland, CO). Total ammonia nitrogen, nitrite, and nitrate were measured weekly with test kits (Hach Company, Loveland, CO). Separate rearing trials were completed to investigate: 1) prey selectivity through development, 2) removal of 1st instar *Artemia* (rotifers to 2nd instar *Artemia* offered at 5 dph, rotifers to 2nd instar *Artemia* offered at 7 dph, traditional HSWRI protocol: rotifers to 1st instar *Artemia* to 2nd instar *Artemia*), 3) clay as a greenwater replacement (clay with continuous feeding, clay with batch feedings, algae paste with batch feedings), and 4) passive larval transfers (passive larval transfers and no transfers).

The stocking density was set at 50 eggs/L for each trial, with each treatment being assigned four experimental tanks. Tanks were supplied with temperature-controlled (22°C) recirculating seawater at 1.5 – 2.0 L/min. A containment screen was placed in the center of each tank along with an aeration ring to maintain good water circulation. From 2 – 10 days post hatch (dph) 300 μm containment screens were used followed by 500 μm screens from 10 – 14 dph. Fluorescent lights were placed 0.7 m above the tanks for illumination (Lithonia, Conyers, GA). Light intensity (800 to 1,500 lux) was set at the surface by using a light meter (EXTECH Instruments, Waltham, MA), and light duration was set at 24 hr. Tanks were cleaned once per week for the duration of the trial and screens were changed as needed.

Live prey was added four times daily at 07:00, 10:00, 13:00, and 16:00. For each trial, except the prey selectivity trial, live prey were added to achieve densities of 20 rotifers/mL or 5 *Artemia*/mL. The prey selectivity trial used all three prey types (enriched rotifers, 1st instar *Artemia*, enriched 2nd instar *Artemia*) offered together from 3 to 20 dph, and live prey densities were kept at 5/ml for both rotifers and *Artemia*. Prey densities were measured twice daily in each tank by taking a 10 – 20 ml sample of seawater from each tank, and counting prey from three 1 mL aliquots of the total sample. Algae paste (Reed Mariculture, Campbell, CA) was used to green the water at a level of 300,000 – 500,000 cells/mL with each feeding. The greenwater replacement trial was standardized using secchi disc readings (30 – 40 cm) for the bentonite clay addition (New Mexico Clay Company, Albuquerque, NM).

Larval growth was measured every two days in the prey selectivity trial starting at 3 dph and ending at 20 dph. For all other trials, growth was measured at 0, 4, 8, and 12 or 14 dph on subsamples of 50 larvae per replicate tank. Larvae were euthanized with MS-222 prior to measuring. Twenty larvae were placed under a microscope (Leica MZ16 Macro, Bannockburn, IL), and digitally photographed (Image Pro Plus, Media Cybernetics, Bethesda, MD) for notochord length (NL) measurements (to the nearest 0.1 mm). All 50 larvae were then rinsed with de-ionized water and dried on glass filter paper dishes in an 80°C oven for 48 hrs. Dishes were weighed before adding larvae and again after the 48-hr drying period to the nearest 1 μg. To calculate the individual dry weight (DW), the total dry weight was divided by the total number of larvae sampled (N = 50).

Bacterial analysis was carried out in both the greenwater replacement and passive larval transfer trials. For both trials, 200 mL samples were collected in sterile cups from each replicate tank for each sample period. Using a micropipetter fitted with a sterile tip, 0.02, 0.1 and 1.0 mL sub-samples were extracted. All samples, along with 10 mL of sterile

saline, were individually vacuumed filtered through a column fitted with a 0.45 μm cellulose-nitrate filter membrane. Once the sample was pulled through the filter column, the membrane was removed using sterile tweezers and placed onto 100 mm thiosulfate-citrate-bile salts-sucrose (TCBS) agar plates. Plates were incubated for 24 hours at 30°C and colony forming units (CFU) were enumerated. Bacterial colony counts (CFU/ml) were used for data analysis. For the greenwater replacement trial, water was sampled from the rearing tanks at 2, 6, 9, and 11 dph, and for the passive larval transfer trial, water was sampled from the rearing tanks at 1, 2, 5, 6, 9, 10 and 12 dph. Sampling frequency was increased in the passive larvae transfer trial to better understand the rate of bacterial colonization for both treatments.

Statistical analyses were performed using Statistica 6.1 (SatSoft, Inc., Tulsa, OK). ANOVAs were used to determine differences among treatments ($P < 0.05$). Arcsine square root transformation was used in the analyses of percentage data.

RESULTS

Water quality measures for all experiments were similar and within an acceptable range for marine fish larvae. Water temperature was maintained at 21.5 ± 0.3°C and salinity was 34.5 ± 0.5 ppt. Dissolved oxygen ranged from 8.0 to 8.6 mg/L (92 – 96% saturation), and mean total ammonia nitrogen and unionized ammonia levels were < 0.01 and 0.0028 mg/L, respectively.

The prey selectivity trial (Figure 1) showed that the larvae selected 1st instar Artemia as early as 3 dph (4.5 mm NL) and 2nd instar Artemia as early as 5 dph (4.6 mm NL). The trial removing 1st instar Artemia showed no significant differences in growth (NL
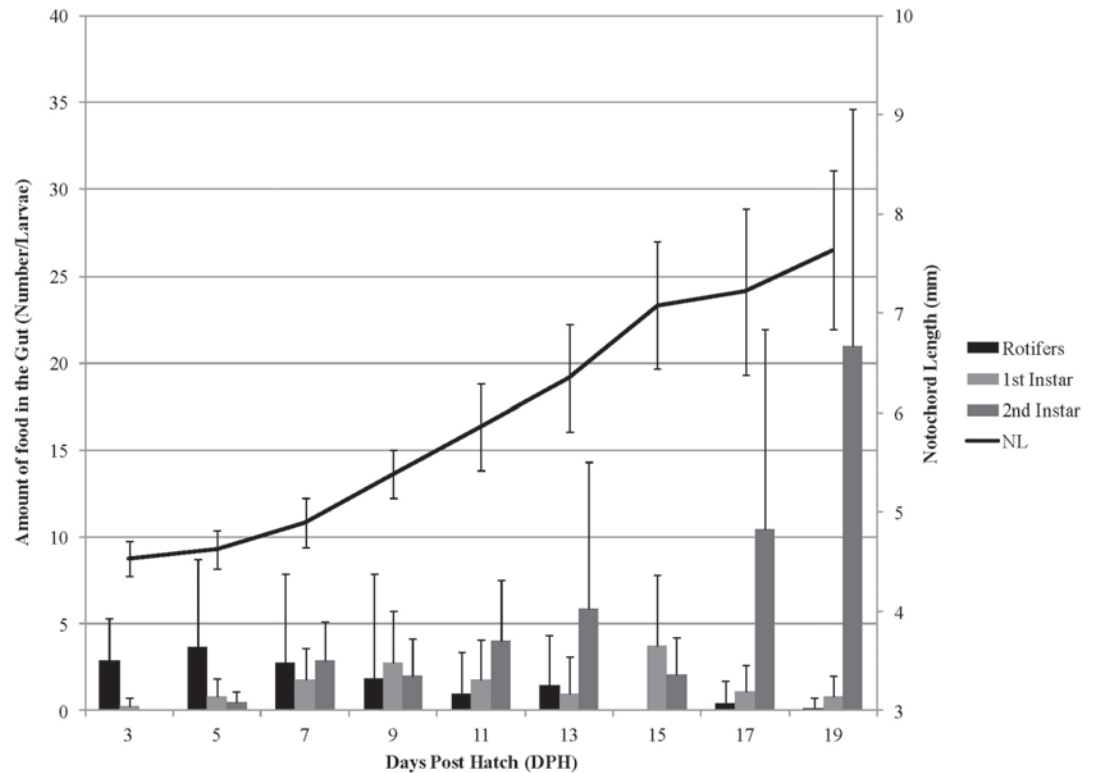

Figure 1. Prey selectivity for CYT offered rotifers, 1st instar *Artemia* and 2nd instar *Artemia*.

and DW) or survival between the traditional HSWRI protocol (rotifers to 1st instar *Artemia* to 2nd instar *Artemia*), rotifers to 2nd instar *Artemia* offered at 5 dph, and rotifers to 2nd instar *Artemia* offered at 7 dph (Table 1).

The greenwater replacement trial showed no significant differences in growth (NL and DW) among any of the treatments (Table 1). However, larvae in the clay with continuous feeding treatment had significantly higher survival (14.1 ± 2.6%) than the clay with batch feeding treatment or the algae paste with batch feeding treatment at 2.3 ± 0.5% and 2.8 ± 1.5%, respectively, at 12 dph. The bacterial colony counts were significantly higher in the batch fed algae paste treatment at 9 and 11 dph (Figure 2).

Table 1. Summary of performance measures for various larval rearing trials conducted on CYT.

Trial	Treatments	End of Trial (dph)	DW (µg ± SD)	NL (mm ± SD)	Survival (% ± SD)
Removal of 1st Instar	2nd instar *Artemia* offerred at 5 dph	14	725 ± 117[a]	6.83 ± 0.68[a]	34.5 ± 8.4[a]
	2nd instar *Artemia* offerred at 7 dph	14	660 ± 91[a]	6.86 ± 0.57[a]	24.6 ± 4.9[a]
	Rotifers to 1st instar to 2nd instar *Artemia*	14	855 ± 102[a]	7.05 ± 0.65[a]	27.6 ± 11.6[a]
Passive Larval Transfer	Passive transfer	12	365 ± 89[a]	6.49 ± 0.59[a]	43.9 ± 13.5[a]
	No transfer	12	510 ± 42[b]	6.78 ± 0.53[a]	23.1 ± 6.3[b]
Greenwater Replacement	Clay with Continuous Feeding	12	425 ± 106[a]	6.59 ± 0.67[a]	14.1 ± 2.6[a]
	Clay with Batch Feeding	12	450 ± 84[a]	6.41 ± 0.72[a]	2.3 ± 0.5[b]
	Algae Paste with Batch Feeding	12	445 ± 19[a]	6.51 ± 0.93[a]	2.8 ± 1.5[b]

Values within each experimental column sharing superscripts are not significantly different.

Finally, larval survival was significantly higher (43.9 ± 13.5%) in the passive larval transfer treatment, but there were no significant differences in growth among the treatments until 12 dph, when larvae in the non-transfer tanks were significantly larger (Table 1). Bacterial colony counts were significantly higher at 6, 9, and 10 dph in the non-transfer tanks (Figure 3).

DISCUSSION

Larvae offered all prey types with each feeding preferentially selected larger food items throughout development. At 4.5 mm NL (3 dph), the larvae began selecting 1st instar *Artemia*, and when the larvae were 4.7 mm NL (5 dph), they began selecting 2nd instar *Artemia*. Hamasaki et al. (2009) showed that *S. dumerili* will also select larger prey items as larvae increase in size, and state that greater amberjack larvae larger than 4.5 mm NL could prey on *Artemia* nauplii. While this trial demonstrated possible live feed transitions for CYT, it does not accurately portray the consumption potential for the species (Stuart unpublished data). Stuart and Drawbridge (2012) showed that CYT should be offered 20 rotifers / mL throughout the early stages of development (2 - 10 dph). By offering a lower prey density at first feeding (5 rotifers / ml) here, larvae may have been compromised. *S. lalandi* larvae are vulnerable to short period starvation
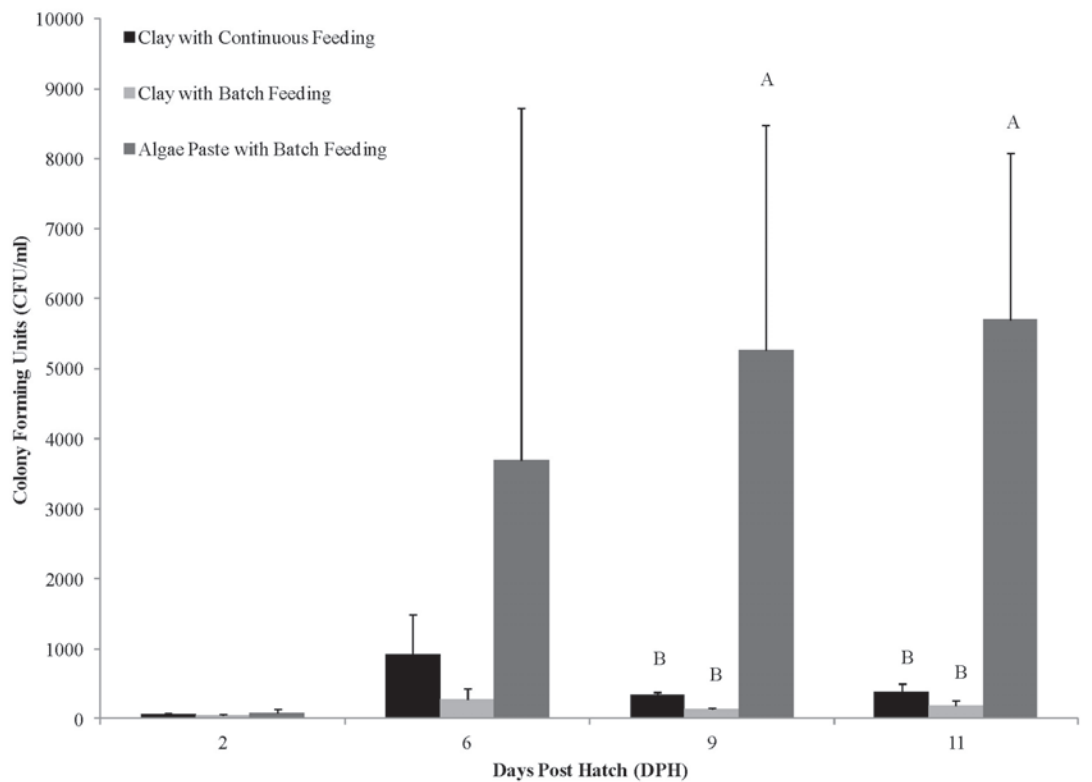

Figure 2. Bacterial colony counts at various time intervals under treatments of clay with continuous feeding, clay with batch feedings, and algae paste with batch feedings. Values sharing letters within ages are not significantly different.
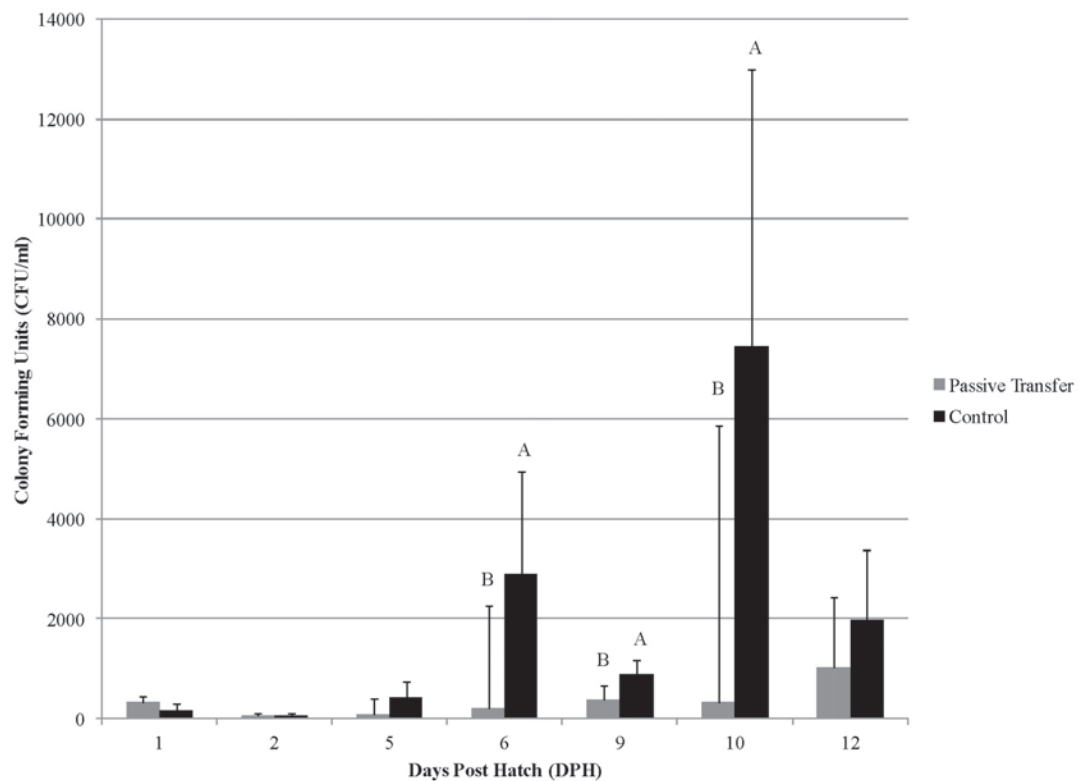

Figure 3. Bacterial colony counts from the passive larval transfer trial. Passive larval transfers were conducted at 1, 5, and 9 dph. Values sharing letters within ages are not significantly different.

at an early age, 2 to 5 dph, and starvation should be avoided until after metamorphosis (Chen et al. 2007). Because the food ration was not optimal for this species in the current trial, future trials should compartmentalize the life stages to offer appropriate food rations in order to determine age specific consumption rates.

There was no treatment effect when comparing growth and survival for larvae beginning the feeding transition onto 2nd instar *Artemia* at 5 dph or 7 dph or larvae that were offered the traditional HSWRI feeding protocol (rotifers, 1st and 2nd instar *Artemia*). An important aspect of larval nutrition is providing adequate levels of highly unsaturated fatty acids (HUFAs) including arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid (Sargent et al. 1999, Faulk and Holt 2005). Since 1st instar *Artemia* nauplii are unable to be enriched (no functional mouth or digestive tract), eliminating that stage allows for a constant supply of enriched food items for the larvae, which may result in higher quality juveniles. Reducing opportunistic bacteria is a key element in microbial control of the rearing environment for marine fish larvae (Vadstein et al. 1993). The positive effect of microbial mitigation is greatest when larvae are stressed, particularly after hatching. Due to their still developing immune systems, newly hatched fish larvae are vulnerable to infections by opportunistic bacteria (Vadstein et al. 1993, Vadstein et al. 2004, Attramadal et al. 2012). Methods to limit the microbial load in rearing systems include reducing organic inputs (i.e. live feeds and algae) and increasing the removal of organic matter (Attramadal et al. 2012). The addition of bentonite clay and the use of passive larval transfers reduced the abundance of potentially harmful *Vibrio spp.* and theoretically, other opportunistic bacterial species as well. Bentonite clay did not affect growth or survival of CYT making it useful as a green water replacement and bacterial control agent.

CONCLUSION

We have determined that combining continuous feeding with clay as a turbidity source and multiple tank transfers throughout development appears to benefit both the growth and survival of CYT larvae. These studies have greatly improved our understanding of the culture requirements for this high performance species, highlighted important areas for future research, and provide a knowledge base to enable successful commercial culture of CYT in southern California.

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Intensive Juvenile Production of Yellowtail Amberjack *(Seriola lalandi)* in Southern California

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ABSTRACT

Hubbs-SeaWorld Research Institute (HSWRI) has been culturing yellowtail amberjack (*Seriola lalandi*) at its laboratory in San Diego, CA since 2003. In 2007, intensive-level production began at the Mission Bay research facility in order to refine methodologies at a commercial scale. Initial results yielded survival levels to one gram juvenile between 0.2 to 5.0%, with varying degrees of skeletal malformation (often greater than 40%). Moreover, larval swim bladder inflation rates have been highly variable, an issue that poses potential problems for cage-based production of the species. In 2012, live feed quality and larval rearing microbial management improvements were made to production protocols and systems to increase juvenile quantity and quality. Many of these changes were based on observations made during HSWRI larval rearing trials coupled with advancements in the global aquaculture industry.

Live feed [rotifers (*Brachionus placatilis*) and *Artemia* (*Artemia franciscana*)] quality was enhanced through both improvement of water quality, and limitation of bacterial loading in the live feeds culture systems. Larval rearing systems were improved through modifications to filtration/disinfection equipment and husbandry protocols.

Ultimately, utilizing improved systems and protocols, three *Seriola lalandi* production runs were completed in the summer of 2012. Resulting production yielded a combined total of over 360,000 weaned juveniles in 2012. Survival rates were 34%, 7%, and 14%, respectively. Skeletal malformation rates were relatively low (< 5.0%) compared to previous years' production. Although these results have been encouraging, larval swim bladder inflation rates were still problematic.

INTRODUCTION

Seriola spp. fingerlings are being produced at various capacities in the United States (*S. rivoliana* and *lalandi*), Chile (*S. lalandi*), Mexico (*S. rivoliana* and *lalandi*) and Ecuador (*S. rivoliana*). In all cases, the organizations involved in these operations are practicing closed life-cycle production; wild and/or F1 broodstock are spawned in captivity and juveniles are reared from their eggs. Juveniles are then either sold nationally or internationally and/or fish are raised to market size within land-based tanks or open-ocean cages.

Being the only *S. lalandi* hatchery in the United States, Hubbs-SeaWorld Research Institute (HSWRI) has been developing commercial scale production techniques for California yellowtail since 2003, when the institute first spawned wild broodstock in captivity. Through the years, various methodologies have been developed via replicated experimentation and larger-scale production run trials. Although results prior to 2012 have shown a steady improvement in production efficiency, average survival rates from egg to one gram juvenile were only as high as 5.0%, with skeletal malformation rates often over 40%.

In 2012, a number of significant changes were made to yellowtail production systems and methodologies, primarily dictated by information gleaned from prior experiments and production-scale runs. Results from the 2012 yellowtail production season were much improved due to these modifications, yielding over 360,000 total weaned juveniles. Survival rates were 34%, 7%, and 14%, respectively, and skeletal malformation rates were low (< 5.0%). Although these survival and malformation results were considered to be a major milestone for commercial-scale *S. lalandi* hatchery production, swim bladder inflation levels were poor, resulting in inflation rates of < 1.0%, 23%, and 46%, respectively.

This report discusses the modifications made to HSWRI systems and protocols that led to this improvement in production. Focus was placed on a) management of bacterial populations within live feeds and larval tanks through improved water filtration systems and passive larval transfer techniques, b) maintenance of live feed quality through improved

cold-storage equipment, and c) delivery of live feeds to larval fish through continuous rotifer/*Artemia* addition. Finally, the topic of swim bladder inflation will be discussed with reference to potential solutions to this production bottleneck for the 2013 season.

Bacterial Management

The bacterial ecology within aquaculture systems has been clearly linked to the successes and failures of marine fish larval rearing (Munro et al. 1995, Olafsen 2001, Ringø and Birkbeck 1999 and Rotman et al. 2005). Leading up to and during the 2012 yellowtail production season, HSWRI scientists began an initiative to better manage bacterial populations within the culture systems.

Vibrio spp. were used as indicators for the presence and quantity of opportunistic bacterial strains. Using thiosulfate-citrate-bile salts-sucrose agar (TCBS) and a quantitative sampling method described by Hernández-López et al. (1995), water quality samples were tested for *Vibrio* spp. within live feeds cultures, larval cultures and incoming water sources. Four key areas were identified as bacterial "hot-spots" within HSWRI culture systems. The following are descriptions of these areas and associated actions taken to mitigate opportunistic bacterial growth.

1) *Incoming water sources* – prior to 2012, water added to larval rearing, rotifer, and *Artemia* systems all tested positive for the presence of *Vibrio* spp. To mitigate bacterial levels in the incoming water, high-capacity cartridge filters were installed as microparticulate removal systems followed by high-output UV sterilization units, yielding kill-rates of $\geq 50,000 \, \mu Ws/cm^2$. With these improvements, TCBS bacterial counts in incoming seawater shifted from as high as 400 colony forming units (CFUs) per ml to non-detectable levels.

2) *Rotifers* – Traditional methods for culturing rotifers, commonly referred to as "batch cultures," have a tendency to encourage the proliferation of opportunistic bacteria (Rotman et al. 2011). This is, in part, due to an overabundance of feed and also to constant "disturbance" of the culture when it is harvested and re-stocked, allowing opportunistic bacteria to regularly outcompete potentially beneficial bacteria. To mitigate sources of opportunistic bacteria, rotifers were raised within a continuous culture recirculation system. Continuous culture systems have the benefit of creating a stable environment that encourages the growth of harmless, if not beneficial, bacteria.

Rotifers sourced from the HSWRI continuous culture system had non-detectible levels of *Vibrio* spp., whereas rotifers raised in traditional batch culture systems commonly have over 1000 *Vibrio* spp. CFUs/ml of culture water.

3) *Artemia* – *Artemia* have long been identified as a source of potentially harmful bacteria in hatcheries (Olafsen 2001). Recent advances in *Artemia* culture methods and products now allow hatcheries to produce "clean" *Artemia*. Hatch Controller and the SEP-Art cyst separation system (INVE Aquaculture, Inc., Salt Lake City, UT) were used for the 2012 production season. Moreover, *Artemia* culture tanks, harvesters, and culture water were disinfected daily with a chlorine solution. Results yielded *Artemia* with *Vibrio* spp. counts less than 0.5 CFUs/*Artemia* nauplius. Prior to these improvements, *Artemia* bacterial assessments often yielded *Vibrio* spp. counts greater than 300 CFUs/*Artemia* nauplius.

4) *Passive larval transfers* – Traditionally, marine fish larval rearing methods include daily hand-siphoning of tank bottoms. This is done to remove organic debris (feces, uneaten feed, dead larvae, etc.) that acts as a growth media for a variety of potentially harmful microorganisms. Although it is necessary to keep larvae in clean tanks, hand-siphoning can result in a disturbance of the bottom debris that lifts some proportion of this material into the water column where it contacts larval fish. Moreover, since algae is added as a contrast agent for improved larval fish feeding, it is often difficult to see the tank bottoms well enough to properly hand-siphon. Lastly, hand-siphoning does not remove potentially harmful biofilm growing on tank walls, standpipes and other objects within the larval tank. As an alternative to hand-siphoning, 2012 production protocols included passive larval transfer techniques. Utilizing a 3" diameter hose, larval fish were transferred from "dirty" to "clean" tanks, resulting in an overall reduced exposure of larval fish to opportunistic bacteria. Depending on the specific run, larval fish were transferred 3–5 times during early life stages (0–14 dph). A concurrent experiment comparing this method to standard larval rearing methods showed significantly lower *Vibrio* spp. growth using passive larval transfer techniques.

Live Feed Quality

Traditional rotifer and *Artemia* culture protocols include a final enrichment prior to feeding them to larval fish. This enrichment typically includes an

array of nutrients essential for proper larval fish development that does not naturally exist in the live feed organisms, such as highly unsaturated fatty acids (HUFAs), amino acids, vitamins, and minerals. Because both rotifers and *Artemia* rapidly metabolize this enrichment, it is common practice to preserve enrichment levels by chilling live feeds down to cooler temperatures (4-10ºC) in what are referred to as "cold banks" or "cold storage"(Lavens 1996).

A common method of cold storage is placing concentrated live feeds into insulated flat-bottom coolers along with some kind of cold source (frozen ice bottles, gel-packs), oxygenation, and aeration (Moretti et al. 1999). However, since both rotifers and *Artemia* cease to swim at these cooler temperatures, they can accumulate in piles, creating low dissolved oxygen areas potentially resulting in > 50% mortality. This mortality then translates into elevated bacterial levels and sub-optimal enrichment profiles that lead to poor larval performance. Lastly, temperature in this traditional method of cold storage is difficult to control without constant monitoring.

To mitigate the variation associated with cold storage, HSWRI staff developed a self-contained cold storage unit. By adding a secondary thermostat to a standard chest freezer, target temperatures were maintained without the need for continuous monitoring. Additionally, the use of aerated conical tanks inside this chest freezer for live feeds minimized settling and yielded rotifer and Artemia survival rates consistently greater than 90% after 24-hours of storage.

Live Feed Delivery
Traditional larval rearing protocols involve "meal" feeding strategies during rotifer and *Artemia* phases. This typically involves 4-8 feedings/day and results in fluctuating feed density within larval tanks (Moretti et al. 1999). Previous HSWRI trials have demonstrated that *S. lalandi* require 15-20 rotifers/ml to effectively feed. If rotifers are at less than 15/ml, larvae cannot effectively find prey and feed intake is diminished.

To address daily variation in live feed densities, HSWRI staff explored the use of a "continuous feeding" strategy. Using high-quality peristaltic pumps, rotifers and *Artemia* were continuously dosed into larval rearing tanks between 20 to 24 hours per day. Live feeds were extracted directly from cold storage and injected into a water line leading to the larval tanks. Rotifer densities were maintained at 20/ml ± 5/ml. *Artemia* densities were maintained between 2-5/ml. Larval gut observations demonstrated that

larvae will feed 24-hours per day. Moreover, the automated feeding protocol ultimately required less labor to execute than standard meal feedings.

Swim Bladder Inflation
Swim bladder inflation (SBI) is a critical developmental milestone in early larval development (Woolley et al. 2012). Although the HSWRI 2012 larval rearing survival and skeletal deformity rates were markedly improved over previous years, SBI rates were poor, with the three runs yielding < 1.0%, 23%, and 46%, respectively. Throughout the 2012 production season, a series of un-replicated, short-term, production-level trials were carried out in attempt to better understand and address the source(s) of poor SBI. Three major variables were examined as potentially contributing to this problem.

1) *Larval rearing tank surface cleanliness* – It is known that *S. lalandi* larvae first inflate their swim bladder at 3-6 dph by "gulping" a bubble of air from the water surface. If there is excess oil or other detritus on the surface, this process could be hindered. To address this issue, two variables were examined:
   a. More frequent cleaning of surface skimmers – Surface skimmer cleaning shifted from 3-4 times per day to once every two hours.
   b. Algae paste type – Because the algae paste used to provide contrast during larval rearing may have been a source of surface oil, prior to the third run of the season, the algae paste was switched from the "oilier" Rotigrow Plus® to Nanno 3600® (Reed Mariculture, Campbell, CA).

2) *Feeding regime* – It was theorized that SBI might have been hindered by the new 24-hour feeding methods. A production-scale comparison was executed comparing 24-hour feeding to "meal" feeding.

3) *Photoperiod* – Some species of marine fish have been shown to require a dark-period to initiate swim bladder inflation, and it was theorized that the 24-hour photoperiod used during the 2012 production season might have somehow inhibited SBI. A production-scale trial was executed comparing 24-hour photoperiod to 14 light: 10 dark.

Results from these trials only demonstrated marginal improvements. Although no marked differences

were found between different feeding regimes and photoperiod, the SBI increase in the third production run may have been due to using a lower-oil algae paste and more frequent surface skimmer cleaning.

CONCLUSIONS

Although SBI rates were still sub-standard, improvements made to the HSWRI *S. lalandi* production systems and methods yielded improved larval survival and reduced skeletal deformity rates. With over 360,000 weaned juveniles produced out of three 8,000 liter tanks resulting from changes made in 2012, it is clear that sufficient quantities of fingerlings can be produced to support commercial-scale growout. Indeed, if grown to a market size of 2 kilos, 360,000 fingerlings could yield up to 720 metric tons of harvestable fish.

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Effects of Artificial Lighting Intensity and Wavelength on the Growth and Survival of Juvenile Flatfish

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ABSTRACT
In this study, juveniles of three flatfish (spotted halibut *Verasper variegatus*, slime flounder *MIicrostomus achne*, and Japanese flounder *Paralichthys olivaceus*) were studied to elucidate the effects of varying the intensity and wavelength of artificial lighting conditions. We conducted rearing experiments for each developmental stage monitoring growth, survival, and feeding status (feeding incidence, average number of prey in the digestive tract) as an indicator of the optimal light environment. Light intensity: In spotted halibut that live in very shallow waters, feeding status was better, and growth and survival rate was improved in brighter conditions and fish were observed to feed even in dark conditions. On the other hand, for slime flounder that live in the deep sea feeding status was better in dark conditions exhibiting improved growth and survival. Wavelength: As a result of rearing experiments under various wavelengths for spotted halibut and flounder, feeding status under green light (518 nm) was better than under white light. It has been suggested that the optimal light environments are closely related to the habitat depth for each fish species at each developmental stage.

INTRODUCTION
It is widely known that certain light-response mechanisms are closely associated with the life history of fish. Light control technology has been essential for manipulating the spawning cycle in broodstock management. In addition, the light environment can significantly affect first-feeding success in various fish species during hatchery seed production. Rearing techniques that optimize light conditions to the target species are essential for efficient production of healthy seed.

In the present study, juvenile specimens of three important flatfish species for fisheries in northern Japan, the spotted halibut (*Verasper variegatus*), slime flounder (*Microstomus achne*), and Japanese flounder (*Paralichthys olivaceus*), were studied to determine the optimal artificial light intensity and wavelength. We conducted rearing experiments for each developmental stage, and measured growth, survival, and feeding status (feeding incidence, average number of prey in the digestive tract) as indicators of the optimal light environment.

MATERIALS AND METHODS
Test Fish
In this study, three flatfish species (spotted halibut, slime flounder, and Japanese flounder) were examined. Pelagic larvae of the spotted halibut are initially distributed on the surface layer of the ocean and thereafter settle in shallow coastal water seeming to prefer bright conditions (Wada et al. 2007; 2012). On the other hand, slime flounder larvae are distributed from the surface layer to a depth of 150 m and seem to prefer darker environments (Ishito and Hashimoto 1993; Wada et al. 2010). The Japanese flounder is intermediate between these two species ecologically (Kuwahara and Suzuki 1982; Hasegawa et al. 2003). In Japan, seed production technology of the Japanese flounder is the most highly developed.

Fish were reared to various developmental stages at the Miyako Station of the Tohoku National Fisheries Research Institute. We conducted rearing experiments for each of three developmental stages to better understand the ecological changes associated with growth. The stages were: stage C: preflexion larva, the onset of mouth opening and feeding, stage E: flexion larva, just before the metamorphosis stage, and stage I: juvenile, metamorphosis completed. The shift from pelagic to benthic habit occurs at this stage. (Minami 1982; Aritaki et al. 2001; Aritaki and Tanaka 2003). Light intensity experiments were conducted on stages C, F and I; wavelength experiments were performed on stages C and F. Information on rearing conditions (capacity of the rearing tank, light conditions, water temperature, and feeding regimes) is summarized in Table 1.

Species	Capacity of rearing tank (kL)	Light condition	Water temp. (°C)	Rotifer feeding (DAH*)	Artemia feeding (DAH*)
Spotted halibut	2-15	Ambient	16	8-23	22-47
Japanese flounder	0.5-50	Ambient	16	1-22	19-37
Slime flounder	0.5-2	Ambient	15	3-26	10-96

*DAH: Day after hatching

Rearing experiments: light intensity and wavelength
Larvae and juveniles of the three flatfish species from matching developmental stages were used for both experiments. Twenty (Stage C and F) or ten (Stage I) individuals from each species were randomly selected from rearing tanks, transferred into a 1L glass beaker filled with 1L of filtered sea water and allowed to acclimatize for 15 h in utter darkness. Mild aeration using an air stone was provided for each beaker. In the light intensity experiment, the distance from the light source (fluorescent lamp) to the beakers was adjusted by moving the beakers. Thus, each beaker was exposed to five different intensities: 22.8, 12.6, 2.3, 0.2, and 0 $\mu mol \cdot m^{-2} s^{-1}$. Light intensity was measured just above the water surface. In the wavelength experiment, the light sources were 5-color LEDs with different wavelengths (blue: 464, blue-green: 497, green: 518, red: 635 nm, and white). Light intensities were set to the optimum value for each species as determined in the previous experiment. After acclimatization in utter darkness for 15 h, test fish were acclimatized for 1 h at the specified light intensities and wavelengths.

Four replicates were conducted to investigate feeding incidence and the number of prey in the digestive tract on the first day of the experiment, and four additional replicates were conducted to investigate total length and survival rate at 10 (Stage C) or 15 (Stage F and I) days after the start of the experiment. Prey organisms were rotifers (*Brachionus plicatilis*) (Stage C) or *Artemia* spp. (Stage F and I). Rotifers and *Artemia* were provided at a density of 10 and 1 ind./mL, respectively. Once a day, 200 mL of sea water with excrement and dead individuals were removed from each beaker using a pipette and 200 mL of filtered sea water were added to avoid deterioration of water quality. The photoperiod was configured to 12L:12D. Details of the light intensity and wavelength rearing experiments are summarized in Table 2.

Table 2. Summary of the experimental setting.

Species	St.*1	DAH	TL*2 (mm)	No. of ind. (ind./L)	Prey organism*3	Term (days)
Spotted halibut	C	10	6.8±0.2	20	Ro	10
	E	25	9.2±0.7	20	Ar	15
	I	65	22.9±1.6	10	Ar	15
Japanese flounder	C	7	5.0±0.2	20	Ro	10
	E	20	8.5±0.5	20	Ar	15
	I	40	17.9±1.7	10	Ar	15
Slime flounder	C	7	7.0±0.2	20	Ro	10
	E	33	11.5±0.3	20	Ar	15
	I	95	27.1±2.3	10	Ar	-*4

*1 St.: Developmental stage
*2 TL: Total length, values are expressed as mean ± S.D.
*3 Ro: Rotifer, Ar: *Artemia*
*4 In stage I for slime flounder, feeding status was only investigated on the first day of the light intensity experiment

Figure 1. Feeding incidence (a) and number of prey in digestive tract (b) on the first day of the experiment, and total length (c) and survival rate (d) at the end of the experiment under various light intensities for spotted halibut *Verasper variegatus*.

RESULTS AND DISCUSSION
Light intensity
Feeding incidence and the number of prey in the digestive tract on the first day of the experiment, as well as total length and survival rate at the end of the experiment under various light intensities are summarized in figures 1 – 3 (Fig. 1: spotted halibut, Fig. 2: Japanese flounder, Fig. 3: slime flounder).

Stage C spotted halibut displayed the highest values in the test group for feeding status (feeding incidence and
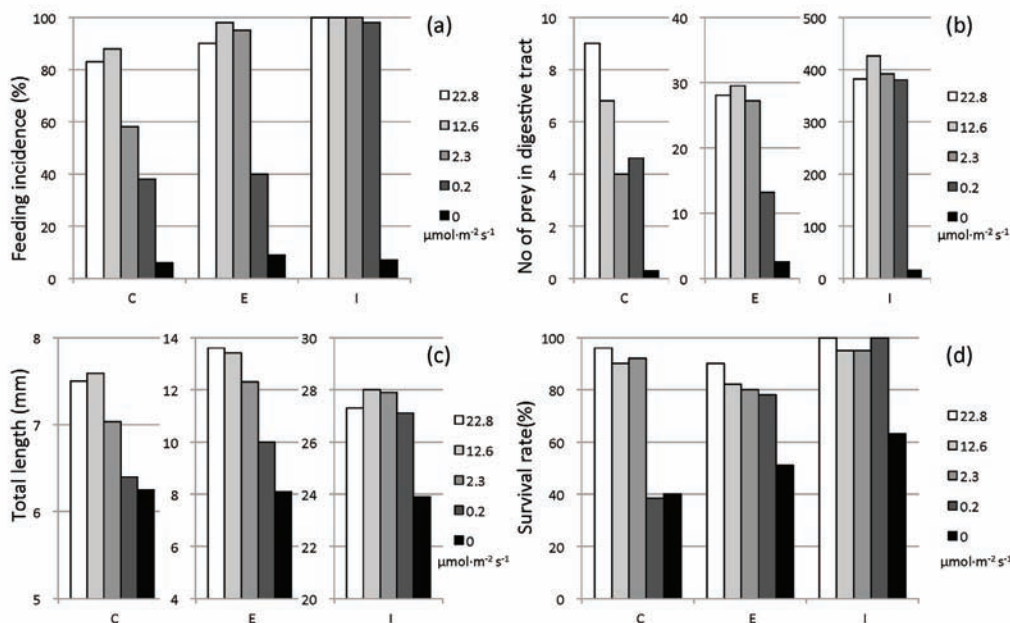
Figure 2. Feeding incidence (a) and number of prey in digestive tract (b) on the first day of the experiment, and total length (c) and survival rate (d) at the end of the experiment under various light intensities for Japanese flounder *Paralichthys olivaceus*.
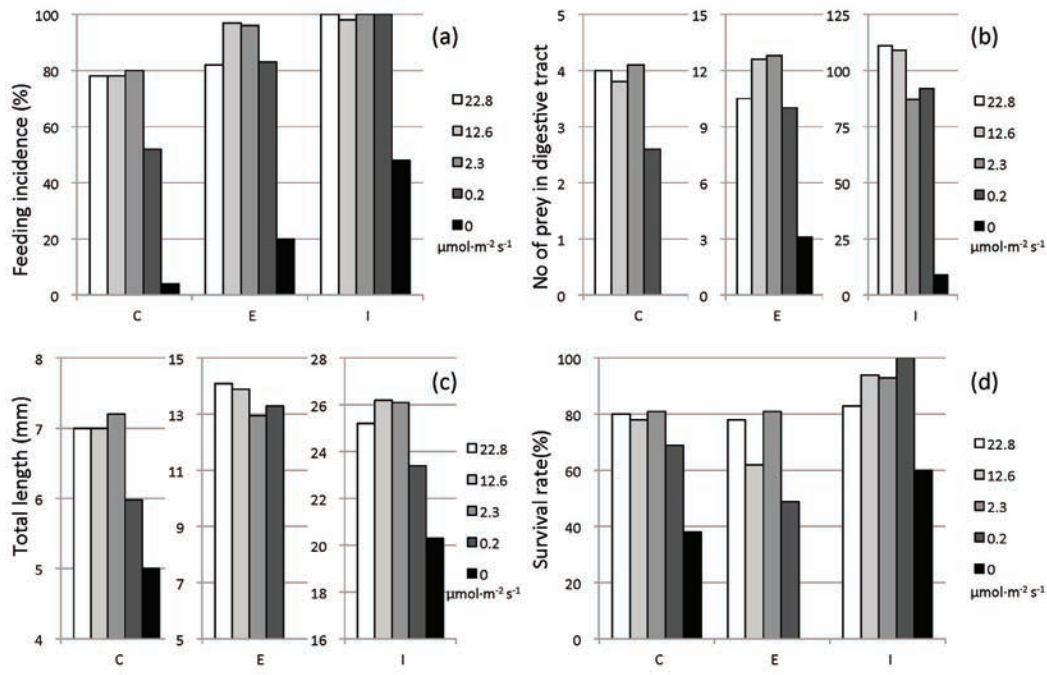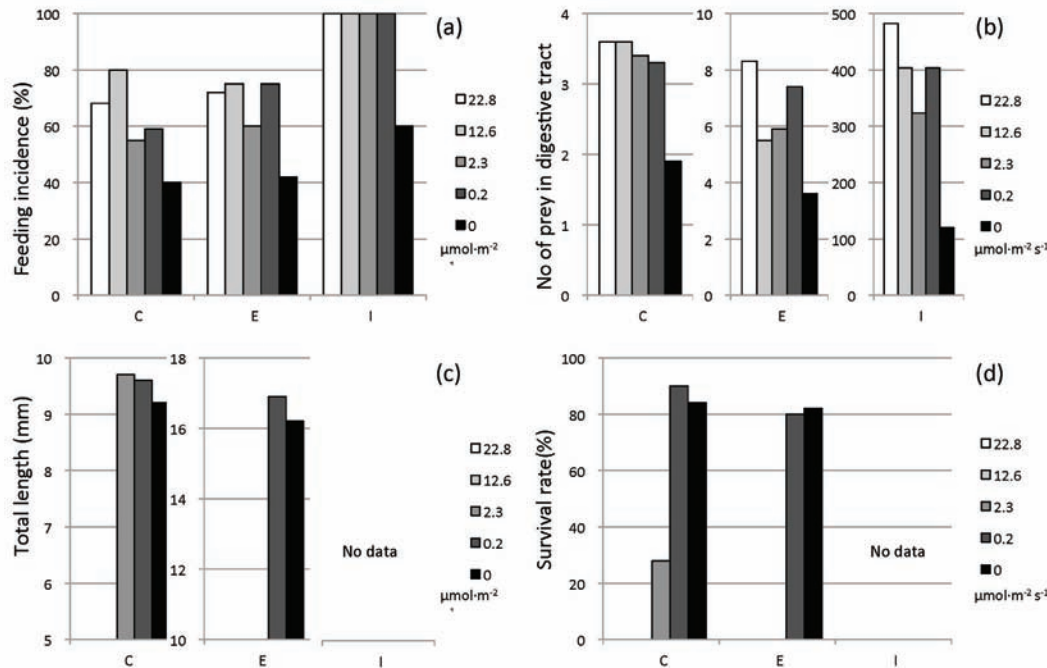

Figure 3. Feeding incidence (a) and number of prey in digestive tract (b) on the first day of the experiment, and total length (c) and survival rate (d) at the end of the experiment under various light intensities for slime flounder *Microstomus achne.*

number of prey in the digestive tract) at light intensities of more than 12.6 μmol·m^{-2}s^{-1}; growth and survival rate were also improved. Similarly, Stage E and I spotted halibut also showed the highest values among the test group at light intensities greater than 2.3 and 0.2 μmol·m^{-2}s^{-1}, respectively. From the above results, feeding status was better, and growth and survival rate were improved in brighter conditions for spotted halibut, which typically live in shallow waters. In addition, their growth showed that they feed even in dark conditions (Fig. 1).

For Japanese flounder, which live in deeper waters than spotted halibut, high values in the test group for feeding status, growth, and survival rate for Stage C were observed with light intensities greater than 2.3 μmol·m^{-2}s^{-1}. Similarly, Stage E and I Japanese flounder had the highest values within the test group at greater than 0.2 μmol·m^{-2}s^{-1}. These results suggest that Japanese flounder can be reared in darker conditions than spotted halibut (Fig. 2).

For slime flounder, which live in the darkest environment, no experiments were performed for Stage I. Overall, slime flounder were able to feed better in dark environments compared to spotted

halibut and Japanese flounder. Additionally, they were able to feed in utter darkness (0 μmol·m^{-2}s^{-1}). In stage C and E, they were able to feed under bright conditions of 2.3 μmol·m^{-2}s^{-1} or more, but they could not survive until the end of the experiment. Slime flounder larvae in bright environments sank to the bottom to avoid the light, and then died. From the above results, slime flounder, which live in the deep sea, were shown to be able to feed in dark conditions, and their growth and survival rate were improved under such conditions (Fig. 3).

Wavelength

Feeding incidence and the number of prey in the digestive tract on the first day of the experiment, as well as total length and survival rate at the end of the experiment under various wavelengths are summarized in figures 4 - 6 (Fig. 4: spotted halibut, Fig. 5: Japanese flounder, Fig. 6: slime flounder). Light intensities in the wavelength experiment were set to the optimum values determined in the previous experiment: those for spotted halibut and Japanese flounder were 12.6 μmol·m^{-2}s^{-1}, and that for slime flounder was 0.2 μmol·m^{-2}s^{-1}.

Figure 4. Feeding incidence (a) and number of prey in digestive tract (b) on the first day of the experiment, and total length (c) and survival rate (d) at the end of the experiment under various wavelengths for spotted halibut *Verasper variegatus*.

Figure 5. Feeding incidence (a) and number of prey in digestive tract (b) on the first day of the experiment, and total length (c) and survival rate (d) at the end of the experiment under various wavelengths for Japanese flounder *Paralichthys olivaceus*.
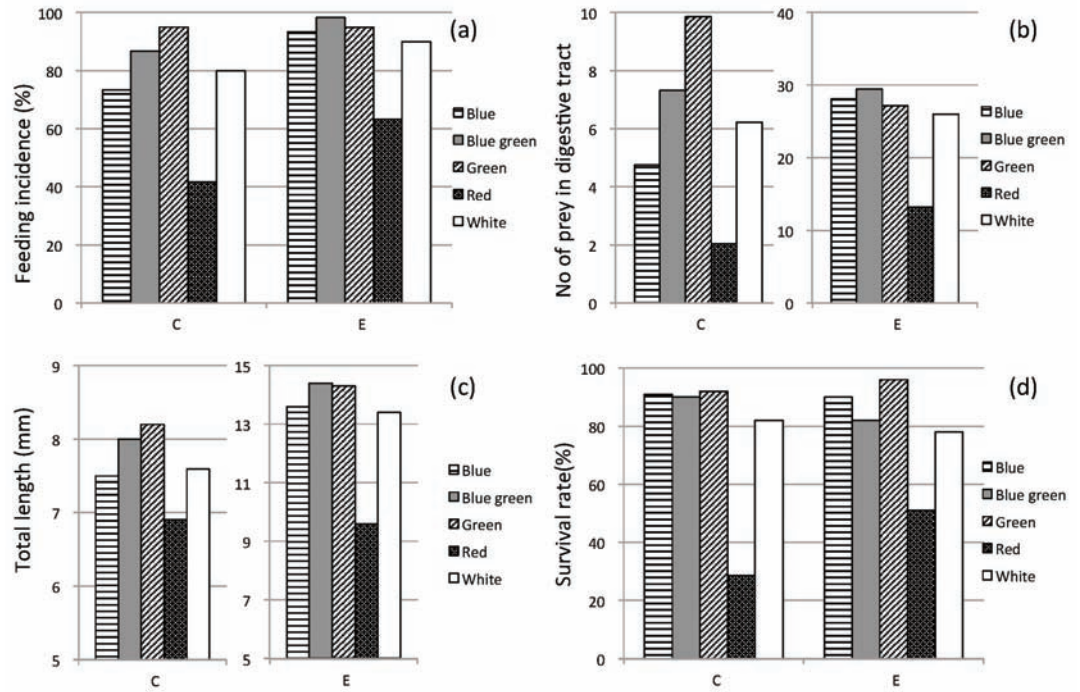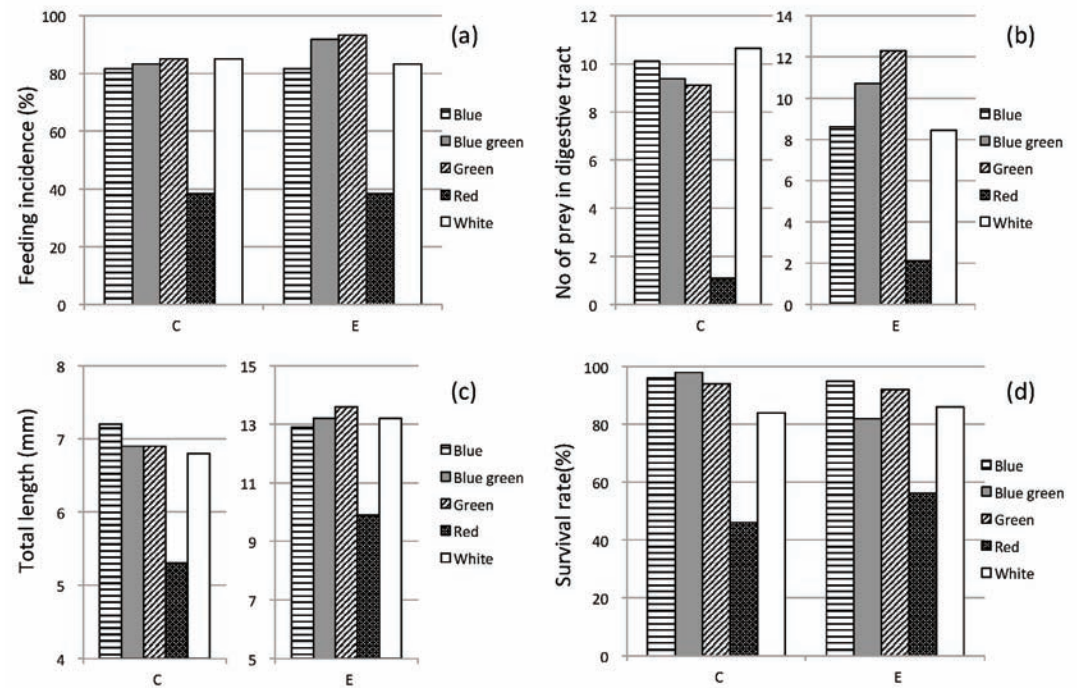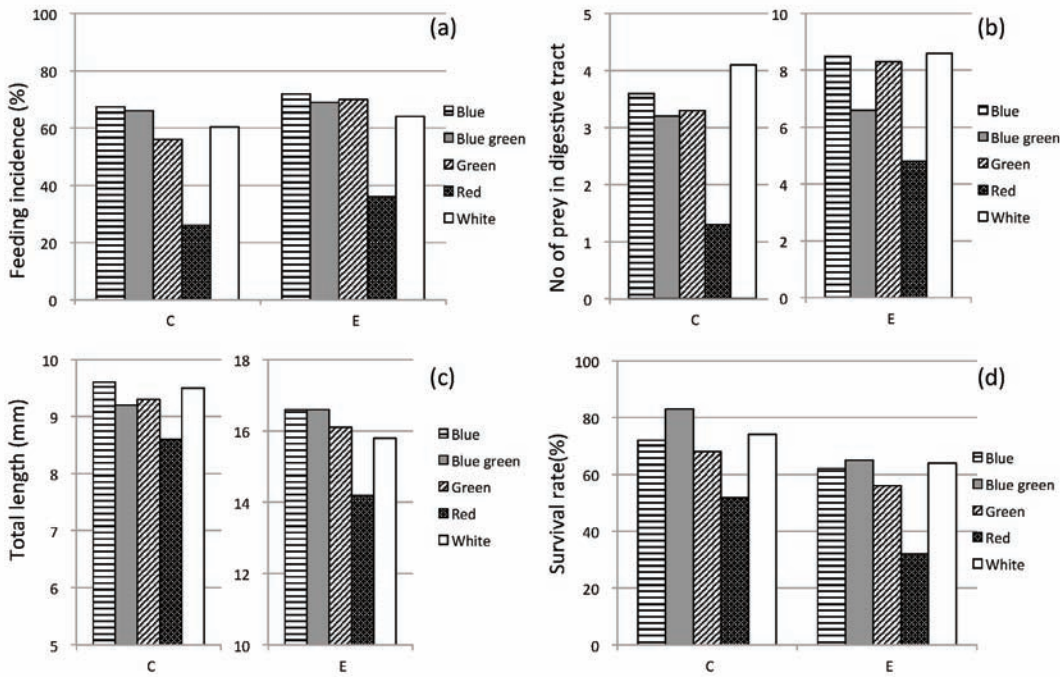
Figure 6. Feeding incidence (a) and number of prey in digestive tract (b) on the first day of the experiment, and total length (c) and survival rate (d) at the end of the experiment under various wavelengths for slime flounder *Microstomus achne.*

For Stage C spotted halibut, feeding status (feeding incidence and number of prey in the digestive tract) in the green (518 nm) and blue-green (497 nm) wavelengths were higher, and in the red (635 nm) light was lower, compared to that in the white light. In Stage E, there were no appreciable differences between the blue (464 nm), blue-green, green, or white light. For Stage C and E Japanese flounder and slime flounder, there were no appreciable differences between the blue, blue-green, green or white light groups. Overall, the growth and survival rates reflected feeding status.

Data from electro-intraretinal-retinogram in adult fish of these species showed the peak spectral sensitivity for spotted halibut was 521 nm, that of Japanese flounder was 511 nm, and that of slime flounder was 489 nm, all of which is supported by the results of the rearing experiments. The above results suggest that the optimal light environment is closely related to the habitat depth for each fish species and each developmental stage.

Application to Mass Production
We have carried out mass production experiments applying the results of this study with spotted halibut. Currently, to control mortality in the early larval stage we have manipulated photoperiod and found that the use of 24-hour continuous lighting for 10–15 days after hatching promotes larval feeding and improves both growth and survival. According to a recent study, metamorphosis-related malformation in seed production results from a difference in the speed of growth and development from that of normal or wild fish. To control metamorphosis-related malformation we implemented photoperiod control and adjusted the rearing temperature so larvae were reared at a similar developmental speed to that of wild fish. This allowed the larvae to undergo normal metamorphosis, and resulted in production of the maximum number of normal fish.

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Withering Syndrome in Abalone in Japan

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ABSTRACT

Withering syndrome (WS) is known as a chronic wasting disease in abalone caused by *Candidatus xenohaliotis californiensis*, a Rickettsia-like organism (RLO) and has been prevalent on the west coast of the United States since the mid-1980s. WS is listed as notifiable disease in the International Aquatic Animal Health Code of the Office International des Epizooties (OIE). In Japan, the WS-RLO was first detected in 2011 in farmed, juvenile Japanese black abalone (*Haliotis discus discus*; 14 – 34mm in shell length). Those abalone experienced monthly mortality rates from 3 to 10% with a cumulative mortality of 32.8%, and histopathological changes and pathogens were not found except for basophilic, intracellular bacterial colonies within the epithelium of the digestive tract. Morphological characteristics and target tissues containing the bacterial inclusions were consistent with those of the WS-RLO. High intensity of infection by the RLO was observed in the epithelium of the posterior portion of the esophagus, the intestinal epithelium and, to a lesser extent, in the epithelium of transport ducts in the digestive gland. The RLOs observed were conclusively identified as the agent of withering syndrome by the additional investigation of PCR and sequence analysis. Preventive measures against WS implemented in Japan will be shown.

Abalone Withering Syndrome: Distribution, Impacts, Current Diagnostic Methods, and New Findings

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Keywords: abalone, disease, PCR, withering syndrome

ABSTRACT

Withering syndrome (WS) is a fatal disease of abalone caused by a Rickettsiales-like organism (WS-RLO). The causative agent, "*Candidatus* Xenohaliotis californiensis," occurs along the eastern Pacific margin of North America in California, USA and Baja California, Mexico. However, as infected abalones have been transported to Chile, China (People's Rep. of), Taiwan, Iceland, Ireland, Israel, Spain, Thailand, and most recently Japan, and possibly other countries, the geographical range of the etiological agent is suspected to be broad, especially where California red abalones, *Haliotis rufescens*, are cultured or in areas where native species have been exposed to this species. Susceptibility varies among species with up to 99% losses of black abalone, *H. cracherodii*, in lab and field studies in the USA, to no losses among the small abalone, *H. diversicolor supertexta*, in Thailand. Some populations that have suffered catastrophic losses due to WS have developed resistance to the disease. In addition, a newly identified phage hyperparasite of the WS-RLO may reduce pathogenicity and dampen losses from the WS-RLO. Diagnosis of WS requires the identification of infection with the pathogen (WS-RLO detected via *in situ* hybridization or histology coupled with PCR and sequence analysis) accompanied by morphological changes that characterize this disease (e.g. pedal and digestive gland atrophy, and digestive gland metaplasia). A quantitative PCR (qPCR) assay was developed and may be useful in quantifying amounts of pathogen DNA. Confirmation of infection by the WS-RLO cannot be done by PCR analysis alone as this method only detects pathogen DNA, but can be used as a proxy for infection in areas where the agent is established. Control measures include avoidance, culling infected animals, cooler temperature and, as per federal regulations, oral or bath treatment with oxytetracycline. Avoidance is best accomplished by the establishment of a health history and multiple health examinations prior to movement of animals. Although histology or *in situ* hybridization are required to confirm infection, PCR is able to detect small amounts of pathogen DNA and is recommended for inclusion in health examinations.

INTRODUCTION

Abalones are primitive marine vetigastropods of the genus *Haliotis* that inhabit the near shore intertidal and shallow subtidal zones. They are ecologically important in engineering habitat by grazing on micro and macroalgae, thereby maintaining open areas for recruitment of conspecifics and other benthic organisms (Geiger & Groves 1999, Roberts 2001), and also support economically valuable fisheries and aquaculture production throughout the world (Gordon & Cook 2001, 2004, Cook & Gordon 2010). Of the over 50 *Haliotis* species world-wide, eight inhabit the north-eastern Pacific (Haaker et al. 1986). They include subtidal species such as the commonly cultured red abalone (*H. rufescens*) and northern or pinto abalone (*H. kamtschatkana*) found in cool waters, the pink (*H. corrugata*) and green (*H. fulgens*) abalones of warmer waters, and the intertidal-shallow, subtidal black abalone (*H. cracherodii*). Fishing pressure and disease threaten abalone populations globally (Hobday & Tegner 2000, Rothaus et al. 2008, Tan et al. 2008, Travers 2008). Currently, five California species experiencing population declines receive varying levels of federal protection ranging from "Species of Concern" (pinto, green and pink abalones) to "Endangered" (white and black abalones). These and other species tested to date are all susceptible to the primary established abalone disease in California, withering syndrome (WS; OIE 2012).

WS is a fatal bacterial disease characterized by a severely shrunken body and infection with a Rickettsiales-like organism (RLO; Fig. 1). Friedman et al. (2000) identified and characterized a gastrointestinal RLO provisionally named "*Candidatus Xenohaliotis californiensis"* (WS-RLO) as the pathogen causing WS. The WS-RLO is an obligate, intracellular bacterium that infects abalone

digestive epithelia and causes severe morphological abnormalities within the digestive gland, resulting in physiological starvation followed by anorexia, absorption of pedal musculature, lethargy and death (Friedman et al. 2003, Braid et al. 2005). Transmission of the WS-RLO is likely fecal-oral (Friedman et al. 2002) and initial infections are located in the post-esophagus (Fig. 1D) and, to a lesser extent, the intestine of host abalone. Subsequently, metaplasia (the substitution of one mature tissue type for another, Fig. 1F) and infection occur in the digestive gland. These digestive gland changes are associated with depletion of glycogen reserves followed by pedal catabolism, atrophy (Fig. 1H) and, finally, death (Friedman et al. 2000, Braid et al. 2005). The severity of WS-RLO infection in juvenile red abalone has been directly correlated with negative physiological functions such as decreased feeding rates, metabolism, production of feces, and energy available for growth (Kismohandaka et al. 1993, González et al. 2012).
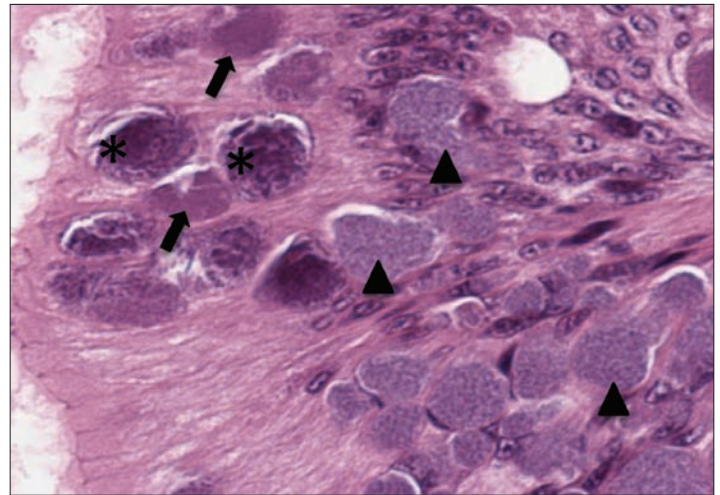

Figure 2. Light micrograph of WS-RLO (withering syndrome; arrows), ST-RLO (stippled; arrowhead), and RLOv (variant; asterisk) inclusions infecting the posterior-esophagus epithelium of California abalone. Individual rod to pleomorphic shaped RLOv (phage-infected WS-RLO) are visible by light microscopy while individual WS-RLO and ST-RLO are not. Note differential hematoxylin and eosin (H&E) staining properties of RLOs. 1000x magnification.
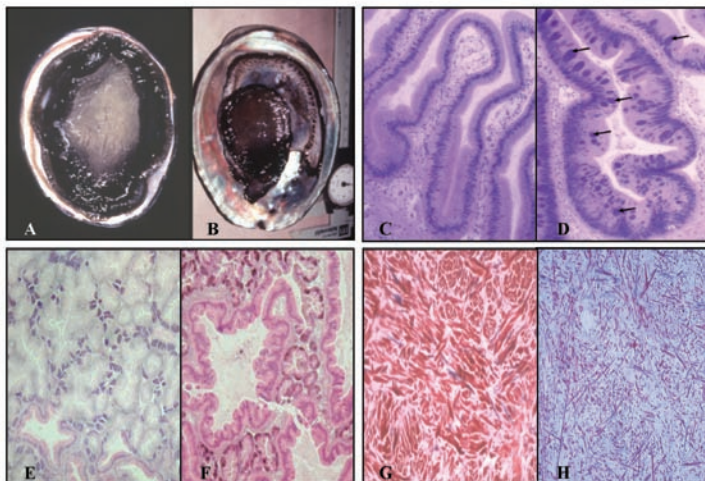

Figure 1. Effects of withering syndrome (WS) on abalones. A: Uninfected black abalone. B: Severely withered WS-RLO infected black abalone. C-H: Light micrographs of abalone tissues stained with hematoxylin and eosin (H&E). C: Normal post-esophagus. 200x magnification. D: WS-RLO infected post-esophagus with arrows indicating WS-RLO cytoplasmic inclusions (bacterial colonies). 200x magnification. E: Normal digestive gland. 100x magnification F: Metaplastic digestive gland. 100x magnification G: Normal pedal musculature. 400x magnification. H: Pedal atrophy. 400x magnification.

Two RLOs are known to infect California abalones: the WS-RLO and the stippled RLO (ST-RLO; Fig. 2, Table 1). Only "*Candidatus* Xenohaliotis californiensis" (WS-RLO) is known to cause WS (Friedman et al. 2000, 2003, Moore et al. 2001), while the ST-RLO appears, to date, to be non-pathogenic and is typically observed at low levels (Friedman et al. in manuscript). The WS-RLO infects all members of the genus *Haliotis* examined to date, including black abalone (Friedman et al. 1997, 2002), white abalone (Moore et al. 2002, Friedman et al. 2007), red abalone

(Moore et al. 2000, 2001), pink abalone (Álvarez-Tinajero et al. 2002), green abalone (Álvarez-Tinajero et al. 2002), the small abalone (*H. diversicolor supertexta*; Wetchateng et al. 2010), Japanese black abalone (*H. discus discus*; Kiryu et al. 2013), and the European abalone (*H. tuberculata*, Balseiro et al. 2006) in the wild or culture facilities, as well as flat abalone (*H. wallalensis*, Friedman unpubl. obs.) and Japanese abalone (*H. discus-hannai*, Friedman unpubl. obs.) in laboratory challenges. WS-RLO has not been identified in any non-haliotid hosts, including limpets and snails cohabiting with WS-RLO infected abalone (Moore et al. 2002, CDFW unpubl. obs.).

Table 1. Light microscopy: rickettsial morphologies (mean ± s.d) and hematoxylin and eosin (H&E) staining properties (Friedman & Crosson 2012).

RLO	H&E Staining	Cellular Location	Histology Fixative	Inclusion Width[a]	Inclusion Length[a]	Bacterial Width	Bacterial Length
WS	Violet	Apical	Davidson's	14.2±5.3	23.2±10.4	TSTM	TSTM
	Violet	Apical	1G4F	15.4±7.0	26.3±11.0	TSTM	TSTM
RLOv	Navy blue	Apical	Davidson's	16.7±7.8	24.1±10.7	2.6±1.0	3.4±1.0
	Navy blue	Apical	1G4F	21.3±7.7	26.1±13.3	1.5±0.7	3.2±1.3
ST	Light blue	Basal	Davidson's	15.26±7.32	19.34±5.49	TSTM	TSTM
	Light blue	Basal	1G4F	ND	ND	ND	ND

s.d standard deviation, *WS* withering syndrome, *TSTM* too small to measure, *ST* stippled, *ND* not determined
[a] Measured to the nearest 0.01 μm

WS was first observed in black abalone populations on the south shore of Santa Cruz Island, CA, USA in 1985 shortly after the strong 1982-83 El Niño-Southern Oscillation (ENSO) event, and subsequently spread to new locations and other abalone host species. From 1986-1989, black abalone population declines and WS were seen at Anacapa Island followed by losses on Santa Cruz, Santa Rosa, Santa Barbara and San Miguel islands (Davis et al. 1992, Tissot 1995, 2007).

WS associated declines in black abalone were first observed at San Nicolas Island (SNI) in 1992 and, as in other affected areas, resulted in markedly increased population declines as compared to pre-WS losses (VanBlaricom et al. 1993, Ruediger 1999). In 1988, WS was observed in Diablo Canyon (Steinbeck et al. 1992) but was not observed elsewhere along mainland California until its discovery north of Point Conception (Altstatt et al. 1996). By 1992, evidence of infected black abalone was reported at all southern California islands except Santa Catalina, especially during seasonal warm water events (Haaker et al. 1992, Tissot 1995, Raimondi et al. 2002).

The 1997-98 ENSO was associated with enhanced clinical signs of disease in wild abalones and also coincided with severe losses in cultured red abalone (Moore et al. 2000, Friedman et al. 2002). From the late 1980s to the present, WS spread naturally and via anthropogenic movement of farmed red abalone (Friedman & Finley 2003, OIE 2012) throughout southern California, into the warmer waters of Baja California, Mexico (Casares-Martinez & Tinoco-Orta 2001, Álvarez-Tinajero et al. 2002, Garcia-Esquivel et al. 2007) and northward (Lafferty & Kuris 1993, Alstatt et al. 1996, Miner et al. 2006). Both clinical disease and the WS-RLO were observed as far north as Point San Pedro (San Francisco County, CA) by 1999 (Friedman & Finley 2003). Clinical WS continues to spread in a northward direction (Miner et al. 2006) and is strongly associated with declines in abalone abundance co-occurring with increasing coastal warming and El Niño events (Tissot 1995, Alstatt et al. 1996, Raimondi et al. 2002). The WS-RLO is considered to be continuously distributed along the west coast of North America from Baja California, Mexico, to southern Sonoma County, California, including the Channel and Farallon Islands (Álvarez-Tinajero et al. 2002, Friedman & Finley 2003, CDFW unpubl. obs.). During a 1999-2000 sampling event, WS-RLO was identified in two red abalone populations in northern California, Van Damme State Park and Crescent City (Friedman & Finley 2003), but has not been detected at those locations since, including histological examination of over 700 red abalone from Van Damme during 2001-2009 (CDFW unpubl. obs.). The anthropogenic introductions at these locations may have failed to become established because of low temperatures. As infected abalones have been transported to Chile, China (People's Rep. of), Taiwan, Iceland, Ireland, Israel, Spain, Thailand (Wetchateng et al. 2010), and most recently Japan (Kiryu et al. 2013), and possibly other countries, the geographical range of the etiological agent is suspected to be broad where California red abalones are cultured or in areas where native species have been exposed to this species.

Climatic changes and short term ocean temperature increases have the potential to significantly alter host-parasite dynamics in abalones infected with bacterial pathogens such as RLOs and make WS one of the most severe threats to abalone populations (Neuman et al. 2010). Temperature can modulate both the transmission and development of WS (Moore et al. 2000, Braid et al. 2005, Vilchis et al. 2005). Thermal induction and increased disease expression have been documented in both lab challenged and field RLO-infected animals including red (Vilchis et al. 2005, Moore et al. 2000, 2011) and black (Tissot 1995, Friedman et al. 1997, 2002) abalones. WS-RLO transmission and subsequent WS development in red abalone were nearly negated at 12.3°C (only 1% transmission and no clinical signs of disease), while up to 94% transmission and extreme clinical signs were observed at 18.7°C (Braid et al. 2005). Although a relationship between food availability (fed or complete starvation) and WS-RLO transmission was observed (Braid et al. 2005), under more realistic feeding conditions (100%, 30% and 10% feeding rates) food availability and WS-RLO transmission were not correlated, further illustrating the importance of temperature in the ecology of this disease (Vilchis et al. 2005).

Temperature appears to have a significant influence on WS in the field. Since initial observation after the 1982-1983 ENSO, WS has been repeatedly associated with seasonal or decadal thermal events (Haaker et al. 1992). Steinbeck et al. (1992) investigated mortality of black abalone within, and adjacent to, the discharge plume of the Diablo Canyon Power Plant during 1988-1989 and found that animals with clinical signs of WS were located exclusively in the thermal discharge zone where water temperatures measured up to 11°C above ambient. Lafferty & Kuris (1993) also discovered a significant correlation between WS mortality rates and warmer locations. Tissot (1995) suggested high temperature was the most important factor limiting black abalone population recovery on Santa Cruz Island. Subsequently, during the severe 1997-1998 ENSO, when markedly elevated seawater temperatures occurred throughout southern and central California, up to 70% of black abalone at surveyed field sites showed clinical signs of WS (Raimondi et al. 2002, Friedman et al. 2003). High daily temperature variability may also increase the susceptibility of black abalone to WS infection, although disease expression was not seen in abalone

until temperatures exceeded thresholds known to facilitate infection (Ben-Horin et al. 2013). Since the mid-1970's decadal regime shift, thermal anomalies have been more common and of longer duration than during the previous 25 years (NOAA 2008). ENSO-neutral conditions were predicted through middle 2009. However, given that annual seasonal thermal maxima in southern California typically reach 17-19°C, temperatures known to augment WS, understanding the role of both seasonal and anomalous ocean warming is crucial to understanding the ecology of marine diseases (NOAA 2008).

Differential Susceptibility & Disease Resistance

While the WS-RLO infects all haliotids tested to date, susceptibility varies among species. Levels of WS range from little effect and no mortality (e.g. wild green and pink abalone: Álvarez-Tinajero et al. 2002, Moore et al. 2009) to moderate mortality (e.g. red abalone: Moore et al. 2000, 2001), to catastrophic impacts with up to 99% population mortality over a span of several years on large spatial scales (e.g. black abalone: Altstatt et al. 1996, Moore et al. 2002, Raimondi et al. 2002, Miner et al. 2006, Friedman et al. 2007), conferring significant alterations to marine nearshore biodiversity (Haaker et al. 1992, Tissot 1995, Friedman et al. 2000, Miner et al. 2006). Vilchis et al. (2005) conducted a long-term study in which development of clinical WS was observed in red but not green abalone at elevated temperatures. These results agreed with a similar study conducted by Moore et al. (2009) in which green abalone exposed to WS-RLO were relatively resistant to disease expression under ENSO conditions. However, thermal modulation remains a key factor as demonstrated by Garcia-Esquivez et al. (2007) who observed green abalone held at elevated laboratory temperatures of 25°C experienced more clinical signs of WS and higher mortality then those held at 20°C. A survey of wild abalones from Baja California showed 32% of the pink and 27% of green abalones had clinical signs of WS. Little (<7%) to no abalones had advanced signs of WS and clinical signs of WS did not correlate with WS-RLO presence (**Álvarez-Tinajero** et al. 2002).

In contrast, white and black abalones are highly susceptible to WS-RLO infection: up to 100% mortality (Fig. 3; Friedman unpubl. data) and 99% (Friedman et al. 2002, Raimondi et al. 2002), respectively. White abalone captive rearing programs have experienced substantial losses after ~2-3 years of culture when animals succumbed to WS-RLO infections and most died from the disease (Friedman et al. 2007). A laboratory study was conducted to compare the

susceptibility of white and green abalones to WS when held at 18°C and few losses (<20%) were observed in green abalone over the course of 26 weeks, while 100% of white abalone died within 13 weeks (Fig. 3; Friedman unpubl. data). Initial losses of green abalone were attributed to handling stress, not WS, as no green abalone died after the first 10 weeks of study when few individuals were infected.
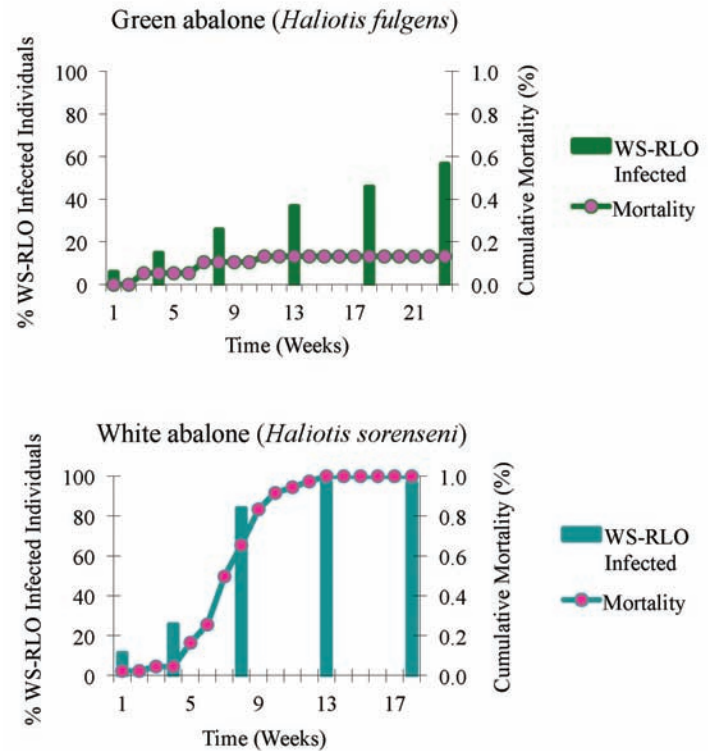

Figure 3. Differential susceptibility of green and white abalones to withering syndrome (WS). Bars represent the percent of WS-RLO infected individuals, while circles represent cumulative proportion mortality (Friedman, unpubl. data).

In addition to differences in disease susceptibility among species, it was recently observed that different populations of a single species appear to respond differently to the presence of WS. Black abalone from San Nicolas Island (SNI) have been under significant WS pressure for over 20 years (Fig. 4A; VanBlaricom et al. 1993). Between 1992-2001, a 99.2% decline in black abalone density (11.22 to 0.095/m², respectively, in permanent plots sited purposely in high-density patches of abalone) occurred on SNI due to WS. From 2002 to 2012, abalone densities on SNI increased over 200% from the minimum in 2001, via recruitment events and apparently improved survival rates despite presence of the WS-RLO (Fig. 4B; VanBlaricom unpubl. data). Thus despite catastrophic losses, a small number of black abalone survived to reproduce. The observation of population increases on SNI suggested that the 1-2% of black abalone that survived WS epidemics were more resistant to WS than populations

not experiencing disease pressure (Friedman et al. in manuscript). It was hypothesized that survivors were able to resist infection by mounting a sufficient immune response and/or resisting bacterial secretions thought to induce host metaplasia.
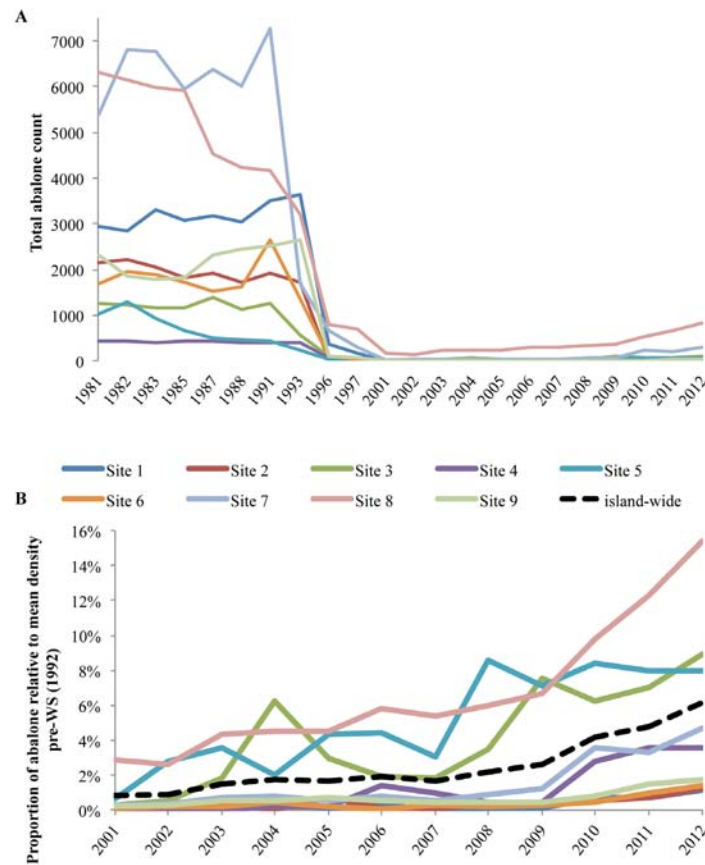

Figure 4. Black abalone population trends on San Nicolas Island (SNI), CA. A: Total abalone counts from 1981-2012. Note sharp population declines after withering syndrome (WS) was first observed in 1992. Declines at some sites prior to 1992 are likely due to overfishing. B: 2001-2012 only. Proportion of abalone relative to mean abalone density pre-WS epidemics (1992). Note strong recruitment event at Site 8, where abalone for disease resistance testing were collected (VanBlaricom 1993, unpubl. data).

The hypothesis that abalone populations under disease pressure selected for the development of disease resistance was tested in the laboratory using progeny of surviving abalone from SNI (Site 8 animals, Fig. 4B) and "naïve" black abalone from Carmel, CA that had not been exposed to WS epidemics, and thus were not under selection for improved tolerance (Friedman et al. in manuscript). Upon RLO exposure at 19°C, decreased mortalities were observed in SNI abalone compared to those from Carmel (Friedman et al. in manuscript). Significant differences in survival were observed among treatments (p<0.001); more RLO exposed abalone from Carmel died than did those from SNI (p<0.05), while no differences in survival were observed between control groups

(p>0.05). All RLO exposed abalone that died had clinical signs of WS and microscopic examination suggested that resistance to WS might be more related to the host response to initial infection than to the ability to resist infection, as resistant abalone showed significantly less metaplasia and a corresponding lower RLO infection intensity in the digestive gland (Fig. 5; Friedman et al. in manuscript). Analysis of WS-RLO DNA by quantitative PCR (qPCR) of feces from both populations showed that more WS-RLO DNA was excreted from Carmel abalone compared to those from SNI suggesting that abalone from SNI (survivors of high disease pressure) express a trait or have some characteristic that decreases the ability of RLOs to proliferate in the digestive gland (Fig. 5; Friedman et al. in manuscript). Clearly a distinct difference in disease resistance exists among black abalone populations independent of temperature. Whether or not the observed differences are of genetic origin is currently being explored (Crosson et al. in manuscript).
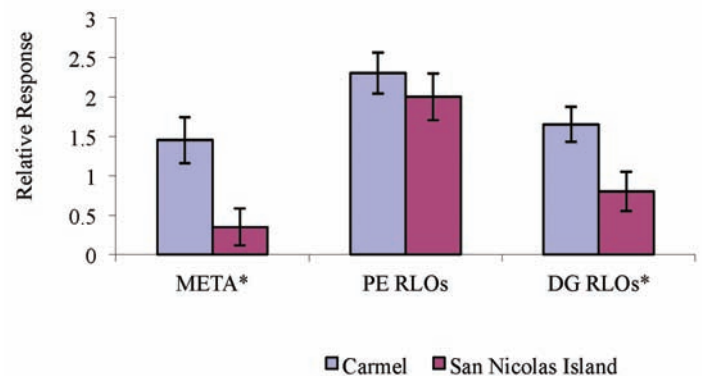

Figure 5. Microscopic observations of hematoxylin and eosin (H&E) stained black abalone tissues. Significant differences between San Nicolas Island (WS resistant) and Carmel (WS "naïve") animals are noted (asterisk). Error bars represent standard error. Meta: Metaplastic response. PE RLOs: Combined RLO intensity in the posterior esophagus. DG RLOs: Combined RLO intensity in the digestive gland (Friedman et al. in manuscript). See Friedman et al. 2002 for relative response scoring.

Phage Hyperparasite
Friedman & Crosson (2012) recently observed a morphological variant of the WS-RLO infecting red abalone from central California and used a combination of light and electron microscopy, *in situ* hybridization, and 16S rDNA sequence analysis to compare the WS-RLO and the RLO variant (RLOv). WS-RLO morphology has been consistent with its original taxonomic description (Friedman et al. 2000) and forms oblong inclusions within the abalone posterior esophagus (PE) and digestive gland (DG) tissues that contain small rod-shaped bacteria; individual bacteria within inclusions, which appear

light purple upon hematoxylin and eosin (H&E) staining, cannot be discerned by light microscopy (Table 1, Fig. 2; Friedman & Crosson 2012). Like the WS-RLO, the RLOv forms oblong inclusions in the PE and DG but contain large, pleomorphic bacteria that stain dark navy blue with H&E (Table 1, Fig. 2; Friedman & Crosson 2012). Transmission electron microscopy (TEM) examination revealed that the large pleomorphic bacteria within RLOv inclusions were infected with a spherical to icosahedral-shaped phage hyperparasite (Fig. 6; Friedman & Crosson 2012). Binding of the WS-RLO-specific *in situ* hybridization probe to the RLOv inclusions demonstrated sequence similarity between these RLOs. In addition, sequence analysis revealed 98.9-99.4 % similarity between 16S rDNA sequences of the WS-RLO and RLOv. Collectively, these data suggest that both of these RLOs infecting California abalone are "*Candidatus Xenohaliotis californiensis,*" (WS-RLO) and that the novel variant is infected by a phage hyperparasite that induced morphological variation of its WS-RLO host.
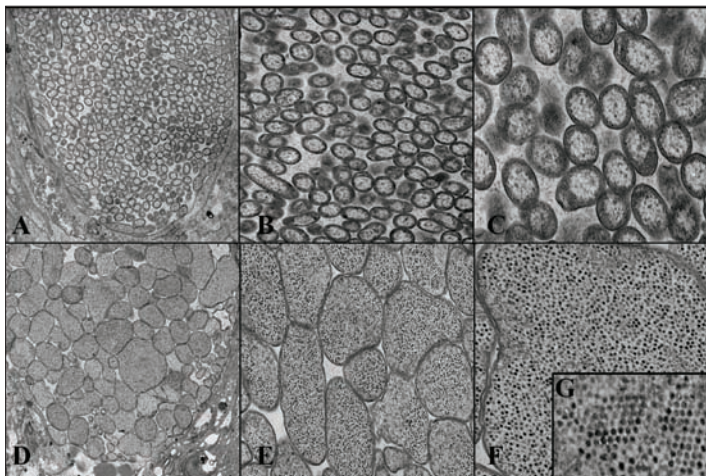

Figure 6. Transmission electron micrograph (TEM) of abalone rickettsial inclusions following preservation in Karnovsky's solution. WS-RLO at (A) 6800x, (B) 13000x, and (C) 30000x magnification. RLOv (phage-infected WS-RLO) at (D) 4800x, (E) 13000x, and (F) 30000x magnification. G: Inset illustrating phage morphology and virions in paracrystalline array (Friedman & Crosson 2012).

The presence of a phage hyperparasite exhibits interesting properties that appear to affect the host-pathogen relationship between the WS-RLO and abalone. For example, in a recent experiment with juvenile black abalone, both WS-RLO and phage-infected inclusions were statistically related to tissue pathology and mortality (p<0.05; Friedman et al. in manuscript). Like the WS-RLO, the phage-infected inclusions appeared to increase in prevalence and intensity with increasing temperature (Crosson et al. in manuscript). Curiously, mortalities of abalone

infected with all RLO types appeared to be delayed and significantly reduced relative to previous studies with the WS-RLO alone (Friedman et al. in manuscript). When black abalone were exposed to WS-RLO, ST-RLO, and RLOv in combination, the trial lasted 17 months during which 48% of the animals died. However, when abalone were exposed to the WS-RLO alone, they experienced 71% mortality in only 7 months. In addition, when the phage (RLOv) was absent, WS-RLO loads were higher and host metaplastic response was ~2x that observed when the phage was co-occurring (Friedman et al. in manuscript). It is likely that the presence of the phage is attenuating WS disease development and consequences of infection will vary among host species and with temperature. Current studies are underway to discern if the phage infections alter physiological processes, such as virulence and pathogenicity, in its WS-RLO host. Also of interest, red abalone farms in California experiencing seasonal losses due to WS since ~ 1990 (e.g. Moore et al. 2000) and with confirmed RLO infected animals are currently reporting decreased losses in product-sized abalone relative to previous trends prior to the observation of the phage hyperparasite (R. Fields pers. comm.).

Diagnostic Methods

Diagnosis of WS requires the identification of infection with the pathogen (WS-RLO via *in situ* hybridization or via histology coupled with PCR and sequence analysis) accompanied by morphological changes that characterize the disease (e.g. pedal and digestive gland atrophy, and digestive gland metaplasia). Definitive diagnosis of WS must be conducted according to World Organization of Animal Health (OIE) standards in the Manual of Diagnostic Tests for Aquatic Animals (OIE, 2012). *In situ* hybridization is the method of choice for WS-RLO confirmation because it allows visualization of a specific DNA probe hybridized to the target pathogen. The *in situ* hybridization technique for WS-RLO developed by Antonio et al. (2000) is extremely useful in visualizing initial stages of infection in sub-clinically infected abalone. Although this method was not formally validated, tests for specificity using several bivalve and fish RLOs suggested the test was specific for WS-RLO only (Antonio et al. 2000).

A conventional PCR assay that specifically amplifies a 160 bp segment of the WS-RLO 16S rDNA sequence available in GenBank (AF133090) was developed by Andree et al. (2000) and allows for greater sensitivity than histology alone. A quantitative PCR (qPCR)

assay was also developed to specifically identify and enumerate bacterial loads of WS-RLO in abalone tissue, fecal, and seawater samples based on 16S rDNA gene copy numbers (Friedman et al. in manuscript). Both PCR assays designed to detect DNA of the WS-RLO were formally validated according to OIE (2012) standards (Friedman et al. in manuscript). The conventional PCR assay limit of detection was 300 gene copies and 3 gene copies for qPCR. Thus qPCR was over 100x more sensitive than conventional PCR in detecting target DNA (Friedman et al. in manuscript). Also, the ability of qPCR to detect and quantify very small amounts of WS-RLO gene copies in a variety of sample types will enable researchers to better understand WS transmission dynamics in both farmed and natural environments while providing a useful, non-lethal tool for WS monitoring. However, it is important to note that DNA-based PCR assays do not detect a viable agent or infection and serve only as a proxy for infection or exposure. Histological examinations remain the gold standard and show clear evidence of infection but may not enable one to discern the taxonomy of the agent (Burreson 2008, OIE 2012). Both conditionally independent tests should be used collectively for proper WS diagnosis.

Control & Recommendations

The most effective prevention of WS is avoidance of the pathogen. Avoidance is best accomplished by the establishment of a health history and multiple health examinations prior to movement of animals. Although histology or *in situ* hybridization are required to confirm infection, PCR is able to detect small amounts of pathogen DNA and is recommended for inclusion in health examinations. Good husbandry practices are essential for control of any bacterial disease and include reducing stocking densities, avoiding grading or mixing of disparate groups or families, and rinsing hands and equipment in fresh or iodinated water between groups/tanks. Holding abalones at cooler temperatures (<15 °C) may also reduce WS-RLO transmission (Braid et al. 2005). Infected groups should be isolated and culled or administered oral or bath treatments with oxytetracycline as per federal regulations (Friedman et al. 2003, 2007).

The ecology of RLOs in abalone disease warrants further investigation. It will be critical to identify the ST-RLO and newly observed WS-RLO phage, understand the host-parasite-environment relationships, and characterize their roles in abalone disease (i.e. competition with the WS-RLO) for successful restoration and management of all abalone species. Much research on host-parasite relationships involves interactions between a single host and one parasite/pathogen. However, evidence from a wide variety of systems suggests that mixed infections involving two or more parasite genotypes or species in a single host are becoming more common and, in some cases, may be the rule. Multiple pathogen infections have been examined in numerous host systems including a variety of invertebrates such as oysters (Stokes & Burreson, 2001), crustaceans (Tang et al. 2003) and abalones (Hine et al. 2002, Balseiro et al. 2006). Understanding the role of abalone-RLO relationships under varying environmental conditions will be imperative to abalone resource management in the face of global climate change. To achieve protection and sustainable use of abalone resources, we must also understand interactions among wild and farmed animals and their potential impacts on disease transmission dynamics, especially in declining and endangered species.

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Characteristic Evaluation Method for Benedenia Disease in Yellowtail (Seriola quinqueradiata)

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Keywords : Benedenia disease, yellowtail, breeding, characteristic evaluation method

ABSTRACT

Benedenia is a parasitic disease caused in *Seriola* species by *Benedenia seriolae*. This parasite can cause growth reduction and external injuries in yellowtail, increasing the risk of secondary viral or bacterial infection. The main method of parasite removal is to soak the fish in a freshwater bath. However, this method requires a great deal of time, cost, and effort. We have been studying DNA Marker-Assisted Selection (MAS) breeding, to select for resistance to Benedenia disease. Three components ("Reproduction technology", "Character evaluation", and "DNA analysis") are critically important to promote MAS breeding success. We focus on one of the key components, "Characteristic evaluation method" relating to Benedenia disease in yellowtail.

INTRODUCTION

Yellowtail (*Seriola quinqueradiata*) is one of the most important species in marine fishery resources and aquaculture in Japan. Production of *Seriola* species occupies more than 50% of Japanese marine finfish aquaculture, and reaches 15,000 tons/year (Figure 1). At present, yellowtail aquaculture is dependent on naturally occurring juveniles as the source of seedlings. It is therefore of concern that aquaculture production is dependent on seasonal and overall natural stock abundance, and that capture for aquaculture could impact wild stocks. Closing the production loop with hatchery-raised seedlings is crucial for the sustained aquaculture of yellowtail.

Requirements by farmers for the introduction of artificial seedlings include not only a 'scheduled and stable supply' but also 'seedlings with value added.' 'Value added' refers to seedlings with characteristics such as rapid growth and disease resistance.
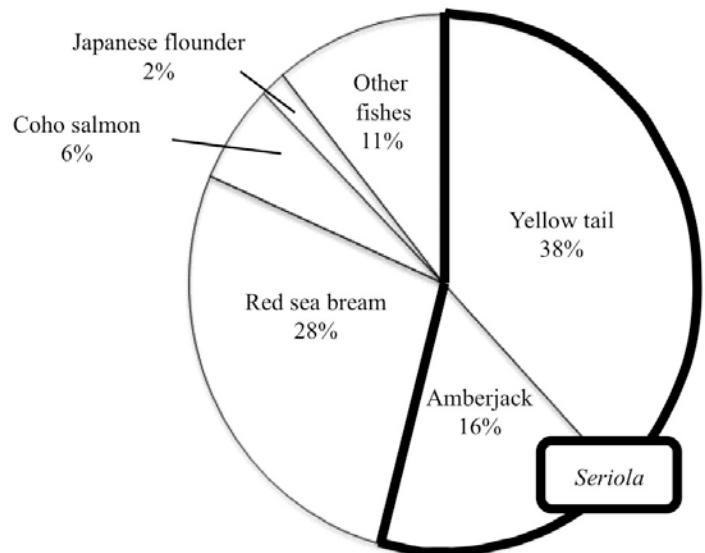

Figure 1. The ratio of marine finfish aquaculture production in Japan 2010 (Source: Fisheries Agency).

Benedenia is a parasitic disease caused in *Seriola* species by *Benedenia seriolae*. This parasite can cause growth reduction and external injuries, increasing the risk of secondary viral or bacterial infections. The most common treatment is to soak affected fish in a freshwater bath. Unfortunately, this method is costly and requires a great deal of time and effort.
We have been studying "marker-assisted selection breeding" with Benedenia disease resistance in yellowtail as the target trait. Three components are important in conducting this study: "Reproduction technology" such as egg collection, initial rearing, and breeding into parent fish; "Characteristic evaluation" of the target trait in a clear and quantitative form; and "DNA analysis" using DNA markers that have been developed rapidly in recent years. This study focuses on the key component "Characteristic evaluation" and methods relating to quantifying Benedenia disease prevalence as a proxy for Benedenia susceptibility in yellowtail.

METHODS

Characteristic evaluation of the target trait 'Benedenia prevalence' is conducted using data on the number of Benedenia parasites found on the fish body. Two different data collection methods are described below, dependent on fish body size: one for large fish (i.e. total length greater than 20cm) and one for small fish (i.e. total length under 20cm). Results of Benedenia prevalence studies in large fish were used to select individuals to breed Benedenia-resistant and Benedenia-susceptible families. Seedlings from these crosses were evaluated for susceptibility using the methods described for small fish.

Method for Large Fish

At the start of the experiment, a tag was inserted into each fish for individual identification. Subsequently, fish were bathed in fresh water to eliminate Benedenia on the body surface. Fish were kept in a net cage during the experimental period. The first investigation was conducted 3–4 weeks after starting the experiment.

During the investigation, yellowtail from the net cage were taken individually using a bag made of Tetoron Russell Weave Net (Tanaka Sanjiro Co.,Ltd., Ogori, Fukuoka, Japan). The bag and fish were placed in a tank containing fresh water for 3–5 minutes. The fish was then identified by its tag, and the parasites were counted as they become clouded and visible on the body surface. Parasites living on the body surface and the number that came off in the bag were summed to obtain the total number of parasites found on each fish. After the investigation, fish were returned to the net cage and maintained so that the investigation could be repeated, for a total of five times over the course of this study.

Characteristics of this methodology are that individual data can be collected because the Benedenia parasites are counted individually. Data can be collected more than once from the same individual because individuals are maintained after each investigation. Benedenia parasitism can be regarded as a genetic trait based on the obtained data, which are useful for breeding. This method cannot be used with small fish because it cannot be performed without inserting a tag for identification, and few samples can be treated because large-scale facilities are necessary as a result of the size of the large fish. Finally, the experimental design is dependent on the natural abundance of Benedenia parasites in the surrounding environment.

Method for Small Fish

In small fish, the investigation was conducted in three steps: (1) Benedenia culture, (2) infection test, and (3) counting. Benedenia are parasitic and cannot be cultured without a host. For that reason, yellowtail parasitized by Benedenia were kept in a tank to serve as a source of eggs for infection. Net fabric was hung down into the tank so that eggs laid by Benedenia became caught in the fabric. After collection, the net fabric was incubated in running water and protected from light until the infection test.

During the infection test the net fabric with entangled Benedenia eggs was placed into a beaker containing seawater, and hatching was promoted by illumination. The net fabric was removed from the beaker after 15 min, after which a sample was taken. The Benedenia larvae in the sample were fixed in formalin and counted under a microscope. Next, 400 yellowtail seedlings were transferred to a 1-kL tank, to which the Benedenia larvae were added. The density of parasites to hosts was 100 Benedenia / 1 yellowtail. After the addition, parasitism was promoted with still water and protection from light for 4 hours. The tank and fish were maintained for a further 10 days.

During the counting procedure, yellowtail seedlings that had been kept for 10 days were individually placed into a 1-L bottle containing fresh water and ice. The bottle was then stirred to remove Benedenia from the fish body, after which the fish was carefully removed. A fin clip from the seedling was taken as a sample for DNA analysis, and the Benedenia that came off in the bottle were retained for visual counting.

Characteristics of this methodology for small fish are that individual data can be collected because the Benedenia parasites are counted individually in the same way as those in the method in large fish. Tag insertion is unnecessary because the lines are distinguishable using DNA analysis, allowing tests to be conducted with small seedlings. Many samples can be treated because the test can be conducted in small-scale facilities. Finally, Benedenia parasitism can be regarded as a genetic trait based on the obtained data, which are useful for breeding.

RESULTS

An example of data from Benedenia susceptibility investigations on large fish is shown below (Figure 2). 100 fish grown from naturally occurring seedlings were evaluated for the prevalence of Benedenia parasites three times before egg collection. Results

show differences between individuals in the number of Benedenia parasites. Further, some individuals were more susceptible to Benedenia than others. Two more Benedenia prevalence counts were conducted after egg collection. Results suggest that wide individual variation in resistance to Benedenia parasitism exists. We hypothesize that this variation in susceptibility might be related to genetic traits.

Figure 2. Number of parasites on large fish. Red lines are susceptible fish. Blue lines are resistant fish.
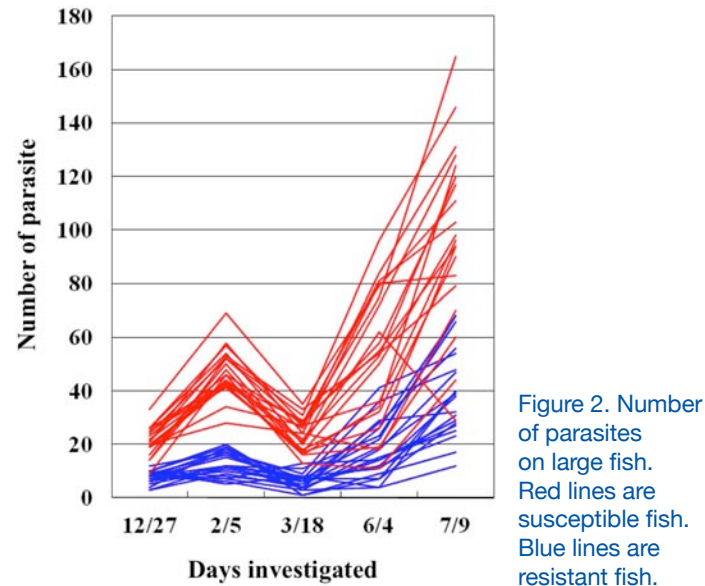
An example of parasitism data collected from small fish is shown below (Figure 3). Fish selected from experimental investigations of Benedenia susceptibility in large fish as described above were used to produce seedlings. One family (Figure 3; Blue) is a Benedenia-resistant family resulting from a cross of two less-susceptible individuals. The other family (Figure 3; Red) is a susceptible family from crossing more-susceptible individuals. Results of infection tests show lower counts of Benedenia parasites in the resistant family than in the susceptible family. A significant difference was also observed (Student's t-test).

Figure 3. Number of parasites on small fish.
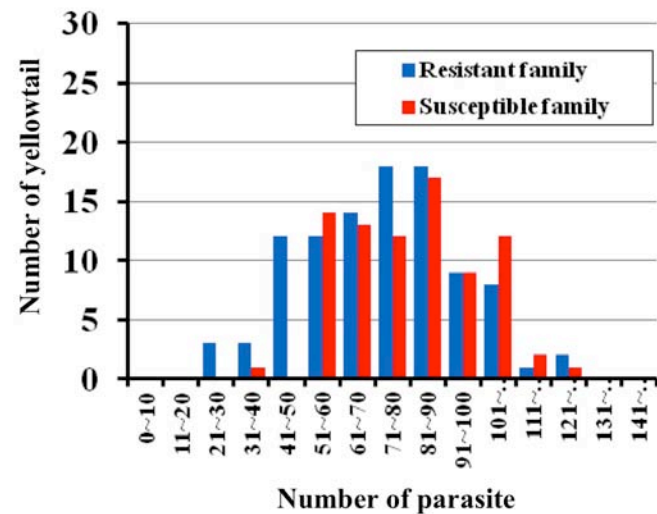
CONCLUSION

As described above, these two methods can reliably quantify the target trait of Benedenia prevalence. Therefore, they are useful as methods for evaluating the trait related to Benedenia disease in yellowtail. Research of yellowtail breeding promoted use of trait evaluation, reproduction techniques, and DNA analysis, and these methods can contribute to the future of the yellowtail aquaculture industry.

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The National Center for Stock Enhancement (NCSE, formerly Japan sea-farming Association), of the Fisheries Research agency, introduced the stock enhancement program for yellowtail (*Seriola quinqueradiata* and *Seriola lalandi*) in 1977. Technical developments in induced spawning as well as larval and juvenile rearing techniques have increased the population of this species to 1 million juveniles per year at NCSE. This project faced three major drawbacks: high mortality of larvae, cannibalism, and the smaller size of released juveniles in comparison with their wild counterparts. The high mortality of larvae was overcome by utilizing strong aeration during the early larval stage, while cannibalism was controlled by grading juveniles. The two-month delay in the spawning season of reared broodstock (the usual spawning season is late April to early May), which caused the smaller size of released juveniles, was solved by developments in advanced spawning techniques. Photoperiod and water temperature manipulations were used to produce eggs in February, thus producing yellowtail juveniles that can be released into the wild at a size similar to that of the wild stock.

Ohara, E., T. Nishimura, Y. Nagakura, T. Sakamoto, K. Mushiake, and N. Okamoto. 2005. Genetic linkage maps of two yellowtails (*Seriola quinqueradiata* and *Seriola lalandi*). *Aquaculture* 244 : Pp41-48.

The yellowtails *Seriola quinqueradiata* and *Seriola lalandi* are the most important species in marine fishery resources and aquaculture in Japan. A genetic linkage map is needed to improve efficiency of breeding by marker-assisted selection (MAS) and for the identification of commercially important genes. Therefore, we have constructed a genetic linkage map for the yellowtails using microsatellite makers. Microsatellites were isolated from the agenomic DNA library of *S. quinqueradiata*. Segregation of 217 microsatellites were studied in 90 progeny from a cross between *S. quinqueradiata* and *S. lalandi*. These were used to construct separate linkage maps of a female (*S. quinqueradiata*) and a male (*S. lalandi*). Twenty-five linkage groups were distinguished in the female (*S. quinqueradiata*) map, which spanned 473.3 centiMorgans (cM) with an average intermaker distance of 2.7 cM (total length estimated: 901.7 cM), and 21 linkage groups were distinguished in the male (*S. lalandi*) map, which spanned 584.3 cM with an average intermaker distance of 4.8 cM (total length estimated: 1715.3 cM). The microsatellite loci and genetic linkage maps will increase the efficiency of selective breeding programs for yellowtails.

Nagakura. Y., T. Yoshinaga, T. Sakamoto, K. Hattori, and N. Okamoto (2010). Susceptibility of four families derived from two Seriola species to the monogenean parasite (*Benedenia seriolae*) using a new challenge method. Journal of Fisheries Technology 3: Pp21–26 (in Japanese) with English abstract

Four families of two *Seriola* species were experimentally challenged with oncomiracidia of *Benedenia seriolae*. One family was produced by crossing wild yellowtails (*S. quingueradaiata*), two families were crosses between yellowtail of the 4th or 5th generation passaged under laboratory conditions, and one hybrid family was a cross between yellowtail and goldstriped amberjack (*S. lalandi*). Four hundred juveniles, 100 per each of the four groups and ranging 108-111 mm in mean total length, were exposed to about 40,000 oncomitacidia of *B. seriolae* within 30 min after hatching in a 1000L tank in darkness for 4 hours. On day 10 post-challenge, the number and size of parasites were estimated on each fish. Infection intensities significantly differed in any combination of families. A significant difference was also detected in worm body length in a combination of two families. The results suggest that families of fish resistant to *B. seriolae* can be produced through breeding. The advantages of the method employed in this study for evaluating fish susceptibility against *B. seriolae* are also discussed.

Advances in the Diagnosis and Management of Amyloodiniosis in Intensive Fish Culture

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ABSTRACT

Amyloodinium ocellatum is a parasitic dinoflagellate that infects a wide variety of warmwater marine and estuarine fishes and causes one of the most serious diseases in warmwater marine fish culture. The life cycle of *A. ocellatum* is direct and consists of three stages – the trophont which is parasitic on the gills and skin, the free-living reproductive tomont, and the infective dinospore. This parasite is particularly troublesome for two reasons. First, current diagnostic methods rely mainly on the microscopic identification of parasites on the skin or gills; therefore, infections often go undetected until massive mortality occurs. Second, the direct life cycle and the fact that each tomont can produce up to 256 infective dinospores results in the rapid build-up of heavy infections particularly in recirculating systems. Although the general course of infection is well understood, no quantitative understanding of the survival and reproductive rates of the parasite that might lead to improved control is known.

Our program has focused on strengthening our ability to address these shortcomings in diagnosis and management of amyloodiniosis. With respect to diagnosis, we developed a novel, highly sensitive, and specific diagnostic tool based on the Loop-mediated Isothermal Amplification (LAMP) reaction to detect both the free-swimming stages of *A. ocellatum* and those parasitic on the fish. The advantages of LAMP assay compared to the PCR are that it is quicker and does not require sophisticated equipment or skilled personnel. No amplification was detected using DNA from related dinoflagellate species, demonstrating the specificity of the assay. The LAMP assay also proved to be more sensitive than the PCR. The established LAMP assay provides a useful tool for the rapid and sensitive detection of *A. ocellatum* in gill and water samples, which could assist in the early detection and control of *A. ocellatum* infections in rearing systems.

With respect to management, we experimentally estimated the vital rates that determine the population growth of the parasite, and used that information to develop a population model for evaluating the relative effects of changes in vital rates on the population growth rate. A schematic representation of the parasite's life cycle was developed and a laboratory challenge model was used to quantify each process in the life cycle in two species of hosts – the spotted seatrout (*Cynoscion nebulosus*) and the red snapper (*Lutjanus campechanus*). We showed that although there were no significant differences in infection rates between the two species, trophonts grew larger and remained attached longer to red snapper than spotted seatrout and spotted seatrout tolerated a higher load of trophonts than red snapper. The model demonstrated that the number of dinospores produced per tomont had the largest effect on parasite population growth. Although the model suggests that control strategies should continue to focus on limiting the number of dinospores in a system, it provides a framework for evaluating the relative contribution of various factors to the parasite's population growth rate under a variety of conditions.

INTRODUCTION

Amyloodinium ocellatum Brown, 1931 (hereafter referred to as AO) is an obligate ectoparasitic marine dinoflagellate that parasitizes a wide variety of marine and estuarine fishes causing a condition commonly known as "marine velvet disease" (Noga 2010). The disease causes high mortalities in the culture of tropical marine and estuarine fishes and is one of the limiting factors in the development of marine aquaculture, particularly for closed, recirculating systems. At the University of Southern Mississippi's Thad Cochran Marine Aquaculture Center, we intensively culture a variety of marine fishes in closed, recirculating systems including spotted seatrout, *Cynoscion nebulosus*, and red snapper, *Lutjanus campechanus*, primarily for stock enhancement purposes. Despite quarantine and biosecurity protocols, periodic outbreaks of amyloodiniosis have plagued our production system.

The life cycle of the parasite is direct and includes three different stages (Brown 1934, Nigrelli 1936): 1) the fish-parasitic feeding stage, or trophont, that attaches to the epithelium of gills and skin; 2) the free-living, encysted, dividing tomont stage that develops after trophonts detach from the host; and 3) the free-swimming, infectious dinospore stage that hatches from the dividing tomont (up to 256 dinospores can come from a single tomont) (Brown 1934, Nigrelli 1936). Although a specific PCR (Levy et al. 2007) is available, diagnosis typically occurs through gross examination following the onset of morbidity, the result of which is that subclinical infections often go undiagnosed until the infection has progressed to the point that treatment is of limited benefit. Treatments include chemical baths using copper sulfate, formalin, chloroquine diphosphate, or hydrogen peroxide (Lawler 1977, Paperna 1984, Lewis et al. 1988, Montgomery-Brock 2000, Montgomery-Brock 2001, Noga and Levy 2006). However, these affect only the dinospore stage (Lawler 1980, Paperna 1984). As a result, long-term treatment regimes are required and recurrences are common. Advances in the control of this important parasite will require improvements in both early detection and identification of critical points in the life cycle that could be interrupted.

The loop-mediated isothermal amplification (LAMP) reaction is now widely used in the diagnosis of several important human diseases such as malaria and African sleeping sickness (Fu et al. 2011) as well as some important fish parasites such as whirling disease (El-Matbouli and Soliman 2005). LAMP provides a highly specific and efficient test that offers a couple of advantages over standard PCR in that through turbidity or the addition of intercalating dyes, results can be obtained visually in the reaction vial in about 1 hr. without thermocycling.

Epidemiological modeling offers a method of conceptualizing ideas about the transmission of diseases and formulating mathematical expressions for parameters that can be measured experimentally. With this, epidemics can be simulated to examine the effects of parameter changes on the progress of an epidemic. This approach has been used for several aquatic diseases including White Spot Syndrome Virus (Lotz and Soto 2002), Taura Syndrome Virus (Lotz et al. 2003), and necrotizing hepatopancreatitis (Vincent et al. 2004).

In this paper, we summarize our work (Picón-Camacho et al. 2013, Masson et al. 2013) developing 1) a LAMP assay for detecting AO and 2) a population model for identifying critical control points in the parasite's life cycle.

MATERIALS AND METHODS
Culture of parasite
A laboratory culture of AO was established using laboratory-reared spotted seatrout, laboratory-reared red snapper, and naturally infected fishes including striped mullet, *Mugil cephalus*, Atlantic croaker, *Micropogonias undulatus*, and spot, *Leiostomus xanthurus,* collected from the Mississippi Sound in the Gulf of Mexico. Details of the culture procedure and procedures for collection and quantification of tomonts and dinospores for use in the experiments may be found in Masson et al. (2011) and Picón-Camacho et al. (2013).

LAMP
For LAMP development, tomonts and dinospores were used in separate experiments to spike a series of 500-ml autoclaved artificial seawater (AASW) with 10,000, 5,000, 2,000, 1,000, 500, 100, 10, and 1 individuals per sample. DNA was extracted from water samples by filtration onto a 47 mm diameter 3-μm pore size Nucleopore filter (Nalgene Nunc International, Rochester, NY, USA). The filter was then placed into 200 μl of ATL lysis buffer with 20 μl (10 mg/l) of Proteinase k (Qiagen, CA, USA) and incubated for 30 min at 55°C with gentle shaking at 450 rpm (Eppendorf, NY, USA). DNA was extracted using a QIAmp DNA Stool Mini Kit (Qiagen) following Auderman et al. (2004) modifications. AO- specific primers AO18SF (5'-GACCTTGCCCGAGAGGG-3') and AO18SR (5'-GGTGTTAAGATTCACCACACTTTCC-3') were used for PCR amplification of a 248 bp segment of the 3' end of the LSU rDNA gene according to Levy et al. (2007). PCR conditions, sequencing, and detection were accomplished as described in Picón-Camacho et al. (2013). LAMP primer design (see Fig. 1 in Picón-Camacho et al. (2013)), determination of optimum conditions, detection, and assays for specificity and sensitivity were conducted.

To examine the effect of possible inhibitors in natural plankton communities in environmental water samples, a series of eight 500-ml seawater samples collected from the pier of the Gulf Coast Research Laboratory (16 psu) were filtered using a 125 μm mesh followed by a 90 μm mesh and then spiked with 10,000, 5,000, 2,000, 1,000, 500, 100, 10 and 1 tomont of AO. DNA was then extracted as described above. Samples were run in duplicate. An environmental water sample without AO was used as a negative control in addition to the negative control for the LAMP assay. To determine if substances in gill/fish tissue affected the efficacy of the reaction, gill tissue

from seatrout infected with AO was examined to confirm the presence of AO trophonts and establish the exact number of trophonts present per gill arch. Five samples of gill tissue, each weighing approximately 100 mg and containing 77, 52, 23, 14 and 4 trophonts, were collected. In addition to these samples, 100 mg of gill tissue collected from a healthy uninfected Atlantic croaker was spiked with a single trophont. Samples were first homogenized using a hand held tissue homogenizer and suspended in 200 μl of ATL lysis buffer. DNA was then extracted using the protocol described above. Each sample was tested in duplicate.

Model

Detached trophonts (tomonts) were measured and transferred individually to a 96-well culture plate (Corning Life Sciences, Corning, NY, USA) (i.e., 1 trophont per well) whose wells were previously filled to half their volume (0.16 ml) with artificial sea water (25°C, 33 ppt). The culture plate was incubated in the dark at 25°C. Culture plates were examined once daily in the morning until the tomont had either sporulated or decomposed to assess the survival of the trophonts, tomonts, and dinospores, the number of tomont divisions, the time to sporulation, and the number of dinospores produced by each tomont. Three trials were done. Three observations were discarded due to error; therefore, the sample consisted of 285 trophonts. A diagrammatic representation of the AO life cycle, representing the three discrete life history stages and the vital rates that regulate the transitions from one stage to another, was developed (Fig. 1). Circles represent life history stages and arcs/arrows represent transitions. Conceptual mathematical expressions for the transitions (and the vital rates regulating them) accompany the arcs (see Fig. 1 caption).
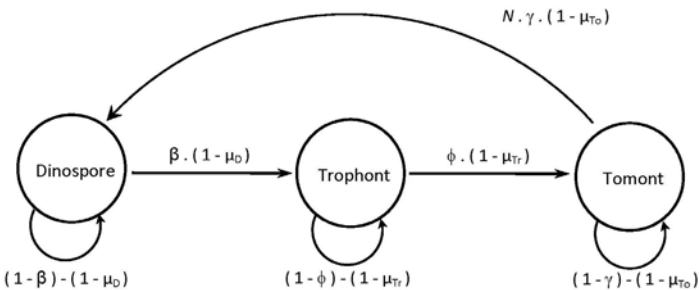

Figure 1. Life cycle graph for an *Amyloodinium ocellatum* population showing the transitions between and within stages and the associated vital rate probabilities; all arcs take one time period to complete. β is the probability that a dinospore infects a fish d^{-1}; μ_D is the probability that a dinospore dies d^{-1}; f is the probability that a trophont detaches from a fish d^{-1}; μ_{Tr} is the probability that a trophont dies d^{-1}; γ is probability that a tomont sporulates d^{-1}; μ_{To} is probability that a tomont dies d^{-1}; and N is the mean number of dinospores produced per tomont.

The vital rate parameters conceptualized in the diagrammatic model were estimated in the laboratory for both spotted seatrout and red snapper. Probabilities of tomont survival, dinospore survival, and tomont sporulation were derived by plotting the number of survivors over time. Data were fit to an exponential function $y = a.e^{b.x}$ (with $1 - e^b$ being the probability that an individual of a given stage undergoes a transition per day) using Systat version 12.0 (Systat 2007). Tomont fecundity was estimated by examining the dividing tomonts once daily until sporulation. The number of tomites just before sporulation was used to determine the number of dinospores produced per tomont (i.e. each tomite produces two dinospores). Based on an examination of the relationship between the number of dinospores produced and tomont size, the number of dinospores produced by tomonts of the mean size was calculated. A previous study (Masson et al. 2011) determined the daily trophont mortality rate, daily trophont infection rate, and the daily trophont detachment rate. Fig. 1 was transformed into an equivalent matrix form to facilitate analysis and simulations of these parameters using Mathcad 13 (Mathsoft 2005). Details of the analysis may be found in Masson et al. (2013).

RESULTS AND DISCUSSION
LAMP

Effective LAMP reactions must be 1) optimized for reaction temperature and time to insure that the maximum detectable result is achieved, 2) specific for the organism in question to insure the absence of false positives, 3) sensitive such that the organism in question is detectable at low levels to insure the absence of false negatives, and 4) uninhibited by normal substances found in field samples. Our results indicate that the optimum temperature for the AO LAMP (AO-LAMP) reaction is 62°C. The optimum time is 25-30 min (Figs. 2 and 3).
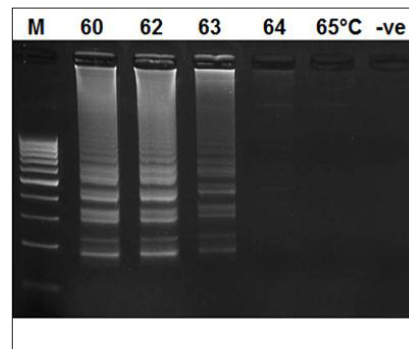

Figure 2. Agarose gel showing the effect of temperature in the AO-LAMP reaction. Amplification time was carried out for 60 min at the different incubation temperatures followed by 80°C for 2 min. M= 100 bp DNA molecular weight standard. –ve= no template negative control.
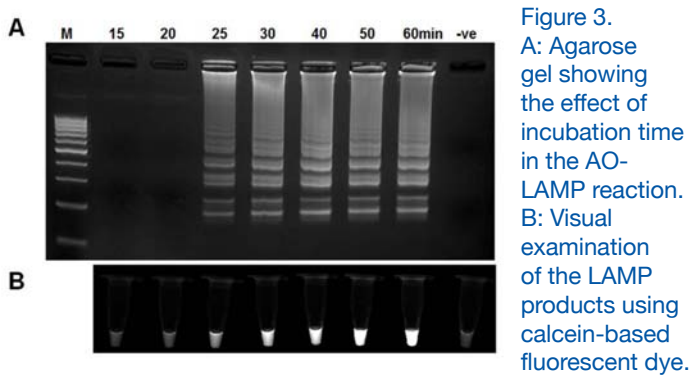
Figure 3. A: Agarose gel showing the effect of incubation time in the AO-LAMP reaction. B: Visual examination of the LAMP products using calcein-based fluorescent dye. Amplification was carried out at 62°C at the indicated incubation times followed by 80°C for 2 min. M= 100 bp DNA molecular weight standard. –ve= no template negative control.

We verified that the AO-LAMP amplifies only AO using three methods:

1) a negative control with no AO DNA to insure that the reaction is not amplifying something else in the reaction vessel,
2) restriction enzyme digestion of amplified LAMP products followed by matching to known sequences of AO DNA to confirm that the amplified product is AO, and
3) controls with DNA from closely related dinoflagellate species to confirm that the reaction does not amplify related species.

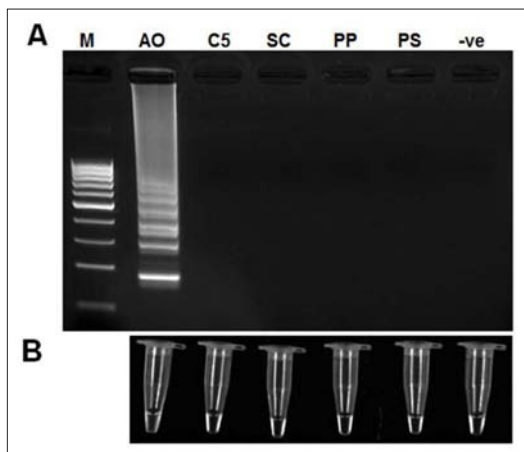No false positives were detected (Fig. 4).

Figure 4. A: Agarose gel showing the specificity of the AO-LAMP assay. B: Visual examination of the LAMP products based on turbidity. LAMP reaction was carried out at 62°C for 1 h followed by 80°C for 2 min. M= 100 bp DNA molecular weight standard, AO= *Amyloodinium ocellatum*, C5= *Cryptoperidiniopsoid* sp. Folly C5, SC=*Dinophyceae* sp. Shepherd's Crook, PP= *Pfiesteria piscicida*, PS= *Pseudopfiesteria shumwayae*, –ve= no template negative control.

We verified the sensitivity of the assay by:

1) serially diluting dinospores to achieve known numbers ranging from 1 to 10,000 and
2) serially diluting the total concentration of DNA to a range of 1fg to 10ng.

The assay detected as few as 10 dinospores and as little as 10 fg of DNA (compared to 100 dinospores and 1 pg of DNA for the currently available PCR). No false negatives were detected (Fig. 5).
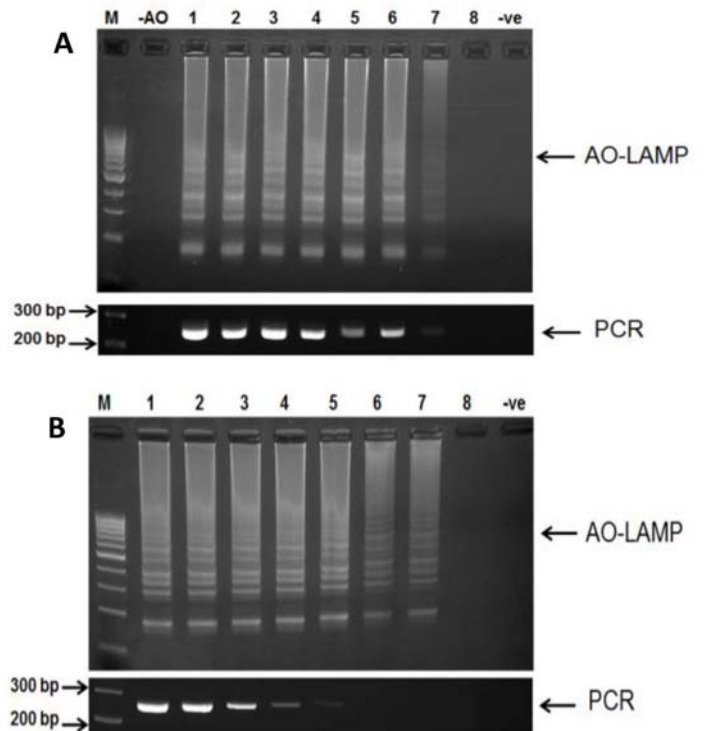
Figure 5. A. AO-LAMP (upper panel) and PCR (lower panel) sensitivity for known numbers of *Amyloodinium ocellatum* dinospores. The amplification by AO-LAMP shows a ladder-like pattern, whereas the PCR shows a single band (248 bp amplification product). M= 100 bp DNA molecular weight standard, AO= Negative water samples (no parasites added to the water), 1= 10,000 dinospores, 2= 5,000 dinospores, 3= 2,000 dinospores, 4= 1,000 dinospores, 5= 500 dinospores, 6= 100 dinospores, 7= 10 dinospores, 8= 1 dinospore, –ve= no template negative control. B. AO-LAMP (upper panel) and PCR (lower panel) sensitivity for serially diluted *Amyloodinium ocellatum* DNA. The amplification by AO-LAMP shows a ladder-like pattern, whereas the PCR shows a single band (248 bp amplification product). M= 100 bp DNA molecular weight standard, 1= 10 ng/µl (initial), 2= 1 ng/µl (1:10 dilution), 3= 100 pg/µl (1:10^2 dilution), 4= 10 pg/µl (1: 10^3 dilution), 5= 1 pg/µl (1: 10^4 dilution), 6= 100 fg/µl (1: 10^5 dilution), 7= 10 fg/µl (1: 10^5 dilution), 8= 1 fg/µl (1: 10^6 dilution), –ve= no template negative control.

We verified that common external factors do not interfere with the reaction by:

1) spiking environmental water samples with known numbers of tomonts ranging from 1-10,000 and
2) collecting gill tissue samples with known numbers of trophonts ranging from 1-77.

In both cases, the assay could detect a single trophont/tomont with no interference (Fig. 6).

Figure 6. A. Agarose gel showing the sensitivity of the AO-LAMP assay when used on environmental water samples spiked with *A. ocellatum* tomonts. LAMP reaction was carried out at 62°C for 1 h followed by 80°C for 2 min. M= 100 bp DNA molecular weight standard, -AO= Negat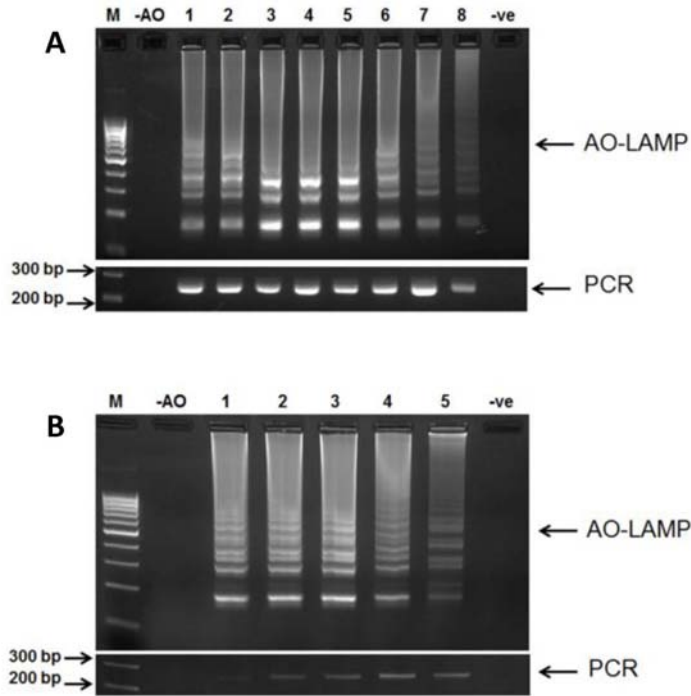ive water samples (no parasites added to the water), 1= 10,000 tomonts, 2= 5,000 tomonts, 3= 2,000 tomonts, 4= 1,000 tomonts, 5= 500 tomonts, 6= 100 tomonts, 7= 10 tomonts, 8= 1 tomont, –ve= no template negative control. B. Agarose gel showing the sensitivity of the AO-LAMP assay when used on gills from seatrout naturally infected with *A. ocellatum*. LAMP reaction was carried out at 62°C for 1 h followed by 80°C for 2 min. M= 100 bp DNA molecular weight standard, -AO= Negative water samples (no parasites added to the water), 1= 77 trophonts, 2= 52 trophonts, 3= 23 trophonts, 4= 14 trophonts, 5= 1 trophont, –ve= no template negative control.

Thus, we have developed an assay that detects all developmental stages of AO and is not inhibited by substances in the environment or gill tissue. The assay is faster than current molecular methods, is highly specific and is more sensitive than the currently available PCR. As a result, we have advanced the diagnosis of AO such that the parasite can be detected before the infection is grossly diagnosable, which provides a greater opportunity to administer treatments.

Model

Growth of trophonts released from fish over time reached an asymptote. Best-fit exponential growth curves for trophonts from both spotted seatrout and red snapper were linearized by reciprocal transformation and shown to have indistinguishable

slopes (see Fig. 2 in Masson et al. 2011). No differences in the overall mean infection rate between spotted seatrout and red snapper were identified (see Masson et al. 2011). Nevertheless, there were differences in some parasite parameters in the two host species. Trophonts grew larger on red snapper than spotted seatrout and were attached longer to red snapper than spotted seatrout (see Masson et al. 2011). Although there was no significant difference in the dinospore dose required to kill 50% of the fish in 48 hrs. (LD_{50}), the trophont lethal load that resulted from the 48-hr LD_{50} exposure (237,243 dinospores/fish for spotted seatrout and 141,010 dinospores/fish for red snapper) was higher in spotted seatrout (178,067) than in red snapper (123,160) (see Fig. 5 in Masson et al. 2011). Solving for the formulas presented in Fig. 1 allows production of a life table (Table 1) that facilitates calculation of the basic epidemiological parameter R_0 (the mean number of offspring produced by an individual in one generation). An $R_0 > 1$ is characteristic of an epidemic state (i.e., the disease organism infects more hosts than is necessary to simply maintain the population). In the case of both spotted seatrout and red snapper, the calculated R_0 indicates a natural epidemic state for AO.

Stage	Lifespan (t_x)	Fecundity (m_x) (dinospores*tomont^{-1}*d^{-1})	Product (t_x*m_x) (R_0) (dinospores*tomont^{-1})
Trophont	1.89 d (1.81-1.99) ss	0	0
	2.07 d (1.93-2.25) rs		
Tomont	2.78d (2.20-4.01)	17.69 (16.92-18.83) ss	49.15 (37.23-75.47) ss
		20.48 (20.11-23.41) rs	56.90 (44.25-93.81) rs
Dinospore	1.67 d (1.57-1.79) ss	0	0
	1.69 d (1.59-1.82) rs		
Σ (generation time)	6.3 d (5.6-7.8) ss		
	6.5 d (5.7-8.1) rs		

Table 1. Life table for Amyloodinium ocellatum. Values are means (95% CI). Ss = spotted seatrout, rs = red snapper.

The equivalent matrix for Fig. 1 is:

$$M = \begin{bmatrix} (1-\beta).(1-\mu_D) & 0 & N.\gamma.(1-\mu_{Tr}) \\ \beta.(1-\mu_D) & (1-\varphi).(1-\mu_{Tr}) & 0 \\ 0 & \varphi.(1-\mu_{Tr}) & (1-\gamma).(1-\mu_{To}) \end{bmatrix}$$

Using the experimentally derived vital rate estimates, analysis of the transition matrices for the life cycle of AO infecting spotted seatrout and red snapper revealed a population growth rate of 1.9988 d^{-1} for seatrout and 2.0461 d^{-1} for red snapper, which suggests that the epidemic state will be attained somewhat faster in red snapper. This matches our incidental observations that infections reach lethal levels faster in red snapper than in spotted seatrout. Matrix analysis also allows calculation of the threshold values for

each vital rate (Table 2). These are the values that the vital rate must obtain to achieve population growth. A comparison with the actual values obtained from the experimental infections reveals that all of the actual values exceed the threshold value, which provides more evidence that AO naturally exists in an epidemic state. A comparison of the differences between actual and threshold values for each vital rate reveals that the threshold and actual values for the dinospore death rate (μ_D) are more similar than the values for the other vital rates. Thus, only a 2.5 (or 2.6 for red snapper) time increase in the dinospore mortality rate would slow or arrest the epidemic. Indeed, the most common strategy for controlling AO outbreaks is the use of copper sulfate which kills the dinospores. Matrix models also allow for simulations that vary the different vital rates and examine the impact on the vital rates and the progress of the epidemic (elasticity and sensitivity analysis) (see Benton and Grant 1999). Table 3 shows the percent change in the AO population growth rate following a 50% change in the various vital rates. The largest changes are related to rates associated with the dinospores. While none of these calculations provide a magic bullet that will solve the problem with AO in aquaculture, the development of the model demonstrates that the disease is different in different hosts and provides a framework for evaluating the relative contribution of various factors to the parasite's population growth rate under a variety of conditions. This method can lead to testing and quantification of different approaches to controlling the parasite.

Vital Rate	Percent increase in the *A. ocellatum* population growth rate for spotted seatrout (λ_{st}) and red snapper (λ_{rs}) due to a 50 % change in a vital rate.	
	Spotted Seatrout	Red Snapper
N	15.34 %	15.30 %
β	13.61 %	13.68 %
γ	11.91 %	12.00 %
μ_D	10.32 %	10.28 %
μ_{To}	0.19 %	0.18 %
μ_{Tr}	0.06 %	0.05 %
ϕ	Not computed due to interference with N	

Table 3. Effect of a 50 % change in a vital rate on the Amyloodinium ocellatum population growth rate for infections in spotted seatrout and red snapper. Refer to Fig. 1 for the definition of symbols.

ACKNOWLEDGMENTS

	Vital rate threshold values for *A. ocellatum* population increase ($\lambda = 1$)					
	Spotted Seatrout			Red Snapper		
Vital Rate	Threshold value	Actual value	Fold difference	Threshold value	Actual value	Fold difference
N	2.814	81.90	29.1	2.885	98.90	34.3
β	0.0078	0.3552	45.5	0.0064	0.3414	53.3
γ	0.0003	0.3534	1178.0	0.0002	0.3534	1767.0
μ_D	0.9652	0.3787	2.5	0.97	0.3787	2.6
μ_{To}	0.911	0.0100	91.1	0.9237	0.0100	92.4
μ_{Tr}	0.9371	0.0027	347.1	0.9416	0.0024	392.3
ϕ	Not computed due to interference with N					

Table 2. Vital rate threshold values for Amyloodinium ocellatum population increase ($\lambda = 1$) and fold difference with their actual values for infections in spotted seatrout and red snapper. Refer to Fig.1 for the definition of symbols.

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Success of Seed Production of Humphead Wrasse *Cheilinus Undulatus* with Improvement of Spawning Induction, Feeding, and Rearing Conditions

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Key words: seed production, spawning induction, initial feeding

ABSTRACT

Humphead wrasse, *Cheilinus undulatus*, is the largest labrid distributed in tropical and sub-tropical regions of the Indo-Pacific and occur around coral reefs. Due to increasing fishing pressure since the early 1990s for the live reef-fish trade, the catch of this species dropped in many countries. Currently, international trade is controlled governmentally with wild catch banned in some countries. Attempts at aquaculture have become important for the sustainable utilization of humphead wrasse. However, although successful seed production was achieved once in Indonesia, consistent production was not established. We examined the method of spawning induction, and initial food and rearing conditions of larvae. Spontaneous spawning was observed from June to September when the water temperature was over 28 °C, and the period was within 1 week of a new moon.. Fertilization occurred in only 10 – 25% of spontaneous spawning events, but fertilization occurred all the time when we drew down the seawater level in the broodstock rearing tank.

Since the mouth width of humphead wrasse larvae was very small (133 μm) at first feeding, larvae were initially fed a variety of different live foods or other small particulate diets.. Two different sizes of monogonont rotifers, *Brachionus rotundiformis* Thai-strain (SS-type) and the more minute Proalid rotifer *Proales similis*, were used as live food, as well as boiled chicken yolk and powdered milk. Larvae preferred *P. similis* to the three other foods during the first seven days after mouth opening. Thereafter, larvae preferred SS-type rotifers. By using *P. similis*,

we produced 22 juveniles at 50 days post hatch [DPH] (survival rate 0.25%) in August 2011. In September 2011, we produced 537 juveniles at 50 DPH (survival rate 10.7%), suggesting that the elucidated rearing method allowed for reproducible seed production of humphead wrasse.

INTRODUCTION

Humphead wrasse, *Cheilinus undulatus*, is the largest labrid, with a maximum size exceeding 2 m total length (TL) and 190 kg body weight (Sadovy et al. 2003). They are distributed in tropical and sub-tropical regions of the Indo-Pacific and occur around coral reefs (Sadovy et al. 2003; Colin 2010). They are known as proterogynous species as all fish are initially females and then change sex to be male, but their age at maturity is relatively older than other labrids (Choat et al. 2006). This is one of the reasons for the difficulty in conserving this species.

Humphead wrasse is an important species as a fisheries resource, and is considered a luxury species in Hong Kong, China, and Southeast Asian countries. Since the early 1990s, humphead wrasses have been heavily exploited in Southeast Asian countries (Sadovy et al. 2003). This species was listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) in 2004. After that, international trade is limited, and some of the countries in Southeast Asia and Oceania banned harvest of this species. In Japan, a local spear fishery exists in the Ryukyu Islands. This fish is not expensive in Okinawa, and catch is consumed domestically by local customers or shipped to luxury Chinese restaurants in the Tokyo metropolitan area.

Attempts at aquaculture, broodstock management, and seed production for sustainable production are being conducted. However, seed production is very difficult. The successful seed production of this species happened only once in Indonesia in 2003 (Slamet and Hutapea 2005). There are challenges in broodstock management and larval rearing that must be solved for successful seed production of humphead wrasse. Therefore, we first investigated methods for the induction of spawning and the collection of fertilized eggs. Then we examined the larval rearing method,

with special attention to food for initial feeding and rearing condition. Finally, we worked on juvenile production.

MATERIALS AND METHODS

Humphead wrasse captured from the Yaeyama islands area were reared at the Yaeyama laboratory Research Center for Subtropical Fisheries, Seikai National Fisheries Research Institute, Fisheries Research Agency, Ishigaki Island, Okinawa, Japan. Fourteen to sixteen fish were divided into four broodstock lines (broodstock-A, B, C, D). Each contained one male and two or three females held in an indoor octagonal concrete tank (60 kL, maximum diagonal 5.8 m, depth 2.5 m) with flowing sand-filtered seawater. These indoor tanks had natural lighting through glass windows and transparent slate on the roof. Fish were fed cuts of fish and squid (1.5% of body weight) three times a week, and clam (2% of body weight) twice a week.

During the years from 2008 to 2011, spawning of humphead wrasse was observed from June to September or October. Spawned eggs were collected by an egg collection net (mesh size: 250 μm) placed in a tank under the over-flow pipe. When spawning was observed, eggs were collected and the total number and the number of fertilized eggs was recorded. During the spawning period, water temperature was continuously monitored every hour using a data logger (HOBO®, Water Temp Pro v2, Onset Computer Co., MA, USA).

To collect fertilized eggs systematically, the effect of drawdown of rearing water in the stimulation of spawning was examined with broodstock-A from 2009 to 2011. The drain was opened to discharge water, and the water level was lowered from the full water level (2.5 m depth) to a low water level (0.5 m depth) over 20 min. During the low water level the water was aerated for at least 30 min. When the courtship behavior was observed, a portion of the eggs were collected to confirm fertilization. Fertility was calculated by the proportion of eggs with cleavage cells (2 - 8 cell stage) as fertilized eggs in over 100 collected eggs. All eggs and/or hatched larvae were collected within 20 hours after fertilization to calculate fecundity and hatching rate.

To find the appropriate initial food, hatched larvae were fed two different sizes of monogonont, SS-type rotifer *Brachionus rotundiformis* Thai-strain, and more minute Proalid rotifer *Proales similis.* Boiled chicken yolk and powdered milk were also examined as

candidates for diet at initial feeding. Newly hatched larvae were placed into seawater at 28 °C in a 100 L *Artemia* hatching tank and the number of fed larvae for each food were counted at 2 DPH and 3 DPH. We demonstrated the reproducible juvenile production in July and August, 2011. The aeration and water temperature were in accordance with the previous study (Hirai et al. 2012). Newly hatched larvae were placed in semi-static seawater at 30 °C in a 500 L polyethylene tank. Larvae were fed *P. similis* from 2 DPH to 11 DPH, SS-type rotifers from 6 DPH to 29 DPH, and S-type rotifers from 28 DPH to 50 DPH. The number of fish was calculated every day from 0 to 7 DPH, and then at 10, 14, and 21 DPH, using a 500 mL column sampler, and all fish were counted at 50 DPH. Total length of fish was measured from 0 to 7 DPH, and then at 10, 12, 14, 21, 28, 35, 37, 42, and 50 DPH.

RESULTS AND DISCUSSION

Spawning was observed from June to September in natural water temperature. Spawning occurred when water temperature was 26.3 to 29.9 °C, and more than 91% of spawning was found when water temperature was higher than 28°C (Fig. 1). Spawning also occurred in October when the temperature of the rearing water was maintained above 28°C. In the wild, the water temperatures of the spawning sites are 28.5 to 30.0 °C (Colin, 2010). More than 73% of spawning occurred in the week before and after a new moon (Fig. 2). In many tropical species including rabbit fish (Rahman et al. 2000; 2003), groupers (Samoilys and Squire 1994; Lee et al. 2002; Teruya et al. 2008), and surgeonfish (Bushnell et al. 2010) sexual maturation and reproduction are cued to lunar cycles. Spawning of humphead wrasse also occurs within 1 week before and after the new moon in Indonesia (Slamet et al. 2003). These results suggest that the lunar-cycle is also related to the spawning rhythm of humphead wrasse in Ishigaki Island when the water temperature is optimal for spawning (Fig. 1 & Fig. 2).
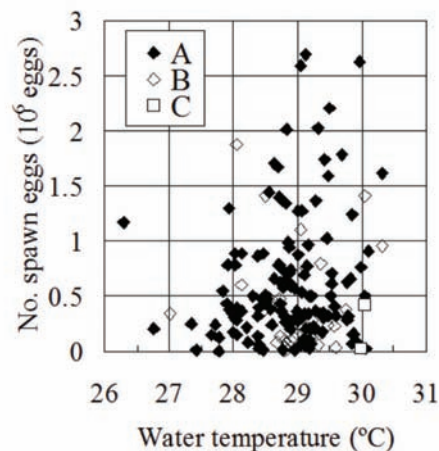

Figure 1. Relationship between number of spawned eggs and water temperature in humphead wrasse reared at Yaeyama laboratory from 2008 to 2011.
♦: broodstock-A,
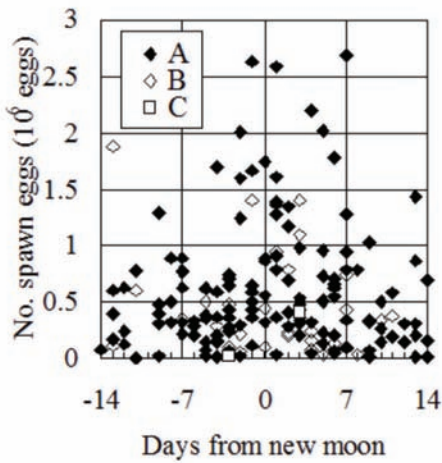◊ broodstock-B,
□: broodstock-C

Figure 2. Relationship between number of spawned eggs and lunar phase in humphead wrasse reared at Yaeyama laboratory from 2008 to 2011.
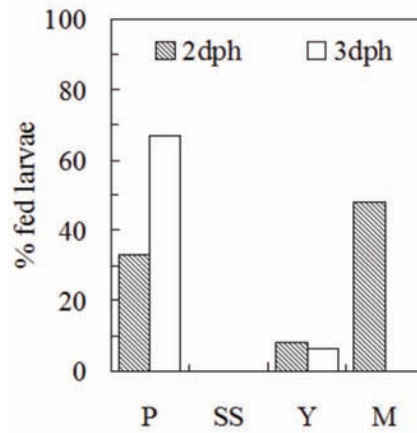♦: broodstock-A,
◊: broodstock-B,
□: broodstock-C

Figure 4. Number of fed larvae when fish were offered different food items at initial feeding. P: *Proales similis*, SS: SS-type rotifer, Y: Boiled chicken egg yolk, M: Powdered milk, Solid bar: 2 DPH, Open bar: 3 DPH.

We found spontaneous spawning in the rearing tank, but the success of fertilization was only 10-25%. In other labrids, the reproductive rhythm is considered to be related to the tidal cycle (Takemura et al. 2008). Although it was unclear why the fertilization rate was so low for spontaneous spawning in reared humphead wrasse, we consider that the lack of tidal change in the tank may have affected the stimulation of spawning and fertilization success. On the other hand, mating behavior and fertilization was found in every spawning when the water in the rearing tank was drawn down (Fig. 3). In our laboratory method, we lowered the water level from 2.5 m depth to 0.5 m depth over 20 min to stimulate spawning behavior. There are two possible reasons why the drawdown of rearing water may stimulate spawning behavior. One is that change of water level stimulates spawning, and the other is that the acceleration of the water current in the tank by flushing stimulates spawning. If spawning is stimulated by the changes in water level, spawning would be found at low tide or in shallow water, but in the wild, spawning in humphead wrasse is seen around the drop-off of the coral reef, and is not observed in the lagoon (Colin 2010). Moreover, spawning occurs mid-way between high tide and low tide when the rip current is fastest (Colin 2010). In our preliminary study, males chased females more rapidly when we created a faster current by aeration in the tank (data not shown). According to these wild and laboratory observations, spawning may be stimulated by the change in speed of the water current.

Diameter of collected eggs was 590-680 µm, and embryos were hatched out within 16-20 hours after fertilization. Total length of hatched larvae was approximately 2.4 mm at 6 hours after hatching. The mouth opening and eye pigmentation was observed at 2 DPH, with mouth diameter of 154 µm, and mouth width of 133 µm.

For the initial feeding, larvae consumed *P. similis*, boiled chicken egg yolk, and powdered milk at 2 DPH. Larvae also consumed *P. similis* and boiled chicken egg yolk at 3 DPH, but an increase in the number of fed larvae from 2 to 3 DPH only resulted from consumption of *P. similis*. Larvae did not feed on SS-type rotifer either 2 or 3 DPH. In another marine teleost, it is reported that seven-band grouper show a higher selectivity for *P. similis* than for SS type rotifer at initial feeding (Wullur et al. 2011). Since larvae of humphead wrasse have a relatively smaller mouth than other marine teleost larvae (Shirota 1970; Sawada et al. 1999; Tanaka et al. 2005), it is suggested that smaller live food is necessary for humphead wrasse larvae for initial feeding than the diet used for general seed production.

Figure. 5 Changes in survival and growth of humphead wrasse larvae and juveniles during successful seed production in 2011. Solid rhombus: Survival (%), Open rhombus: total length (TL:mm).
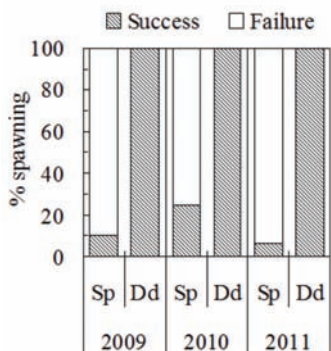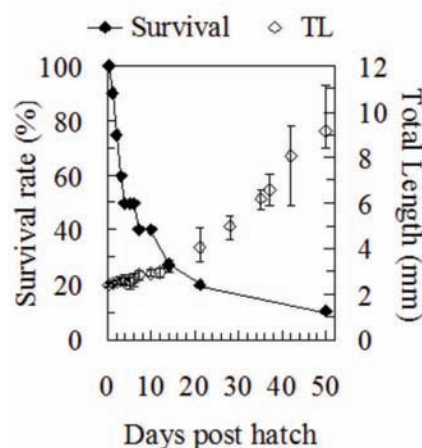

Figure 3. Difference in successful fertilization between spontaneous spawning (Sp) and stimulation of spawning by drawdown of seawater (Dd) in broodstock-A from 2009 to 2011.

Based on the rearing conditions described in Hirai et al. (2012) and using *P. similis* as initial live food, juvenile humphead wrasse were reproducibly produced in August (N=22, survival rate: 0.25%, mean TL: 9.0 mm) and September (N=537, survival rate: 10.7%, TL: 9.1 mm) in 2011. During juvenile production, larvae were fed SS-type rotifers starting 7 DPH. From 11 DPH, the number of SS-type rotifers in larval gut was greater than the number of *P. similis*. On the development and growth of larvae and juvenile humphead wrasse, yolk-sac larvae were found at 0-1 DPH with total length 2.29-2.57 mm. Preflexion larvae were found at 2-28DPH, and all larvae from 2 to 21 DPH were in preflexion phase with a size range of total length 2.46-4.43 mm. Flexion larvae were found only at 28 DPH, with total length 4.65-5.01 mm. Postflexion larvae were found from 28 to 42 DPH, with total length 5.20-6.57 mm. More than 90% of fish at 42 DPH were in juvenile stage. Size range for total length of juveniles at 42 and 50 DPH was 7.23-11.15 mm. The dietary sequence to raise juveniles was *P. similis* for 2-11 DPH, SS-type rotifers for 6-29 DPH, and S-type rotifers for 28-50 DPH. In the culture of other marine teleosts , rotifers, *Artemia*, copepods, and artificial diets have been used during the larval period. However, *Artemia nauplii* were accepted by humphead wrasse juveniles after 50 DPH, suggesting that smaller food is necessary for the seed production of species with small larvae like humphead wrasse.

In this study, we demonstrated reproducible seed production of humphead wrasse. Humphead wrasse juveniles were relatively smaller than other marine teleost juveniles. The first consumption of *Artemia* was found in the juvenile period, but not in the larval period, suggesting that the dietary sequence is different than other marine teleosts for aquaculture. In the future, establishing methods for juvenile culture will be the next stage in the sustainable production of humphead wrasse.

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The authors examined two different sizes of monogononta, SS-type rotifer *Brachionus rotundiformis* Thai-strain and the more minute Proalid rotifer *Proales similis*, as candidates of live food for the first feeding of humphead wrasse. Rearing under the condition of 20 ml/min aeration and oil addition, it was shown that the larvae preferred *P. similis* to *B. rotundiformis* at the first feeding, and survived for 9 days under the provision of *P. similis* until the 6th day. Growth in total length and pigmentation were observed on the 8th day. These results suggest that *P. similis* is suitable for the rearing of humphead wrasse larvae during the initial feeding phase.

Fisheries Research Agency and Nagasaki University. 2011. Japan's first production of humphead wrasse fry – World's first reproducible production – (in Japanese), Press Release, URL: http://www.fra.affrc.go.jp/pressrelease/pr23/231129/index.html

The press release outlines success in efficiently obtaining fertilized eggs through artificially induced spawning. The hatched larvae of this species were found to be very small, with a small mouth, and were unable to be fed general size live foods. Researchers decided to feed larvae with zooplankton *Proales similis* of the family Proalidae, which is not longer than 0.08 mm, and successfully produced 22 juveniles in August 2011. In September 2011, the culture environment was further improved, and 537 juveniles were produced, exceeding the number (120 juveniles) produced in Indonesia. This was the world's first successful reproducible seed production of humphead wrasse.

Wullur S, Sakakura Y, and Hagiwara A (2009) The minute monogonont rotifer *Proales similis* de Beauchamp: Culture and feeding to small mouth fish larvae. Aquaculture, 293, 62-67

This study examined the feasibility of using the minute monogonont rotifer *Proales similis*, with body length and width smaller and narrower than that of *Brachionus rotundiformis*, as live food by analyzing its life history, population growth, and feeding incidence by fish larvae. *P. similis* grew well at temperatures 25 to 35 °C and at salinities of 2 to 15 ppt. Results from feeding experiments confirm that *P. similis* is consumed by seven-band grouper (*Epinephelus septemfasciatus*) larvae. The larvae demonstrated the highest feeding rate at 20 ind./ml of *P. similis*.

Improving the Hatchery Output of the Hawaiian Pink Snapper, *Pristimpomoides filamentosus*

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The opakapaka, one of the highly prized "Deep Seven" bottom fish species, has been branded with an "over fishing" status. This situation has mandated that Hawaii develop a fishery management plan for the bottom fish fishery in the Main Hawaiian Islands. Part of this plan was the development of hatchery protocols where initial efforts have resulted in the only spawning broodstock maintained in captivity. This has offered the opportunity to develop hatchery techniques for their artificial propagation.

To insure that the nutritional requirements of opakapaka larvae are being met, fatty acid profiles of spawned opakapaka eggs were determined. Total fatty acid content in the opakapaka eggs matched that of wild caught copepods. While the total fat content may be relatively low the percent composition of the essential fatty acids, DHA and EPA ($26.9\% \pm 4.3\%$ and $2.7\% \pm 0.4\%$, respectively) are consistent with other reports indicating that both essential fatty acids are critical for survival and growth of the opakapaka larvae. In addition, the composition of essential fatty acids for the various live food organisms were obtained and with the exception of Nanno-fed rotifers, the live feeds employed, have similar profiles from a percent composition standpoint and indicates that protocols that were developed can be adjusted to meet the nutritional requirements of the opakapaka larvae.

Using changes in total length between fed and unfed larvae and monitoring temporal changes in gape size, it is clearly evident that introduction of live food organisms must take place no later than the third day post-hatch. When larvae were presented with a variety of live food organisms either alone or in combination, the results indicate that a copepod nauplius-only diet is a superior feeding regime over the all others tested.

Since adult copepod and nauplius production is dependent on cell density of the phytoplankton being used, the team used these findings to consistently time production of *Parvocalanus sp.* nauplii at densities ranging between 5-10 individuals/ml in a 3,000-L rearing tank by the third day post-hatching. This was achieved by "conditioning" copepod cultures using *Isochrysis galbana* (Tahitian Strain) at cell densities of 4×10^5 cells/ml prior to stocking into the larval rearing tank. Employing this strategy resulted in unprecedented larval survival with a mean of 80% passing the first feeding stages up until 10-12 day post-hatching.

Utilizing video recordings of the foraging behavior of opakapaka larvae revealed that the evasive abilities of adult and copepodite stages of calanoid copepods allowed them to avoid being captured by the larvae. While rotifers could be easily captured the growing body of evidence suggests that the ubiquitous rotifer is not a suitable live food for opakapaka larvae and another transitional live food is needed.

Effects of Docosahexaenoic Acid and Taurine Levels in Rotifers on Growth, Survival and Swim Bladder Inflation of Larval Amberjack *Seriola dumerili*

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Keywords: *Seriola dumerili*; Docosahexaenoic acid; Enrichment; Rotifer; Taurine

ABSTRACT

The amberjack *Seriola dumerili* is a commonly cultured species in southwest Japan, and amberjack aquaculture production together with yellowtail *Seriola quinqueradiata* dominates in that area. Although a stable supply of amberjack juveniles artificially produced in Japan is urgently needed (Mushiake 2006), the current technology for mass seed production is still incomplete. Successful seed production of marine fish requires fortification of live foods with the essential nutrients that are insufficient in the live foods alone. The docosahexaenoic acid (DHA) and taurine contents of wild amberjack larvae were found to be higher than in cultured larvae, and those in *Artemia nauplii* and especially rotifers, which are used as live foods for amberjack larvae during seed production, were markedly lower compared to wild zooplankton (Yamamoto et al., 2008). These findings suggest that DHA and taurine contents in rotifers used for the seed production of amberjack are insufficient for the requirements of this species.
First, we investigated the effects of algae with different DHA contents on the growth, performance, survival, and swim bladder inflation of larval amberjack. The algae were used for rotifer enrichment and were also supplemented to the larval rearing tanks in a static condition. Feeding trials were conducted from 1 to 10 days post-hatch (DPH). Rotifers enriched with *Nannochloropsis* [eicosapentaenoic acid (EPA) rich rotifers] were effective to enhance the growth and survival, but EPA was not essential to improve the swim bladder inflation in amberjack larvae.

Next, we investigated the effect of DHA enrichment of rotifers on larval amberjack. Larvae at 3 DPH were fed rotifers enriched with commercial DHA supplements at 4 levels, and reared for 7 days. DHA enrichment of rotifers was effective to improve the growth, survival rate, and swim bladder inflation of amberjack larvae.

Finally, the effects of feeding rotifers enriched with taurine at various levels on larval amberjack were investigated. Amberjack larvae at 3 DPH were fed rotifers enriched with a commercial taurine supplement at 4 levels (0, 200, 400 and 800mg/l), and reared for 7 days. The growth of fish fed rotifers enriched with the taurine supplement at 800mg/l was improved compared to fish fed the rotifers without taurine enrichment, although there were no significant effects of taurine enrichment on larval survival and swim bladder inflation.

The results of these studies indicate that enrichment of rotifers with DHA is essential for the growth, survival, and swim bladder inflation of larval amberjack, and taurine enrichment to rotifers is important during the early stages of amberjack larvae, contributing to improved larval growth.

INTRODUCTION

The amberjack *Seriola dumerili* (Risso) is a commercially important fish in the Mediterranean region and Japan because of its high growth rate and high commercial value (Mazzola et al. 2000; Nakada 2002). However, aquaculture of this species has been dependent on wild captured juveniles or eggs produced from hormone-induced mature bloodstock obtained from the wild (Shiozawa et al. 2003; Jerez et al. 2006). Although stable supplies of artificially reared amberjack juveniles are urgently needed for the development of successful amberjack aquaculture, the current technology for mass seed production is still incomplete (Papandroulakis et al. 2005; Mushiake 2006).

Successful seed production of marine fish requires the provision of live foods fortified with the essential

nutrients insufficient in the live foods alone. Live foods that are commonly used for marine fish seed production, such as rotifers, are sometimes low in essential nutrients. Therefore, enrichment of live foods with essential nutrients prior to feeding is necessary. The DHA and taurine contents of wild amberjack larvae were found to be higher than cultured ones, and the levels of these nutrients in were higher in wild zooplankton samples than in the live foods fed to amberjack larvae during seed production (Yamamoto et al. 2008). These findings suggest that live foods used for seed production are insufficient for the DHA and taurine requirements of amberjack larvae. However, there is no information on the effect of DHA and taurine on growth performance of amberjack larvae.

In seed production of marine fish, swim bladder inflation is considered to be a critical factor for survival (Battaglene et al. 1994; Takashi et al. 2006; Tanaka et al. 2009). Larvae making contact with the tank bottom due to weak upward swimming ability against gravity and infrequent swimming during nighttime have both been indicated as causes of mass mortality (Takashi et al. 2006; Tanaka et al. 2009). Mortality due to larval contact with the tank bottom is suggested to be associated with the lack of a functional swim bladder (Battaglene et al. 1994). Although a positive relationship between swim bladder inflation and n-3 highly unsaturated fatty acids (n-3 HUFA) content in rotifers was observed in gilthead sea bream *Sparus aurata* (Koven et al. 1990), the effect of n-3 HUFA and especially DHA on swim bladder inflation has not been fully clarified.

The aim of this study was to investigate the effects of rotifers enriched at various levels with DHA and taurine on growth, survival, and swim bladder inflation of amberjack larvae.

MATERIALS AND METHODS
Rotifer Enrichment and Larval Rearing

Three feeding experiments were conducted under identical conditions at Shibushi Station, National Center for Stock Enhancement, Shibushi, Kagoshima, Japan. Rotifers were stocked in 200L tanks (water volume 100L) at a density of 500 individuals/mL, and enriched as stated below for 12h in 100% sea water maintained at 24°C.

In experiment 1, rotifers were enriched with four treatments: 1) freshwater *Chlorella vulgaris* without EPA and DHA (Fresh Chlorella V12, Chlorella Industry Co., Ltd., Tokyo, Japan) "treatment DHA 0", 2) DHA-enriched at a lower level *C. vulgaris* (High grade Chlorella V12, HG, Chlorella Industry) "treatment DHA 0.5", 3) another DHA-enriched at a higher level *C. vulgaris* (Super fresh Chlorella V12, SV, Chlorella Industry) "treatment DHA 1.0", and 4) enriched with chilled *Nannochloropsis* containing EPA but not DHA (Yanmarine K-1, Chlorella Industry) "treatment Nanno".

In experiment 2, rotifers were enriched with four treatments: 1) freshwater *Chlorella vulgaris* "treatment Chlo", 2) a mixture of *Chlorella SV* and DHA-enriched *Chlorella* SV (2:1, v/v) "treatment Chlo+DHA-Chlo", 3) DHA-enriched *Chlorella* SV "treatment DHA-Chlo", and 4) DHA-enriched *Chlorella* SV and a commercial DHA emulsion (Bio Chromis, Chlorella Industry) "treatment DHA-Chlo+emulsion".

In experiment 3, rotifers were enriched for 12 h DHA-enriched *Chlorella* SV and then enriched for a further 9 h with 0, 200, 400 and 800mg/l of a commercial taurine supplement (Aquaplus ET; Marubeni Nisshin Feed Co., Tokyo, Japan) "Treatment Tau 0, Tau 200, Tau 400 and Tau 800". The enrichment of rotifers is summarized in Table 1.

Table 1. Foods used for rotifer enrichment in experiments 1, 2 and 3.

Experiment 1	Food for rotifer enrichment
DHA 0	1.0mL/L V12[a]
DHA 0.5	1.0mL/L HG[b]
DHA 1.0	1.0mL/L SV[c]
Nanno	2.7mL/L YK1[d]
Experiment 2	
Chlo	1.0mL/L V12
Chlo+DHA-Chlo	1.0mL/L of a mixture of V12 and SV (2:1, v/v)
DHA-Chlo	1.0mL/L SV
DHA-Chlo+emulsion	1.0mL/L SV and then enriched for a further 9h with 0.1g/L DHA emulsion[e]
Experiment 3	
Tau 0	1.0mL/L SV
Tau 200	1.0mL/LSV and then enriched for a further 9h with 200mg/Ltaurine supplment[f]
Tau 400	1.0mL/LSV and then enriched for a further 9h with 400mg/Ltaurine supplment
Tau 800	1.0mL/LSV and then enriched for a further 9h with 800mg/Ltaurine supplment

[a] Freshwater *Chlorella vulgaris* (Fresh Chlorella V12, Chlorella Industry Co., Ltd., Tokyo, Japan)
[b] DHA-enriched *Chlorella vulgaris* (High grade Chlorella V12, Chlorella Industry)
[c] DHA-enriched *Chlorella vulgaris* (Super fresh Chlorella V12, Chlorella Industry)
[d] Chilled *Nannochloropsis* (Yanmarine K-1, Chlorella Industry)
[e] Bio Chromis (Chlorella Industry)
[f] Aquaplus ET (Marubeni Nisshin Feed Co., Tokyo, Japan)

reared broodstock at the Komame Branch, Stock Enhancement Technology Development Center, Otsuki, Kochi, Japan. In all experiments, 10,000 larvae at 1 day post hatching (DPH) were stocked in each of twelve (four groups in triplicate) 500L

black polycarbonate tanks (water volume 500L). In experiments 1 and 3, amberjack larvae were reared until 10 DPH without water supply. In experiment 2, larvae were reared in a flow-through water system (0.3 L/min) until 10 DPH. Aeration was provided to each tank at 0.3L/min through an air-stone. Photoperiod was set at 14 h light (6:00-20:00): 10 h dark. Water temperature was maintained at around 24°C using a heater. Newly enriched rotifers were added to the tanks from 3 DPH at around 8:00 and 14:00 to maintain a density of 5 individuals/mL. Rotifer densities in the larval rearing tanks were checked by sampling 5 mL of rearing water from all tanks twice daily (6:00 and 13:00). To avoid starvation of rotifers after being introduced into the larval rearing tanks, the respective *Chlorella* (3.5 mL tank^{-1}) or *Nannochloropsis* (9.5 mL tank^{-1}) used for the enrichment was added twice daily as food for rotifers in each larval rearing tank. A surface skimmer was installed between 3 and 6 DPH to keep the surface free from lipidic films, which is a requisite for swim bladder inflation (Papandroulakis et al. 2005). Twenty fish were sampled using a 200mL polyethylene cup from each tank and anaesthetized with ethyl 3-aminobenzoate methanesulfonic acid (Sigma-Aldrich Japan, Tokyo, Japan) and total length measurements were taken every 2 days during the feeding experiments. The presence of an air inflated swim bladder (%) was determined using a profile projector (V-12BSC, Nikon Corp., Tokyo, Japan) in the same twenty fish 4 and 6 DPH from each tank.

At the end of each rearing experiment (10 DPH), the number of amberjack larvae in each tank was counted and sampled for chemical analyses. Samples of enriched rotifers were taken in each experiment, after being washed with freshwater. The sampling of rotifers in the larval-rearing tanks (tank rotifers) was done by siphoning part of the rearing water at the end of each feeding trial. The rotifer and fish samples were frozen immediately and stored at –80°C until analysis.

Chemical Analysis

Determination of sample moisture content was made by drying 10-h at 110 °C. Lipids from rotifers and fish samples were extracted by the chloroform-methanol (2:1, v/v) method (Folch et al. 1957) containing 0.01% butylhydroxytoluene. The preparation of fatty acid methyl esters and the conditions for fatty acid composition analysis by a gas liquid chromatography (GC-2010, Shimadzu, Kyoto, Japan) were the same as described in a previous work (Matsunari et al. 2012a). Free amino acids including taurine were extracted in 0.6 N perchloric acid (Ogata and Murai 1994). The free amino acid composition was determined by an automatic amino acid analyzer L-8500 (Hitachi, Tokyo, Japan).

Statistical Analysis

Data were subjected to one-way ANOVA and the differences between treatment means were compared using Tukey's test. For all statistical analyses, the SPSS 11.0 microcomputer software package (SPSS, Chicago, IL, USA) was used. In all statistical tests, differences at $P<0.05$ were considered significant.

RESULTS
Chemical Composition of Rotifers

Experiment 1: There were no significant differences ($P>0.05$) in the lipid contents among the treatments (Table 2). The EPA content in the total lipids was highest in the rotifers of treatment Nanno, intermediate in rotifers of treatments DHA 0.5 and 1.0, and lowest in rotifers of treatment DHA 0. The DHA content in the total lipids of rotifers in treatments DHA 0.5 and 1.0 were significantly higher ($P<0.05$) compared to the rotifers of treatments DHA 0 and Nanno.

Table 2. Crude lipid, EPA, DHA and Σn-3HUFA contents (g/100g, DM) in enriched rotifers used for the feeding trial in Expt. 1.

Enriched rotifers	*Chlorella*			*Nannochloropsis*
	DHA 0	DHA 0.5	DHA 1.0	
Crude lipid	13.8±0.5[a]	14.6±0.7[a]	15.3±0.4[a]	14.8±1.0[a]
EPA	0.0±0.0[a]	0.4±0.0[b]	0.7±0.0[c]	2.5±0.4[d]
DHA	0.0±0.0[a]	0.6±0.0[b]	1.3±0.1[c]	0.0±0.0[a]
Σn-3HUFA	0.3±0.0[a]	1.3±0.0[a]	2.5±0.2[b]	3.2±0.4[c]
Tank rotifers				
Crude lipid	9.7±0.4[a]	9.2±0.3[a]	9.4±0.7[a]	11.7±0.3[b]
EPA	0.2±0.0[a]	0.4±0.0[b]	0.6±0.1[c]	2.3±0.1[d]
DHA	0.2±0.1[b]	0.3±0.0[b]	0.8±0.1[c]	0.0±0.0[a]
Σn-3HUFA	0.6±0.1[a]	1.0±0.0[a]	1.8±0.2[b]	3.1±0.2[c]

Mean ± SD, $n = 3$
Values with the same superscript letter within the same row in each treatment are not significantly different ($P > 0.05$)

The total lipid content of rotifers in treatment Nanno was significantly higher compared with the rotifers enriched with the three *Chlorella* products. The EPA and n-3 HUFA contents in the total lipids of rotifers were highest in treatment Nanno. The DHA content in the total lipids was highest in the rotifers of treatment DHA 1.0, intermediate in rotifers of treatments DHA 0.5 and 0, and lowest in rotifers of treatment Nanno.

Experiment 2: Total lipid content was highest in rotifers of treatment DHA-Chlo+emulsion, although there were no significant differences in the lipid contents among the treatments (Table 3). The EPA content in the total lipids of rotifers increased with the amount of DHA-Chlo enriched. The DHA content in the total lipids of rotifers increased with the DHA enrichment from 0.0 g/100g (treatment Chlo) to 1.7 g/100g(treatment DHA-Chlo+emulsion) on a dry matter basis.

Table 3. Crude lipid, EPA, DHA and Σn-3HUFA contents (g/100g, DM) in enriched rotifers used for the feeding trial in Expt. 2.

	Chlo	Chlo+ DHA-Chlo	DHA-Chlo	DHA-Chlo+ emulsion
Crude lipid	11.3±1.4	11.1±1.1	12.3±2.0	13.2±1.2
EPA	0.0±0.0[a]	0.2±0.0[b]	0.5±0.2[c]	0.6±0.1[c]
DHA	0.0±0.0[a]	0.3±0.1[b]	1.0±0.4[c]	1.7±0.3[d]
Σn-3HUFA	0.4±0.1[a]	0.9±0.1[a]	2.0±0.6[b]	2.8±0.4[c]

Mean±S.D., n =4
Values with the same superscript letter within the same row are not significantly different ($P > 0.05$)

Experiment 3: The taurine concentration of enriched rotifers increased with increasing taurine supplementation from 1.5 to 7.2 mg/g dry matter (Table 4). Compared to the enriched rotifers, concentration of taurine in the tank rotifers was generally lower except for treatment Tau 0, although the concentration was higher in the rotifers with higher taurine supplement levels.

Table 4. Taurine concentrations (mg/g, DM) of rotifers freshly enriched and tank rotifers used for the feeding trial in Expt.3.

	Tau 0	Tau 200	Tau 400	Tau 800
Enriched (n = 5)	1.5±0.2[a]	2.7±0.5[ab]	4.2±0.9[b]	7.2±1.7[c]
Tank (n = 3)	1.7±0.7[a]	2.4±0.2[ab]	2.6±0.1[ab]	3.4±0.3[b]

Values are mean ±S.D.
Values with the same superscript letter within the same row are not significantly different ($P > 0.05$)

Growth, Survival and Swim Bladder Inflation of Amberjack Larvae

Experiment 1: The survival of fish fed the rotifers enriched with *Nannochloropsis* was significantly higher than fish fed the rotifers enriched with the *Chlorella* products (Table 5). The survival rates of fish fed the rotifers in treatments DHA 0.5 and 1.0 tended to improve without significant differences among the rotifers enriched with the *Chlorella* products. The growth of fish was not significantly different among the treatments. The swim bladder inflation (%) of fish was not significantly different among the treatments but numerically highest in treatment DHA 0.5, followed by treatment DHA 1.0, DHA 0, and Nanno.

Table 5. Growth, survival rate and swim bladder inflation of amberjack larvae in Expt. 1 , 2 and 3.

	Initial fish (3DPH)	Final fish (10DPH)		
	Total length (mm)	Total length (mm)	Survival rate (%)	Swim bladder inflation (%)*
Expt. 1				
DHA 0		4.7±0.1	4.1±2.6[a]	23.3±9.5
DHA 0.5	3.7±0.0	4.9±0.1	16.0±2.5[a]	39.2±10.4
DHA 1.0		4.8±0.1	14.8±9.9[a]	31.7±29.3
Nanno		5.0±0.1	37.6±3.8[b]	14.2±11.8
Expt. 2				
Chlo		4.7±0.0[a]	14.4±9.6	28.3±8.8[a]
Chlo+DHA-Chlo	3.9±0.1	5.1±0.1[b]	15.3±3.8	54.2±10.1[b]
DHA-Chlo		5.1±0.2[b]	18.7±5.6	59.2±3.8[b]
DHA-Chlo+emulsion		5.0±0.1[b]	22.2±7.7	55.0±13.9[b]
Expt. 3				
Tau 0		4.5±0.1[a]	7.0±4.9	33.3±18.1
Tau 200		4.5±0.0[a]	9.0±3.4	30.0±9.0
Tau 400	3.8±0.1	4.5±0.1[a]	12.3±3.4	34.2±7.2
Tau 800		4.6±0.0[b]	10.7±1.6	44.2±7.6

Mean ± S.D., n = 3
Values with the same superscript letter within the same column in each treatment are not significantly different ($P > 0.05$)
*Values given are the means at 4 and 6 DPH

Experiment 2: The amberjack larvae fed rotifers of treatment Chlo showed the lowest growth, survival and swim bladder inflation. The growth was significantly and similarly improved by the three treatments of DHA enriched rotifers (treatments Chlo+DHA-Chlo, DHA-Chlo and DHA-Chlo+emulsion). The survival rate was improved proportionally with the levels of DHA in rotifers; however, significant differences were not observed among treatments. The swim bladder inflation of fish was significantly improved by feeding the DHA enriched rotifers (treatments Chlo+DHA-Chle, DHA-Chlo, and DHA-Chlo+emulsion).

Experiment 3: Survival rates were not significantly different among the treatments but the value for treatment Tau 400 was numerically highest, followed by treatments Tau 800, Tau 200, and Tau 0. The total length of fish in treatment Tau 800 was significantly higher than those of fish fed the other rotifers at 10 DPH. The swim bladder inflation did not significantly differ among treatments.

DISCUSSION
The nutritional value of rotifers in larval-rearing tanks without water exchange can be maintained by appropriate supplementation of microalgae (Yamamoto et al. 2009). This

observation was reconfirmed in experiment 1 of the present study; the contents of EPA and DHA of the tank rotifers reflected the proportions of the microalgae (*Nannochloropsis* and DHA enriched *Chlorella*) supplemented to the larval-rearing tanks. In the present study, the highest growth and survival rates were observed in amberjack larvae fed rotifers of treatment Nanno (EPA rich rotifers), compared to larvae fed rotifers fed with *Chlorella* products. Thus, EPA effectively improved growth and survival in amberjack larvae as shown in other marine fish larvae such as red sea bream *Pagrus major* (Watanabe et al. 1989), yellowtail *Seriola quinqueradiata* (Furuita et al. 1996a) and striped jack *Pseudocaranx dentex* (Takeuchi et al. 1996).

The larvae of physoclists species are initially physostomous during initial swim bladder inflation. Swim bladder inflation in physoclists species, , often fails in artificially reared fish (Spectorova and Doroshev 1976). The lack of a functional swim bladder has been associated with skeletal deformities such as lordosis (Kitajima et al. 1981) as well as mass mortality (Battaglene et al. 1994). In walleye, initial swim bladder inflation is achieved by ingesting air at the water's surface during a brief and finite period when the pneumatic duct connecting the gut and the swim bladder is open (Rieger and Summerfelt 1998). In larval amberjack, swim bladder inflation has been suggested to be achieved in a similar way (Teruya et al. 2009). In larval amberjack, this period has been suggested to exist between 3 to 6 DPH (Imai et al. 2011). In the present study, the swim bladder inflation at 4 to 6 DPH of fish fed rotifers treated with *Nannochloropsis*, although not statistically different from the others, was inferior to those of the other fish fed rotifers treated with *Chlorella*. In larval red sea bream and yellowtail, EPA is inferior to DHA as an essential fatty acid (EFA) from the viewpoint of vitality (Watanabe et al. 1989; Furuita et al. 1996a, b). Thus, the lower swim bladder inflation of amberjack larvae fed rotifers enriched with *Nannochloropsis* (rotifers contain minimal DHA) could be attributable to the dietary deficiency of DHA, resulting in larvae with poorer vitality and difficulties penetrating or remaining beneath the water's surface.

In experiment 2, growth and survival rate of amberjack larvae fed rotifers enriched with DHA (treatments Chlo+DHA-Chle, DHA-Chlo, and DHA-Chlo+emulsion) improved compared to larvae fed rotifers without DHA enrichment. This indicates that amberjack larvae require DHA as an EFA for normal growth and development, as shown in other marine larval fish such as gilthead sea bream *Sparus aurata* L.

(Mourente et al. 1993), yellowtail *Seriola* (Temminck & Schlegel; Furuita et al. 1996a), and Pacific bluefin tuna *Thunnus orientalis* (Temminck & Schlegel; Seoka et al. 2007).

The swim bladder inflation of fish fed rotifers enriched with DHA-enriched *Chlorella* was significantly superior to that of fish fed rotifers enriched with *Chlorella* but without DHA enrichment. The rotifers were cultured with DHA enriched *Chlorella* containing n-3 HUFA, resulting in an increase of the proportions of not only DHA but also EPA in the total lipids. In terms of EFA, EPA effectively improves survival as well as DHA, but does not improve vitality of larval red sea bream and yellowtail (Furuita et al. 1996a, b). Although the fish size in the present study was not sufficient for examination of vitality, (for example by a stress test exposing the fish out of water) the vitality of larvae is reported to be improved by the elevation of DHA levels in rotifers (Watanabe et al. 1989; Ishizaki et al. 1996). Thus, as discussed above for experiment 1, amberjack larvae fed rotifers without DHA enrichment in experiment 2 might also have difficulties in remaining at the surface or penetrating the water surface due to their poorer vitality. The survival rate of amberjack larvae fed rotifers enriched with DHA improved compared to larvae fed rotifers without DHA enrichment, possibly as a result of decreased mortality due to successful swim bladder inflation.

In experiment 3, the total length of fish in treatment Tau 800 was significantly larger in comparison with the other treatments. Thus, taurine improved the growth of amberjack larvae as shown in other marine fish larvae such as red sea bream (Chen et al. 2004), Japanese flounder *Paralichthys olivaceus* (Chen et al. 2005) , Pacific cod *Gadus macrocephalus* (Matsunari et al. 2005) and Senegalese sole *Solea senegalensis* (Pinto et al 2010). The taurine concentration in larvae is suggested to affect amino acid retention. In Senegalese sole, amino acid retention increased when the larval body had a higher taurine content (Pinto et al. 2010). In the present study, the taurine concentration was highest in fish fed the rotifers of treatment Tau 800. In contrast, concentrations of several free amino acids in fish fed the rotifers of treatment Tau 800 were lower compared to those of fish fed the rotifers without taurine enrichment. The lower amounts of free amino acids in the tissue amino acid pool of the taurine-enriched fish suggest that free amino acids have been more effectively used for muscle protein deposition or energy production. The improved growth performance of fish fed the rotifers of treatment Tau 800 may be attributed to increased amino acid retention.

In the present study, the survival rates of amberjack larvae fed rotifers enriched with taurine seemed to improve up to the 400mg/l level of enrichment compared to larvae fed rotifers without taurine enrichment, although the effect was not significant. Similar results were observed in other marine fish larvae (Chen et al. 2004; Chen et al. 2005; Matsunari et al. 2005; Pinto et al. 2010), which suggests that the effect of taurine on survival depends on fish species and their life cycle. Moreover, there were no significant differences in swim bladder inflation among the treatments in this study unlike the effectiveness of DHA or n-3 HUFA enrichment of rotifers in previous studies (Koven et al. 1990; Matsunari et al. 2012b). The DHA or n-3HUFA concentrations in both enriched and tank rotifers in this study were similar between treatments. This suggests that dietary taurine supplementation during the rotifer feeding stages of amberjack larvae has no effect on swim bladder inflation.

The results of these studies indicate that enrichment of rotifers with DHA is essential for the growth, survival and swim bladder inflation of larval amberjack, and taurine enrichment to rotifers is important during the early stages of amberjack larvae, contributing to improved larval growth.

ACKNOWLEDGEMENTS

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To improve the nutritional quality of live foods and dry feeds ordinarily used for the seed production of amberjack *Seriola dumerili*, the nutrient contents of rotifers, *Artemia nauplii* and commercial feeds used in two larval production stations were evaluated. For comparison of the nutrient contents, artificially produced larvae, wild-caught juveniles, and wild zooplankton samples were also analyzed. The proportions of 22:6n-3 in the polar lipid of the cultured larvae increased by feeding dry feeds. The taurine contents of the cultured larvae reflected the contents of their foods (rotifers < dry feed < *Artemia nauplii*). The taurine content and the proportion of 22:6n-3 in *Acartia* spp. were higher than in foods fed to the larvae. These parameters in the wild juveniles were higher than the cultured ones.

Matsunari H., Hashimoto H., Oda K., Masuda Y., Imaizumi H., Teruya K., Furuita H., Yamamoto T., Hamada K., Mushiake K. 2012. Effects of docosahexaenoic acid on growth, survival and swim bladder inflation of larval amberjack *Seriola dumerili*. Aquac Res doi:10.111/j.1365-2109.2012.03174.x

The effect of DHA on the growth performance and survival of larval *Seriola dumerili* during the rotifer feeding period was investigated. Amberjack larvae at 3 days post hatching were fed rotifers enriched with freshwater *Chlorella* (Chlo), a mixture (2:1, v/v) of Chlo and DHA-enriched *Chlorella* (DHA-Chlo), DHA-Chlo, and DHA-Chlo with commercial DHA emulsion, in triplicate for 7 days. The survival rate was improved by the enrichment of rotifers with DHA-Chlo alone, and DHA-Chlo with emulsion. Growth and swim bladder inflation of fish fed rotifers enriched with DHA-Chlo were significantly improved, however, with increased levels of DHA further improvement was not found. The DHA requirement of amberjack larvae is estimated to be 1.5mg/g on a dry matter basis of rotifers.

Matsunari H, Hashimoto H, Oda K, Masuda Y, Imaizumi H, Teruya K, Furuita H, Yamamoto T, Hamada K, Mushiake K 2012. Effect of different algae used for enrichment of rotifers on growth, survival and swim bladder inflation of larval amberjack *Seriola dumerili*. Aquac Int doi:10.1007/s10499-12-9522-8

The effect of algae with different DHA contents used for the enrichment of rotifers on the growth performance, survival, and swim bladder inflation of larval amberjack *Seriola dumerili* was investigated. Rotifers were fed with freshwater *Chlorella vulgaris* containing 3 levels of DHA (rotifer containing DHA 0.04, 0.60, 1.32g 100g^{-1}) and *Nannochloropsis* (rotifer containing DHA, 0.04g 100g^{-1}; EPA, 2.54g 100g^{-1}). The same algae were supplemented to the larval rearing tanks in static condition. The larvae in each triplicate group were fed the enriched rotifers from 3 days post hatch for 7 days. Growth and survival rate of fish fed the rotifers enriched with *Nannochloropsis* were higher than those of fish fed the rotifers enriched with all three *Chlorella* treatments. Swim bladder inflation was lowest in fish fed the rotifers enriched with *Nannochloropsis*. Although rotifers enriched with *Nannochloropsis* were effective for the growth and survival, DHA is essential for swim bladder inflation in amberjack larvae.

Understanding *Artemia* Biogeography and Its Nutritional Qualities as a Biocapsule to Deliver Micronutrients Related to Hatchery Production and Juvenile Quality

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Key words: larvaeculture, *Artemia*, biogeography, bioencapsulation, nutrients.

ABSTRACT

The culture of fish, mollusk, and crustacean larvae is generally carried out under controlled hatchery and nursery conditions. Developing larvae are usually very small, extremely fragile, and generally not physiologically developed. Proper larval development and survival is one of the major bottlenecks in commercial aquaculture. Many of these problems are related with nutritional diseases such as deformities, loss of pigmentation, and infectious diseases affecting commercial aquaculture with high economic losses. In larvae culture the best sources of nutrition continue to be live food because of its nutritional, natural qualities. *Artemia* are one of the most important live foods in commercial aquaculture because of its content of fatty acids and amino acids. These nutrients are not stored in appreciable amounts in the larvae body, so signs of deficiency and sickness usually appear within weeks in young, rapidly growing fish. Many countries are substituting *Artemia* spp. as a result of high brine shrimp cyst prices. Some of the ingredients in feeds are rice bran, corn bran, soybeans, *etcetera*. Even though these foods could be enriched, larvae cannot digest it easily. These micronized products can also increase water eutrophication and stunt larval development. For these reasons, the first goal of this work is to report updated information on *Artemia* spp. biogeography as potential new resources of *Artemia* spp. cysts. These worldwide, hypersaline ecosystems have been explored and in some, started cyst exploitation or culture. However, these new sites require ecological and population studies of *Artemia* spp, to develop programs of adequate production management and cyst quality. This worldwide increase of *Artemia* spp. cyst locations can potentially decrease market prices. The second goal is to identify and report on important information related to the use of *Artemia* as a "biocapsule." The non-selective consumption of particulate food by brine shrimp enables its use as a vector to deliver specific nutrients and biological protectors.

INTRODUCTION

Importance of *Artemia* to Aquaculture

New technologies in finfish and shellfish nutrition are being developed for commercial aquaculture. Regardless of the vast improvement in fish nutrition, there is still the necessity of live food such as rotifers, copepods and *Artemia* as vectors to deliver micronutrients that are not present in artificial foods. *Artemia* possess unique characteristics, especially in regard to reproduction. *Artemia* females are oviparous and produce dormant stage embryos called cysts. The cyst can be dormant for months or years according to the environmental variations and the process of selection and quality control (Clegg & Conte, 1980; Drinkwater & Clegg, 1991; INVE, 2012). Cysts can be packed in vacuum cans and the nauplii will hatch in 24-48h in optimal conditions, with a hatching rate around 70% to > 90% (Sorgeloos, et al., 1997; Torrentera & Dodson, 2004, INVE, 2012). Nauplii of *Artemia* are very nutritious, containing high levels of essential fatty acids. Brine shrimp hatching and fatty acid content is highly variable by season and often lacking long chain n-3 fatty acids within and between locations (Pearson & Sorgeloos, 1980; Dhont & Sorgeloos 2002, Torrentera & Dodson, 2004).

Another commercially useful property of *Artemia* is nondiscriminatory feeding; *Artemia* are able to ingest small food particles ranging from 1 to 50 μm in size (Dobbeleir et al., 1980), such as microalgae (Sorgeloos et al., 1986), baker's yeast, dried microalgae, and micronized products from the food industry (Dobbeleir et al., 1980; Sorgeloos *et al.*, 1977, Sorgeloos et al., 2001). Hatcheries and nurseries suffer critical economic losses as a result of high larval fish mortality due to lack of micronutrients and bioprotectors. Bioprotectors for fish and shelfish are probiotics (several species of friendly bacteria and yeast) or other naturals resources, such as plant extracts that activate the defense mechanism which protects an organism externally and internally. These nutritional requirements are not present in sufficient amounts in artificial food. However, *Artemia* bioencapsulation of these nutrients enables it to be a vector for micronutrients and bioprotectors, providing fish larvae with required nutrients (Sorgeloos et al., 1977; Léger, et al, 1987; Sorgeloos et al., 2001; Langdon et al., 2008; Touraki et al. (2012; Seenivasan et al., 2012).

History of *Artemia* Cyst Production for Commercial Aquaculture and the Crisis of Lack of Cyst Resources vs. High Market Prices

Artemia spp. are distributed globally in inland salt lakes, ocean salterns, and small alkaline temporary ponds (Persoone & Sorgeloos, 1980, Van Stappen, 1996, 2002, Torrentera & Dodson 2002, 2004, Van Stappen, 2012; Shadrin, et al., 2012). The first harvests of *Artemia* biomass were initiated in the saltern ponds of San Francisco Bay (SFB) during the late 1930's. The salt-workers of SFB found that *Artemia* and their cysts could be harvested as a by-product of solar salt and commercial cyst harvesting began in the late 1950's (Lavens & Sorgeloos, 1996, 2000). Great Salt Lake (GSL), Utah-USA first harvest occurred during the 1950's when adult brine shrimp were harvested to be used as fish food in the aquarium trade in the United States. During the 1960's, the market became dominated by harvest of the cysts, which were used in the commercial aquaculture of shrimp, prawns, and some fish, primarily outside of the United States. As the demand for *Artemia* cysts grew, GSL started formal commercial exploitation. GSL cysts harvest during 1995-96 and 1996-97 seasons were about 15 million pounds gross weight (about half is suitable for final product). These two North American producers accounted for about 80 to 85 % of the total sales of *Artemia* cysts from the 1960's to the1980's (Lavens & Sorgeloos, 1996; Lavens & Sorgeloos, 2000, Brine shrimp and Great Salt Lake Ecology. usgs.gov, 2013). Commercial aquaculture of fish and shrimp started emerging in the early 1960's and has been in continuous development with improved production technologies creating more demand for *Artemia* cysts (Lavens & Sorgeloos, 2000; Sorgeloos, et al., 2001; Van Stappen, 2012). Unfortunately, SFB and GSL are facing severe environmental changes that have diminished *Artemia* cyst production. These two main sources of commercial cysts suffer major environmental problems such as extreme evaporation and sedimentation, and thereby, it is steadily being modified by the encroachment of various human activities, affecting *Artemia* cyst production (Arnow, T., 1985; Arnow & Doyle, 1990; Wurtsbaugh et al., 1990, Wurtsbaugh & Z. M. Gliwicz. 2001).

By the mid 1970's, a dramatic decline of GSL cyst production was reported with the situation worsening as a result of high import taxes in certain developing countries; with prices for *Artemia* cysts rising up to USD $50-100/kg. Between 1994 -1995, studies estimated cyst production of just over 5000 tons (4.54 million Kg/dry weight) for the entire lake, with commercial harvest taking 21% (Wurtsbaugh & Gliwicz 2001). The yield of dry, processed cysts varies annually, but recovery is typically 30-35%, which is not adequate for the aquaculture market demand. As a result of the lack of cysts and high prices, the *Artemia* Reference Center (ARC, at the University of Belgium), in association with other international institutions, started developing a joint project of international exploration of new natural sources of *Artemia*. This included identifying geographical distribution of sites, abundance and cyst quality, and *Artemia* inoculation and culture in salterns of developing countries that are unable to pay high prices for cysts. For example, since the first initiatives of the 1980's, the seasonal culture of *Artemia* in the Mekong Delta has expanded and has been transformed from artisanal salt farms into an important supplier of high quality cysts for local use and for the international market (Nguyen 2011).

Updated *Artemia* Worldwide Biogeography

The brine shrimp, *Artemia,* is a complex group of several sibling species, although *Artemia salina* is the species usually referred, in the commercial aquaculture *A. salina* (Leach 1819) is the main species in the Old World. It is a sexually reproducing species distributed in Europe, Southern Africa, Cyprus, Crimea, Tunisia, and Russia and, Mid-latitudes of Asia, Japan, and introduced in Australia. *A. salina* also cohabits with many other sexual and asexual species, such as *Artemia parthenogenetica,* that is a complex group of parthenogenic species that have variable chromosome numbers (diploids, tetraploids, pentaploids, etc.). *Artemia urmiana* (Günther, 1890) is distributed in the Iranian Lake Urmia, several Crimean lakes, Ukraine, Turkey and Russia. *Artemia sinica* (Cai, 1989) inhabit inland China, Mongolia and Russia. *Artemia tibetiana* (Abatzopoulos *et al.*, 1998) is distributed throughout Tibet. In the New World, the dominant species is *Artemia franciscana* (Kellog, 1906) a sexually reproducing species widely distributed in North and South America and the Caribbean region (Van Stappen 2002, Torrentera & Dodson, 2004). *A. franciscana* cysts were introduced to Europe, Australia, New Zeeland, Brazil and Cuba, most likely for aquaculture purposes or perhaps incidentally from commercial batches (Tizol, *et al.*, 2009; Camara, 2012). Molecular genetics studies of *Artemia* spp. populations from the Yucatan Peninsula, Oaxaca, and Cuba found that the Caribbean-Cuban population was introduced from SFB cysts and that the southern Mexican populations were distinct from both SFB and Cuban *Artemia* breed, forming a separate clade (Tizol et al., 2009). This finding corroborated the results of a previous cytogenetic diversity study of *Artemia* spp. populations in the Yucatan Peninsula (Torrentera & Abreu, 2002).

New reports worldwide have indicated an increase of cases of multiple *Artemia* species cohabiting in the same site. This is the case of several European sites with *A. salina*, and *A. parthenogenetica* (Van Stappen 2002; Abatzopoulos, et al., 2009; Shadrin, et al., 2012). Another case of *Artemia* sites with the presence of two or more sexual and parthenogenic species is in Ukraine and Russia with *A. urmiana*, *A. salina*, and *A. parthenogenetica* growing in the same sites (Van Stappen, et al., 2002; Van Stappen, 2009; Shadrin, et al., 2012; Shadrin & Anufriieva, 2012). The same has been reported in Macaronesia (Hontoria et al., 2012). In Tunisia, there are 49 sites with *A. salina* and *A. parthenogenetica* (Hachem, et al., 2012), as well as some sites in Algeria (Hichem Kara, et al., 2012). India recently reported the characterization of three new populations of sexual brine shrimp (*Artemia* sp. (no defined species) cohabiting with *A. parthenogenetica* (Vasudevan, 2012). In Iraq, 22 sites with *A. salina* and *A. parthenogenetica* populations have been reported (Salman, et al., 2012). Table 1 and Fig 1A show the updated Worldwide Biogeography *of Artemia* species, number of sites by continents, and number of countries explored. Fig 1B shows Lake Cambay in India, a potential site for brine shrimp and cyst production (Van Stappen, 2012).

Figure 1A. Updated worldwide *Artemia* spp. sites as of 2012 (Map modified from Torrentera, 2012). Presently, more than 1000 sites have been recorded, (black dots show the most populated sites).

Figure 1B. Lake Cambay in India, a hypersaline lake of 18 000 km² (ARC, 1995).

Table 1. Number of hypersaline habitats with *Artemia* spp. populations by world region, number of countries explored, and species. Information updated by Torrentera L. 2012. AF (*A. franciscana*), AP (*A. persimilis*), AS (*A. salina*), AU (*A. urmiana*), AS (*A. sinica*), AT (*A. tibetiana*), APC (*A. parthenogenetica* complex) and Asp (*Artemia* sp not defined species). *AF and *AS probable introductions for aquaculture purposes.

Table 1		
NEW WORLD	**Number of sites**	**Artemia Species**
North America		
Canada, USA, Mexico	115	AF Asp
Central America (7 Countries; 2 countries explored)	7	AF Asp
Caribbean (13 countries, including colonies; 6 explored)	28	AF Asp
South America (12 countries; 3 countries explored)	63	AF Asp / AP AF Asp
New World Total sites	**213**	
OLD WORD	**Number of sites**	**Artemia Species**
Europe		
Europe (40 countries; 14 countries explored)	448	AS APC AU *AF
Macaronecia (Norh Atlantic Archipielags)	9	AS APC *AF Asp
ASIA (71 Countries; 9 countries Explored)	119	AS APC AU AT Asp
Africa (61 countries; 12 countries explored)	208	AP AS *AF Asp
Australia and New Zealand	8	**AS *AF
Old World Total sites	**792**	
Worldwide Total sites	**1005**	

Artemia species and respective abbreviation: AF (*A. franciscana*), AP (*A. persimilis*), AS (*A. salina*), AU (*A. urmiana*), AS (*A. sinica*), AT (*A. tibetiana*), APC (*A. parthonogenetica* complex) and *A sp.* (no defined species). *AF and **AS probable introduced for aquaculture purposes.

Speed of Commercial Aquaculture Development, Disease Outbreaks, and Economical Losses

Since commercial aquaculture started to develop in several countries when such as Japan, several countries in Europe, USA, Australia, other parts of Asia, and Latin America. Every year, more than 1,500 metric tons of *Artemia* cysts were marketed worldwide to feed fish and shrimp. Shrimp hatcheries were making up 80 to 85% of global *Artemia* cyst sales (FAO, 1996A; FAO 1996B; Dhont & Sorgeloos, 2002; FAO, 2005). The international trade of live fish and shellfish has increased worldwide translocation of pathogens, resulting in a fast spread of infectious and parasitic diseases affecting commercial aquaculture. As a result of these issues, recently discussed new, sustainable and clean aquaculture practices are been promoted (FAO, 2005). An ecosystem approach to aquaculture is suggested, with the main goals to integrate aquaculture with other sectors, to deal with external and internal problems, dealing with environmental impacts at the farmer level, at the water shed scale and at the global level (FAO 2010A). In Japan, where some of the most advanced aquaculture technologies are utilized, important finfish commercial culture species

such as red sea bream, black sea bream, yellow tail and other marine physoclistous, suffered from nutritional and microbial diseases (Kitajima, Watanabe, & Fujita, 1994, Watanabe & Vasallo2003). Japanese researchers started to investigate possible sources of microbial infection and dissemination in water and fish. In 1987 Muroga, et al. isolated intestinal microflora of farmed red seabream and black seabream at larval and juvenile stages. Results of these studies demonstrated that *Vibrio*, *Moraxella* and *Pseudomonas* were the main causes of these fish mortalities. Their experiments showed total bacterial count of $7.4x10^{\wedge 4}$ and $3.4x10^{\wedge 4}$ (CFU)/fish, from intestinal samples of red seabream and black seabream, respectively. Among the intestinal flora, *Vibrio* accounted for 45% and *Pseudomonas* for 30% of total bacteria for rotifers and brine shrimp, respectively. Bacteria *Pseudomonas* accounted for 22% and *Moraxella* 29% in rotifers and brine shrimp, *Pseudomonas* was the predominant strain with 48%. The incidence of *Vibrio* in water and live diets was 7% and 11%, respectively (Muroga, et al., 1987). The first solutions to contend infectious diseases in larvae and juvenile finfish were the use of antibiotics; however these methods in many cases caused toxicity especially for the delicate larvae. Calculation of antibiotic optimal minimum concentration was a challenge difficult to face (Touraki et al., 1999; Vaseeharan et al., 2004). Alternative approaches to attenuate the severe effects of antibiotics in disease outbreaks included the encapsulation of antibiotics within *Artemia* (Cook & Rust, 2002; Vaseeharan et al., 2004).

Improvement of Live Food in Essential Fatty Acids to Decrease Nutritional Diseases in Larvaeculture.

Fish deformities and lack of pigmentation are frequently encountered in the culture of larvae and juveniles of yellowtail, red seabream, and black seabream in Japan. (Kitajima, et al.1994). Live food enrichment was developed in Japan using modified yeast enriched with omega-3s to feed the rotifer *Brachionus pricatilis*, and the copepod *Tigriopus japonicus*. These enriched organisms were used as nutritious live food to partially substitute the use of microalgae during the 1960's (Fukusho, 1980; Fukusho, et al., 1980; Watanabe & Vass 2003). These live foods enriched with omega-3's decreased larval deformation and mortality and improved finfish development. In the late 1970's, the *Artemia* Reference Center started research to improve *Artemia* nauplii hatching by decapsulation and enrichment with 3-n fatty acids. These practices were very successful and spread out in several countries as a routine technique in hatcheries and nurseries (Sorgeloos, et al., 1977; Sorgeloos, et al., 2001). However new problems started

to arise, such as the presence of pathogenic bacteria in *Artemia* nauplii, and cyst wastes, and culture water, particularly *Vibrio* and *Pseudomonas* (Dobbeleir, et. al., 1980; FAO, 2005).

Artemia Bioencapsulation as a Vector to Deliver Micronutrients and Bioprotectors

New emerging issues with the use of massive culture of brine shrimp nauplii and are the presence of pathogenic bacteria, such as *Vibrio* and *Pseudomonas*, isolated from brine shrimp nauplii (over and within the brine shrimp body) causing high mortalities in hatcheries and nurseries. Researchers of different institutions are developing new techniques to improve the quality of brine shrimp nauplii. For instance, to improve the selection of viable cyst from died nauplii and cyst shells, and the use of *Artemia* bioencaptulation as a vector to deliver micronutrients such as vitamins and pigments and bioprotectors (mainly friendly bacteria and yeast species, also called probiotics), as well as medicinal plant extracts (Langdon, et. al., 2008; Ngo Van Hail et. al., 2009; Touraki, et. al., 2012, among others). To date, companies such as INVE are offering new alternatives to improve the quality of cysts selection and high quality brine shrimp nauplii and gnotobiotic *Artemia* (Li & Gatlin, 2004; Marques, et al., 2006; Siyavash, et al., 2007). With these new techniques to improving *Artemia* it is possible to deliver specific amounts of particulate or emulsified essentials nutrients to cultured fish via bioencapsulation of these products. Nutritional quality of *Artemia* can be further tailored to suit the specific fish and shell fish larvae species' requirements. The feeding mechanism of brine shrimp enables its use as a vector for delivering different substances to aquatic organisms. *Artemia* enrichment has had a major impact on improved larviculture outputs, not only in terms of survival, growth, and success in metamorphosis for many species of fish and crustaceans, but also in regards to a reduction in incidence of malformations, improved pigmentation, and improved stress resistance (Sorgeloos et al., 2001; FAO, 2012).

Another method to control infectious diseases is the use of vaccines and immune-stimulants. Shellfish and finfish larvae cannot rely on an acquired immune system to combat diseases, but have to rely on the innate immune system consisting of cellular and humoral immunity components (e.g. lysozymes, lysosomal, peroxisomes, enzymes, lectins, or other antibacterial components) (Kurtz & Franz, 2003; Little & Kraaijeveld, 2004). Application of vaccines in adult fish with fully developed immune systems is very

successful (McLauchlan et al., 2003; Irie et al., 2005). However, vaccines are not available for all pathogens, usually involve stressful handling of animals, and are ineffective with most invertebrates and early life stages of vertebrates (Olafsen, 2001). Several products, such as β-glucans, chitin, nanoproteins, peptidoglycans, alginates, and bacterial components are being applied in fish and shellfish cultures, to induce protection against a wide range of diseases (Takahashi et al., 2000; Burgents et al., 2004; Skjermo & Bergh, 2004; Wang & Chen, 2005). The yeast *Saccharomyces cerevisiae*, has been found to be a good immune enhancer in some aquatic organisms (Li et al., 2004), and is an excellent source of β-glucans and chitin to overcome the disadvantages of poorly developed immune systems (Marques et al., 2004; 2006).

The improvement and standardization of the *Artemia* gnotobiotic test system has allowed scientists to study the effect of food composition on survival and growth of *Artemia* in the presence or absence of a pathogen, and has provided a framework to acquire knowledge on host-microbial interactions (Marques, et al., 2004), Marques et al. tested gnotobiotic *Artemia* nauplii in the presence of 10 bacterial strains, combined with four different major axenic live food (two strains of *Saccharomyces cerevisiae* and two strains of *Dunaliella tertiolecta*) with differing nutritional values. The combination with dead bacteria exerted a strong effect on *Artemia* survival reducing or even eliminating bacterial numbers on nauplii culture systems when medium to good-quality major feed sources were used, possibly due to improvements in the health status of *Artemia*. Some probiotic bacteria, such as *Cytophaga* improved the performance of *Artemia* nauplii beyond the effect observed with dead bacteria, independent of the food supplied. These approaches can be used to study the exact mode of action of bacteria and its use as immune stimulants in gnotobiotic *Artemia* for larval defense against pathogens like *Vibrio* species (Marques, et al., 2006; Soltanian, et al., 2007; Siyavach, et al., 2007; Hipolito-Moralez,et al., 2009). Table 2 shows some recent studies with applications of *Artemia* as a biocapsule to deliver micronutrients, vaccines, and probiotics.

There is extensive information on the use of bioencapsulated gnotobiotic *Artemia* in several areas of nutrition (vitamins, pigments,), control of pathogens using bioprotectors (dead or attenuated microorganisms, antitoxins, probiotics, microalgae, extracts of green, brown, and red macroalgae), and plant extracts, such as extract of berberine from

Berberis aristata is used successfully in INVE (cysts from Great Salt Lake, Utah EG type; INVE, 2006), Other potential plant extracts are *Plecthranthus amboinicus* and *Schinus terebintheifolliu* which have properties to kill *Aeromonas* and *Vibrio* in finfish (Silveira, 2006). Table 2 provides some examples of application of *Artemia* as a biocapsule.

Table 2. Alternatives in the use of Artemia as a biocapsule delivery technique, with notable applications, target species, results, and references.

Artemia as a Biocapsule	Application	Culture Species	References
Deliver Technique	**Micronutrients**		
wax spray balls with Low-molecular weight, water-soluble nutrients and antibiotics	vitamin A, vitamin E, astaxanthin, selenium, vitamin C and thiamin & Oxytetracyline	Juvenile Zebra fish , *Danio rerio*	Langdon, C, *et al.* 2008
Bacillus subtilis and *Lactobacillus plantarum against vibriosis*	**Vaccines** Oral vaccination agains *Vibrio anguillarum*	*fish larvae Dicentrarchus labrax*	Touraki, *et al.* 2012
	Probiotics		
Live probiotic bacteria on the early development of the digestive tract of gnotobiotic *Artemia*	*Lactobacillus sporogenes*	Postlarvae *Macrobrachium rosenbergii*	Seenivasan, *et al.* 2012
	Probionts isolated from fish gut		
A urmina nauplii enrichment with yeast and two friendly bacteria	yeast which bacteria isolates from fish gut	*Bluga sturgeon*	Dehghan, *et al.* 2011

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ANNOTATED REFERENCES

Seenivasan C., C., P. Saravana Bhavan, S. Radhakrishnan, R. Shanthi. 2012. Enrichment of *Artemia* nauplii with *Lactobacillus sporogenes* for Enhancing the Survival, Growth and Levels of Biochemical Constituents in the Post Larvae of the Freshwater Prawn *Macrobrachium rosenbergii*. Turkish J. Fish. Aqua. Sci. 12: 23-3

The authors in this study designed and experiment to prove that *Artemia* could be used as vector to deliver probiotics to improve larval digestion and growth. They used *Artemia franciscana* naupli enriched with bacterium, *Lactobacillus sporogenes* and fed the freshwater prawn, *Macrobrachium rosenbergii* post larvae (PL). *Artemia* was enriched with different concentrations of *L. sporogenes*. Enriched *Artemia* produced significantly (P<0.05) higher survival and growth when compared with the control fed with un-enriched *Artemia*. M. rosenbergii (PL) biomass increase, total weight gain, specific growth rate increase, condition factor and mean conversion ratio were found to be higher in PL fed *with L. sporogenes* enriched *Artemia* when compared with control. The enhanced growth performance of PL fed with *L. sporogenes* was further confirmed by the lower food conversion ratio recorded. The levels of biochemical constituents, such as total protein, amino acids, carbohydrate and lipid contents were found significantly higher (P<0.05) in *M. rosenbergii* PL fed on L. sporogenes enriched *Artemia* particularly when compared to the un-richer control. The authors concluded that cells of *L. sporogenes* using *Artemia* as

a vector can be considered as suitable probiotic for attaining good survival and growth of *M. rosenbergeii* post larvae.

Soltanian Siyavash, Jean Dhont, Patrick Sorgeloos, Peter Bossier. 2007. Influence of different yeast cell-wall mutants on performance and protection against pathogenic bacteria (*Vibrio campbellii*) in gnotobiotically-grown *Artemia*. Fish & Shellfish Immunology 23: 141-153

In this study was determined the selection of isogenic yeast strains (with deletion for genes involved in cell-wall synthesis) to evaluate their nutritional and immune-stimulatory characteristics for gnotobiotically-grown *Artemia*. A set of experiments showed the nutritional value of isogenic yeast strains (effected in mannoproteins, glucan, chitin and cell-wall bound protein synthesis) for gnotobiotically-grown *Artemia*. Yeast cell-wall mutants were always better feed for *Artemia* than the isogenic wild type (WT) mainly because they supported a higher survival. The difference in *Artemia* performance between WT and mutants feeding was reduced when stationary-phase grown cells were used. These results suggest that any mutation affecting the yeast cell wall make-up is sufficient to improve the digestibility in *Artemia*. The second set of experiments, investigates the use of a small amount of yeast cells in gnotobiotic *Artemia* to overcome pathogenicity of *Vibrio campbellii* (VC). Among all yeast cell strains used in this study, only mnn9 mutant yeast (less cell-wall bound mannoproteins and more glucan and chitin) seems to completely protect *Artemia* against V C. The result with these mutants is of particular interest, as its nutritional value for *Artemia* as comparable to the wild type. The results of this study suggest non-interference of *Artemia* general nutritional effects.

Shadrin Nickolai, Elena Anufriieva, Ekaterina Galagovets. 2012. Distribution and historical biogeography of *Artemia leach*, 1819 (Crustacea: Anostraca) in Ukraine. Int. J. *Artemia* Biol. Vol. 2, No 2: 30-42

In this study the authors described the recent information about the biogeography of *Artemia* in Ukraine. The authors identified the following areas with potential for aquaculture: The Crimean Peninsula; the coastal zone of the NW Black Sea; the north coast of the Sea of Azov; Donetsk Oblast; Zakarpattia Oblast. The authors recognized that there are at least two bisexual species (*A. salina* and *A. urmiana*) and the parthenogenetic complex populations of *Artemia* dwell in the Ukraine. In some lakes (Koyashskoye, Terecly-Konradskoye) they found *A. urmiana* and parthenogens both, but the two species separated in time. In the waters of the Crimea and NW part of the Black Sea brine shrimp are found under salinities from 10-20 ppt (if no other animals present) to 350 - 370 ppt. Larvae and adults of some Coleoptera, Hemiptera, ostracods *Eucypris inflata* (Sars) were found, and probably other invertebrates may consume *Artemia* completely in the Crimean lakes whose salinity is below 60-110 ppt. Exploitation of salt can create new habitat for brine shrimp. Ukraine hypersaline ecosystems are very promising opportunities for industrial harvesting of *Artemia* cysts.

Cohen Rosa Graciela. 2012. Review of the biogeography of *Artemia* Leach, 1819 (Crustacea: Anostraca) in Argentina. Int. J. *Artemia* Biol. Vol. 2, No 1: 9-23.

In this study the author provides updated review of the biogeography *of Artemia* in Argentina and other countries in South America. She describes two species of *Artemia* in these countries: *A. franciscana* widely distributed throughout the Americas and the Caribbean, and *A. persimilis* with a more restricted distribution in southern South America (Argentina, Chile, Peru, and Bolivia). In Argentina *A. franciscana* is located in the Buenos Aires Province. *A. persimilis* is from: La Pampa and Santa Cruz Provinces. The southernmost record of the species corresponds to Laguna de los Cisnes in the Chilean Tierra del Fuego. Temperature is considered as a main factor in the determination of the present geographic distribution of both species in these countries. *A. franciscana* and *A. persimilis* are considered as endemic species to the Americas. However, in recent decades, *A. franciscana* was intentionally introduced in several countries of the New and the Old World, mainly associated with aquaculture. Two African populations recently were assigned as *A. franciscana* suggesting other natural events raising the hypothesis that the original geographic distribution of this species was not only restricted to the Americas. On the other hand, *A. persimilis* was described for the first time in Salinas Grandes de Hidalgo, Argentina and in Europe from the Italian population of Saline di San Bartolomeo, Sardinia. *A. persimilis* (now extinct) co-occurred with *A. salina* in the later location. The presence of several *Artemia persimilis* populations at high-altitude (above 4,500 m) in the South America Altiplano could lead to an hypothesis that suggest that in the geological past,

A. persimilis would have retreated to cooler and higher altitudes and latitudes, leaving isolated populations in Andean enclaves.

Torrentera Laura and Stanley I. Dodson. 2004. Ecology of the brine shrimp Artemia in the Yucatan, Mexico, Salterns. Journal of Plankton Research. Vol 26:(5) 1-8.

In this article the authors described the biogeography and ecology of the brine shrimp Artemia across the Yucatan peninsula. They recorded monthly field data during one year at the North Coast hypersaline ecosystems: temporary pond, pools, salterns and saltmarshes. The Yucatan Peninsula is a great limestone platform projecting from Mexico and Central America northward into the Gulf of Mexico and the Caribbean Sea. The most important ports sampled included: Celestun, at the extreme north west of the peninsula and Chuburna and, Xtampu, at the intermediate area and, Las Coloradas, at the extreme northeastern part of the peninsula. The salinity ranges from that of seawater (35 g/L) to about 150 g/L or more. The locations revealed high extreme alkalinity, salinity and temperature conditions, and hypoxia and in some instances, total desiccation. The four locations were similar in water temperature, nitrites, and phosphates, but differed in salinity, oxygen content, water depth, nitrates, ammonia, silicates, carbonates, sulfates, and pH. The dominant multicellular organism in these habitats is Artemia, the brine shrimp. The brine shrimp in the Yucatan are bisexual and polyploidy populations. The Yucatan brine shrimp show differences in chromosome number and conspicuous morphological differences among them and also between the wide distributed A. franciscana species. Further investigation is needed to determine the taxonomic name of these populations; in the midterm the Yucatan brine shrimp populations has the status of Artemia sp. The Yucatan brine shrimp abundance and population dynamics were significantly correlated with specific environmental conditions. The annual natural production mean value of Artemia Biomass calculated in the sampled area was 450 D. W. (g/square meter). The cyst production mean D. W. value was 733 (g/square meters). The field study provides ecological basis for the exploitation of Artemia as a live food that possess good nutritional value for larvae culture of finfish and crustaceans.

These hypersaline ecosystems should be protected because they are nesting and feeding areas for the Caribbean flamingo and other marine birds.

Ngo Van Hai1, Nicky Buller, Ravi Fotedar, 2009. Encapsulation capacity of Artemia nauplii with customized probiotics for use in the cultivation of western king prawns (Penaeus latisulcatus Kishinouye, 1896). Journal Compilation © Blackwell Publishing Ltd.

In this study the authors take advantage of the progress in the technology of Artemia encapsulation capacity and designed a bioencapsulated Artemia nauplii with customized probiotics; Pseudomonas synxantha and Pseudomonas aeruginosa for use in the cultivation of western king prawns (Penaeus latisulcatus). They set up seven trials to investigate this encapsulation capacity in terms of Artemia survival and probiotic load in Artemia. Newly hatched Artemia nauplii were raised at 250 nauplii /mL, then, they were fed individual probiotics at 0, 103, 105 and 107 (CFU/mL) and mixtures of these two probiotics (105 CFU/mL) at 30:70, 50:50 or 70:30 v/v in a medium of ozonated water (OW), tryptone soya broth (TSB), and a mixture of these media. In their results they found that the appropriate medium for encapsulation of probiotics by Artemia nauplii was the mixture of OW and TSB at 75:25 v/v; whereas, the use of OW or TSB alone was not effective. Artemia nauplii most effectively encapsulated the customized probiotics at 105 CFU/mL. The authors concluded that the encapsulation of Artemia nauplii is optimized by using a combination of P. synxantha and P. aeruginosa at 50:50 v/v in a media mixture of OW and TSB at 75: 25 v/v. Artemia should be harvested at 48 h when survival is still high (78%) and the probiotic load in Artemia is high (3 × 104 CFU nauplius−1).