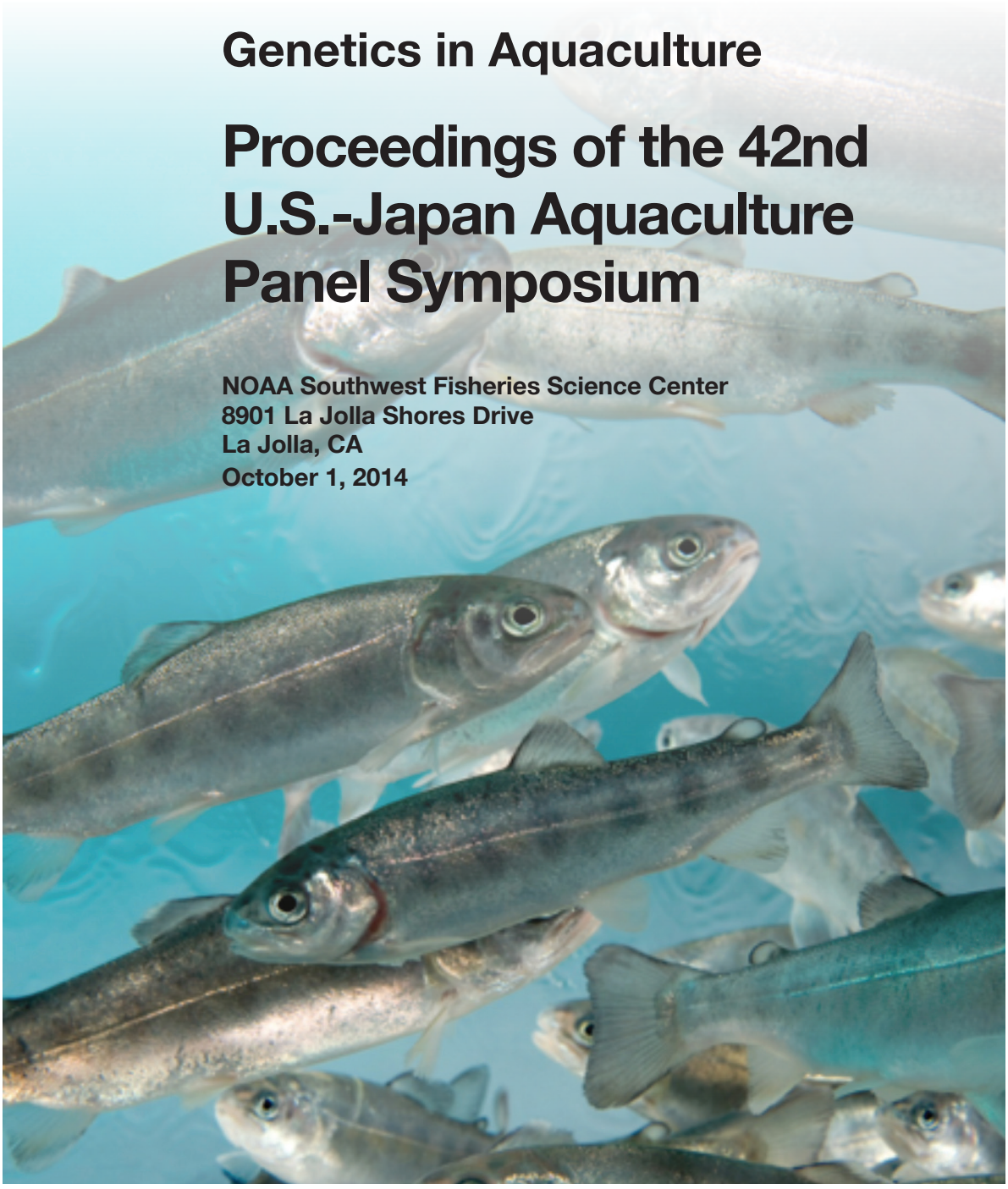

Genetics in Aquaculture

Proceedings of the 42nd U.S.-Japan Aquaculture Panel Symposium

NOAA Southwest Fisheries Science Center
8901 La Jolla Shores Drive
La Jolla, CA
October 1, 2014

U.S. DEPARTMENT OF COMMERCE
National Oceanic and Atmospheric Administration
National Marine Fisheries Service

NOAA Technical Memorandum NMFS-F/SPO-168

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Cover photo of juvenile rainbow trout raised in a tank at the ARS National Center for Cool and Cold Water Aquaculture in Leetown, West Virginia. Scientists there have been working to breed resistance to bacterial cold-water disease into rainbow trout. Courtesy of Stephen Ausmus, USDA Agricultural Research Service. Image Number D1946-4

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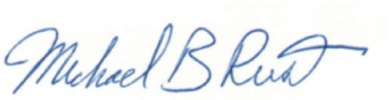
Introduction

Exciting new developments in genetics and genomics contribute significantly to advances in aquaculture production today and will be of even greater importance in the future. Genetic improvements through selective breeding, genetics and health management, understanding genetic interactions of wild and cultured stocks and genetics and climate change are all research priorities of the Japanese Fisheries Research Agency, the National Oceanic and Atmospheric Administration and the United States Department of Agriculture. The primary focus of this symposium will be on solving production problems faced by the aquaculture industries of the two nations using genetic and genomic approaches. This will facilitate development of more competitive new and existing aquaculture industries. Selective breeding has a long history of improving production traits in many livestock species and is having progressively greater impacts in aquaculture; enhancing genetic improvement through application of genomics can significantly accelerate this process. In health management genetic approaches to improve resistance, enhance immune response, better understand pathogens and improve vaccines are all tools to strengthen the aquaculture industry. Climate change will present many challenges to aquacultured species and genetics and genomics will prove to be valuable tools in addressing these. This Symposium will include a workshop component for broad ranging discussions of some of the new approaches being developed and how they might be applied in collaborative research efforts to resolve key bottlenecks and facilitate aquaculture industry expansion in Japan and the United States.



Michael Rust,
NOAA Fisheries Office of Aquaculture
United States Panel Chair

Plans, Challenges and Opportunities for Warm Water Aquaculture and the Role of the Southwest Fisheries Science Center

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ABSTRACT

Each region of the US EEZ provides unique challenges and opportunities for marine aquaculture. Access to markets, regulatory frameworks, competing ocean uses, water quality and prevalence of extreme weather events are all part of considerations for the development of a successful industry. The southwest portion of the western US coastline and adjoining waters in Mexico are characterized by: a. minimal continental shelf habitat, b. low terrestrial runoff and nutrient loading, c. reliable water exchange via currents, upwelling and tidal mixing, and d. low prevalence of extreme weather events. Natural features of the southwest US coupled with a diverse and educated urban population provides economic opportunities for the marketing of high value aquaculture products. It also requires strict adherence to the highest standards of seafood quality and a minimum of adverse environmental impacts. Siting of aquaculture facilities in federal waters (beyond 3 miles) provides access to high quality habitat but is limited by the availability of locations suitable for moorings.

The Southwest Fisheries Science Center (SWFSC) is interested in partnering with private entrepreneurs, academic researchers, and State and international regulatory partners to explore opportunities to increase seafood supply, economic activity and job creation while minimizing the impacts to the marine ecosystem. The SWFSC and academic partners are particularly strong in Fisheries Oceanography, Protected Resources Monitoring, Biotechnology and Economics.

In this presentation we will discuss current and planned research opportunities and how they relate to site selection, impacts evaluation and brood stock improvement. The California Cooperative Fisheries Investigations (CalCOFI) has maintained an ocean-observing program that has routinely sampled water conditions in the California Current since 1949 and is providing baseline information and predictive modeling data for site selection and evaluation of impacts. Likewise the Southern California Ocean Observing System (SCOOS) is providing real-time observations for impacts evaluation. Along with site impacts, successful aquaculture must document and maintain natural genetic structure and genetic diversity while addressing issues associated with disease, parasitism and artificial diet in culture. Genomic approaches are being developed for abalone species and *Seriola* species to provide a baseline record of natural genetic variance and a means of monitoring genetic changes in brood stocks. Economic studies focus on individual economics but more important evaluate systemic impacts and also explorations of the "transfer effect", the ecological and protected resources consequences of our reliance on wild capture seafood imports. Protected resources surveys provide information on migratory pathways and areas of high occupancy and can be used to guide site selection and monitor interactions via passive and active acoustics.

The Strategy for Breeding Study of Fisheries Organisms by Fisheries Research Agency (FRA)

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Keywords: selective breeding, Fisheries Research Agency of Japan, marker assisted selection, assisted reproductive technology, genetic resources, pedigree construction, genetic improvement

ABSTRACT

Recent progresses of seed production techniques and of molecular biological techniques are increasing expectations to genetic improvement of cultured species, that is facing high price of feeds, high cost for disease prevention, low farm gate price and many other problems. For effective and efficient promotion of aquatic breeding studies which lead to accurate measures against problems in actual culture sites, the Fisheries Research Agency of Japan (FRA) announced the "Strategy for breeding study of fisheries organisms" on March 2013, through several discussions and cooperation with prefectural institutes, universities, governmental officials and private corporations. The strategy consists of two parts, namely, "Present situation of research and development of aquatic breeding study" and "Direction and policy for promotion of aquatic breeding study". We introduce the latter parts of the strategy in this paper. As a fundamental concept, we should decide on priority in industrially large-scale and widely spread species, while strains adapting to regional environments are expected to be made by each prefectural institute. Development of fundamental technology, evaluation methods of traits and preservation methods of genetic resources are thought to be necessary as schemes for promotion of aquatic breeding. In addition, improving cooperative system of R&D, provisions for protection of intellectual property and consideration to natural environment are thought to be necessary to enhance improved strains in actual culture sites. We introduced the outline of the strategy for breeding study.

INTRODUCTION AND DISCUSSION

Fisheries Research Agency (FRA) made the strategy for breeding study of fisheries organism in 2013 (Fig. 1). This strategy is expected with dissolving many problem of aquaculture, by proceeding breeding study for improving economic condition of aquaculture farm and activating the industry. Mainly, the scheme of the strategy is consisted of two parts, "Present situation of research and development of aquatic breeding study" and "Direction and policy for promotion of aquatic breeding study" (Fig. 2). At first, we introduce the past and current aquaculture in Japan, and the outline of the strategy for breeding study of FRA.

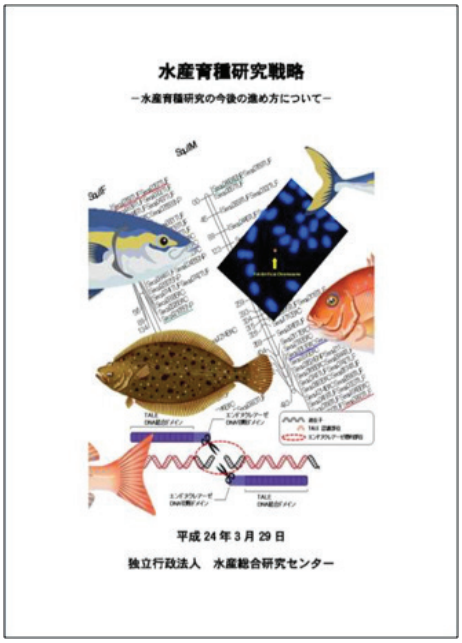

Figure 1. Publication of "The strategy for breeding study of fisheries organisms".

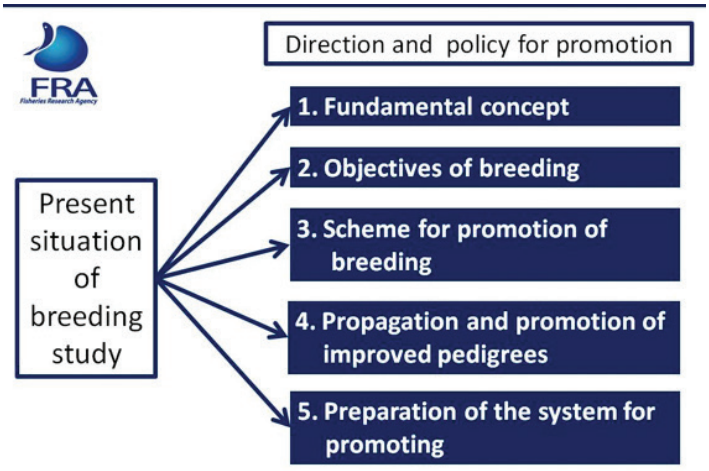

Figure 2. The scheme and contents of "The strategy for breeding study of fisheries organisms".

Past and current aquaculture in Japan

History of fish breeding in Japan

Beginning of fish breeding in Japan could be started, at least from Edo era or before. It has started at introduction of goldfish, and ornamenta carp as Nishiki-goi, in Japanese. These are transferred in Japan from China. Selective and cross breeding of these fish started at least in mid-18th century. Goldfish was getting in popular as a pet during 19th century. For edible fish breeding, it has started by rainbow trout (RT) derived from California, USA, in 1877, after "Meiji Restoration" when it had started to modernize Japan's social system. Selective, cross and polyploidy breeding of RT has been operated to acquire disease resistance, fast growth and several other traits. About salt water fish, in 1960's, almost a hundred years later from the introduction of RT, selective breeding of red seabream *(Pagrus major)* has begun for growth, color and body shape by Kindai University. After over 40 years of selection, the culture period to gain 1kg body weight had reduced to one and half year from three years. Now, this selective strain is the most popular pedigree in Japanese marine aquaculture. Later, many marine and fresh water fish had been started to research for breeding study.

Present situation of aquaculture in Japan

The amount of total aquacultural production was having a peak as 1.43 million ton in 1988, and going down to 1.07 million ton until 2012 (Fig. 3). The reason of this decline was estimated to high price of feeds, high cost for prevention of disease, low farm gate price and severe competition with foreign products. However, the ratio of production amount of aquaculture to total fisheries production is going up to 22 % in 2012, twice as that of 1988 (Fig. 3). Thus, the importance of aquaculture in Japan is getting higher in these years.

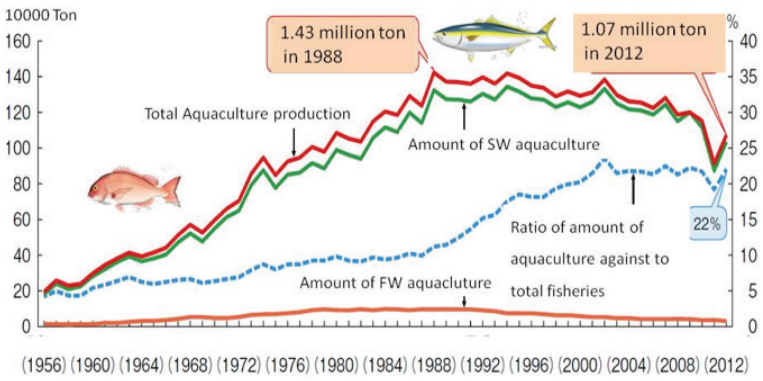

Figure 3. Transition for production amount of aquaculture in Japan, and the ratio of production amount of aquaculture against to total fisheries production including in aquaculture and fishery.

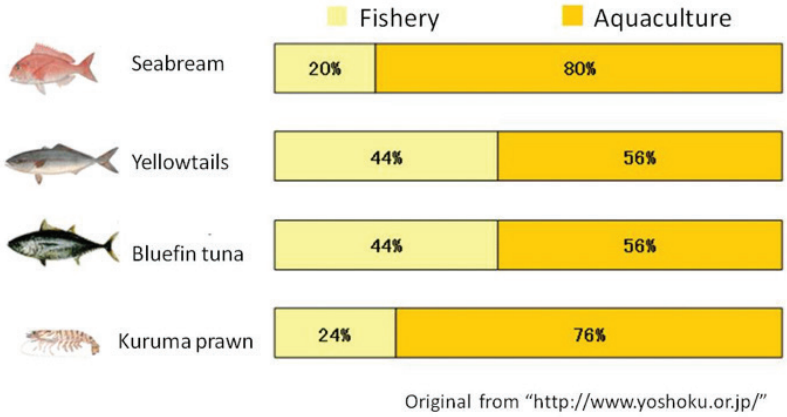

Figure 4. Ratio of production amount of aquaculture and fishery in Japan.

The ratio of production amount of aquaculture and fishery in 2011 was different depending of fish species (Fig. 4). Seabream aquaculture are operated in a full-life cycle. On the contrary, yellowtail and bluefin tuna seeds are almost originated from natural resources. The production amounts of these fish are rather high compare to that of fishery. In 2013, yellowtail *(Seriola quinqueradiata)* occupy 44%, red seabream 23% and greater amberjack *(Seriola dumerii)* 16% in total production amount of aquaculture (Fig. 5). These three species are very important in Japan, because they occupy over 80% of 240 thousand tons of total aquaculture production.

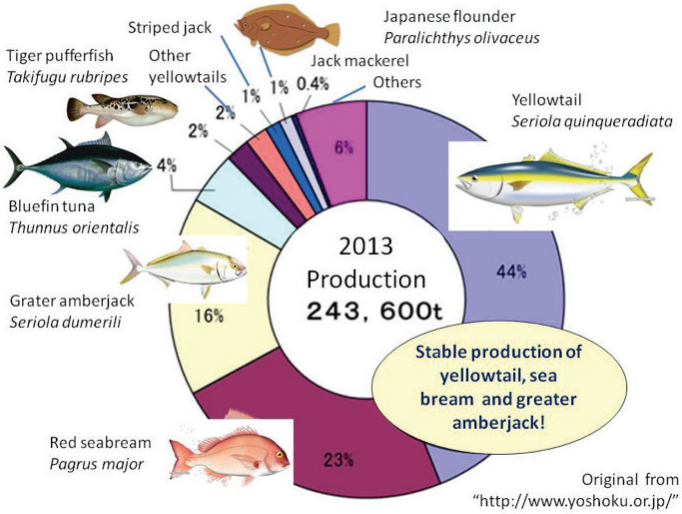

Figure 5. Production amounts of major aquaculture fish in Japan.

However some fish species are still under construction of full-life cycle aquaculture system, e.g. yellowtail, greater amberjack, longtooth grouper *(Epinephelus bruneus)*, bluefin tuna *(Thunnus orientalis)* and Japanese eel *(Anguilla japonica)*. Some of them already established the "Systematic study of seed production on fish farming and aquaculture", e.g. red seabream, Japanese flounder *(Paralichthys olivaceus)* and pufferfish (Takifugu rubripes), which achieved

some "Progress of molecular biological technique". They are expected to fulfill genetic improvement to contribute for aquaculture industry.

Direction and policy for promotion of aquaculture breeding study in Japan

Fundamental concept

About fundamental nationwide concept, we will focus on the priority based on the size of industry or the size of influence, widely or not. In this sense, important objective fish are yellowtails, Japanese flounder, groupers, Japanese eel, bluefin tuna and salmonids in finfish; for seaweeds, Laver as Nori in Japanese name; for shellfish, shortneck clam, pacific oyster and other bivalves; and for shrimp, Kuruma prawn are important species in Japan aquaculture industries. For regional or prefectural breeding, they need to develop strains adapting to regional environment by prefectural institute. In both cases for nationwide and regional or prefectural breeding, they should develop pedigrees quickly in consideration for practical use.

Objectives of breeding

Making a breeding plan for which species and what trait should be improved are very important to start breeding. However, in Japan's aquaculture, there are many species which product amount is small, except for yellowtails or red seabream, there are a few common trait for breeding for all species. Therefore, a breeding plan should be determined in each species depending on the case.

In this part, we categorized fundamental four objectives for breeding.

1. Improvement of administrative character: fast growth, high food efficiency, easy to handle, etc.
2. Improvement of environmental adaptability: tolerance to high temperature, low oxygen concentration, harmful algae, etc.
3. Improvement of economical character: high productivity of flesh, high concentration of functional ingredient, fine appearances, etc.
4. Improvement of disease resistance: strength of fundamental immunity, resistance to several pathogens, etc.

Scheme for promotion of breeding

To efficiently promote aquaculture breeding, we need to research and development of many breeding related methods and technologies, and to prepare rearing and testing facilities. We summarized the important R&D study to promote breeding at the point of a long-term viewing.

1. R&D of fundamental breeding technology
   Marker-assisted selection (MAS) is one of the most powerful and practical methods to promote breeding. Meanwhile, new breeding techniques is been trying to apply vigorously all over the world.

   i) R&D of DNA marker and MAS
   - Development of DNA markers for each species using next generation sequencer (NGS)
   - Making of genetic linkage map, QTL analysis of quantitative traits, analysis of correlation between phenotype and DNA polymorphism, etc.
   - Acceleration of linkage analysis

   ii) R&D of new breeding techniques
   Genome editing using artificial nuclease as TALEN or CRISPR/Cas (defined below) is getting widely and rapidly spread to use every fields of life science. It will be a powerful tool not only to analyze the function of interested gene, but also to make a basic pedigree having a modified gene function for making an applied strain for commercial use by crossing.
   - Development of artificial mutagenesis techniques, e.g. random mutagenesis known as TILLING (Targeting Induced Local Lesions In Genomes), genome editing as TALEN (Transcription activator-like effector nucleases) or CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat)/Cas9 (CRISPR associated protein 9), etc.
   - GMO (Genetically modified organism) is still powerful method to give a new gene function as long as the purpose is for benefits

   iii) R&D of assisting technology for breeding
   - Development of new sterility technique of cultured fish: it could be useful to reduce the risk of breeding strain escaping into natural environment
   - Promotion of surrogate propagation of cultured fish: it will be a useful tool for establishing strains in future

2. Development of evaluation methods for traits
   To make a useful pedigree having a commercial trait, it is important to evaluate a useful trait correctly and ideally.
   i) Evaluation method of fast growth
   ii) Evaluation method of disease resistance
   iii) Evaluation of other trait

3. Development of pedigrees
   Genetic resources for development of new pedigrees in prefectural institute or private company should be bred for their aim by themselves. Especially, marine and fresh water fish will be produced pedigrees with fast growth or disease resistance.

4. Development of preservation methods of resources
   Keeping many genetic resources and evaluating of pedigrees are essential to promote breeding. We focused on the genetic resource species from our main aquaculture species. We will keep rearing such resources, if possible, and develop a method to preserve for the species that cannot be kept as pedigreed lines.
   - Keeping strains for long time is the job of the Public Preservation Center (described below)
   - Developing the method of cryopreservation and restoration techniques of the germ cells

Propagate promotion for improved pedigrees

We just pointed out several essences of propagate promotion for improved pedigrees.

- Adjust role-sharing of each institute or company from the start of development
- Confirm prospective policies of pedigrees as intellectual property
- Patent law, trademark law, measures against outflow, traceability, etc.

Preparation of promoting system for aquaculture breeding

1. Organization of "R&D Center for Aquatic Breeding"
   In FRA, "Research Center for Aquatic Breeding" was established in 2014. To proceed producing useful pedigrees or strains requested from various customer and manufacture needs, we need to promote breeding by widely combining outcomes from genetics, molecular biology, physiology, nutrition, systems engineering and etc. For that purpose, we need an organization and a system including research facility and researchers, like as R&D Center for Aquatic Breeding, to promote such a modern breeding.

2. Organization of "Preservation Center of Genetic Resources"
   To disperse of risks of loss of genetics resources and pedigrees, we need Preservation Center of Genetic Resources, which are settled in multiple places in Japan, because aquaculture species are originated from various environments as in northern (cold) and southern (warm) area, or in marine and freshwater. It is favorable to use FRA research facilities in all Japan for preservation of genetic resources.

3. Improving cooperative system of R&D
   To reinforcement of cooperation with prefectural institute, universities and private company, it is important to make a system to exchange the information of each organization through the department for social cooperation, about how to utilize the products of bred strains, from at the beginning of breeding program to at the stage of transferring the technology to industry.

4. Protection of intellectual property and consideration to environment
   We don't have a law to protect the aquaculture breeder's right except for seaweed. To protect their right, the only way is to make the pedigree sterile, and to register a record to the information of breeders in a public database for breeding information. We need to establish the technology and the database system.

   Also regarding established pedigrees, we need to manage them not to escape to nature, because of their genetic profile should be biased during breeding.
   - Protection of right of breeder
   - Preventive measures against genetic disturbance in natural area

Final notes

Japan has 130 years of history of aquaculture about seeds production and raising techniques. FRA will reinforce the cooperation with prefectural institute, universities and company in accordance with "the strategy for breeding study of fisheries organisms". FRA will promote to develop DNA marker assisted selection, reproduction techniques and new breeding technologies, vigorously.

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Ozaki, A., W. Kai, J. Aoki, K. Nomura, K. Araki, K. Yoshida, T. Tsuzaki, T. Sugaya, M. Sano, K. Kobayashi-Fuji, T. Sakamoto and N. Okamoto (2012). Application to the genetic breeding using by genomics information in Aquaculture. Fish Genetics and Breeding Science, 41, Issue 2, 173-178.

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A sea area of around Japan is one of the fertile areas to produce marine natural resources in the world, the product amount of Japanese fisheries and aquaculture is only covered 60 % of the domestic demand itself. Japanese Fisheries Agency made a plan to raise the food self-sufficiency ratio up to 70% for fin-fish, and 73% for seaweeds in 2022. The domestic aquaculture production is mostly consumed in the domestic consumption, and therefore needs to develop itself to achieve the goal of marine food self-sufficient ratio. However, the business condition of Japanese aquaculture is rather serious, because of hardness to keep fish-meal for feeding from overseas, to save the cost for preventing disease, and to compete against cheaper import marine products. To overcome these serious problems of aquaculture farmer in Japan, Fisheries Research Agency (FRA) made strategy for breeding study of fisheries organisms to achieve genetically improvement, for example, about the adaptation in aquaculture environment, or more suitable products for consumer demand. The establishment of breeding in aquaculture is expected to maintain the amount of domestic production, to enhance international competitiveness and to activate related industries, by reduction of production cost and enhancement of domestic consumption. The strategy is comprised of two parts, one is “Present situation of research and development of aquatic breeding study”, the other is “Direction and policy for promotion of aquatic breeding study”.

Wada, K. T. (2012). Evaluation of parental breeding values in aquaculture – reviews. Fish Genetics and Breeding Science, 41, Issue 2, 87-96. (in Japanese)

The strategies in evaluation of parents and in mating design are important in aquaculture breeding programs. This review deals with topics of evaluating the breeding values of parents in fish and shellfish species of aquaculture. (1) In order to evaluate offspring reared at a communal environment, various chemical or physical tags and molecular markers have been developed for assigning the parentage in mating experiments or in selection programs. (2) Accuracy of selection is a main target in genetic evaluation of parental animals and the breeding value has been estimated in many traits of many species. Methodology like BLUP developed in breeding sciences of livestock can be applied to aquatic animals with some modification. (3) Increased cost and inbreeding rate and decreased genetic diversity are concerns in selective breeding since the number of offspring is much larger in aquaculture breeding program than in those of livestock.

Ozaki, A., W. Kai, J. Aoki, K. Nomura, K. Araki, K. Yoshida, T. Tsuzaki, T. Sugaya, M. Sano, K. Kobayashi-Fuji, T. Sakamoto and N. Okamoto (2012). Application to the genetic breeding using by genomics information in aquaculture. Fish Genetics and Breeding Science, 41, Issue 2, 173-178. (in Japanese)

Ten years ago, genetic breeding study of fisheries organisms was mainly about salmonids because it was the main production of world aquaculture. But now, the aquaculture of marine fish is getting important, the center of breeding study is moving in marine fish. As developing technique of fish seedling, not only the main aquaculture fish species, for example, flounder, puffer fish and yellowtail, but also the hard seedling species like eel and tuna are expected to be applied with breeding study based on genomic information after establishment of complete raising technique in aquaculture farm. We are introducing about studies of Quantitative Trait Loci (QTL) and Marker-Assisted Selection (MAS) in aquaculture fish, and discussing about necessity and possibility of breeding study in aquaculture.

Selective Breeding Research at USDA ARS

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ABSTRACT

There is great potential for intensifying aquaculture production. Selective breeding to improve production efficiency is an important tool and the potential to modify performance traits through selective breeding is considerable. The development of successful selective breeding programs for aquaculture is a long-term commitment. While the gains from selective breeding are permanent and cumulative, initiation of breeding programs often require government support in the early stages, with regular input from industry. The aquaculture program within the US Department of Agriculture, Agricultural Research Service has supported development of numerous selective breeding programs for food fish and shellfish species using a variety of methods including mass selection, family selection and crossbreeding both intra-specific crossbreeding-different strains, same species; and interspecific cross-breeding-different species. I will highlight aspects of the development of genetic improvement programs for catfish, rainbow trout, Atlantic salmon, yellow perch, striped bass and oysters. While growth performance is a trait of primary concern in most programs, other traits have gained importance, too. With catfish, the recent development of methods for mass production of the hybrid catfish (blue catfish male x channel catfish female) has raised the importance of reproductive performance. Atlantic salmon selective breeding programs are some of the most advanced in the world (Norway, Chile, Iceland, Scotland), however, due to their listing as endangered species in North America, continent of origin has become a fundamental criterion for breeding. Disease resistance, the ability to withstand specific pathogens and have higher survival, has been a critical focus for rainbow trout and other species as well. Product aspects such as greater fillet yield are being targeted; and the possibility to selectively breed for greater ability to convert and deposit long chain n-3 fatty acids is an intriguing target for selective breeding, too. In addition to reviewing progress in these projects, I will discuss some of the research and breeding strategies that have been adopted recently to maximize industry relevance and to incorporate genomic information into our genetic improvement programs.

Assessment of Global Population Genetics of *Seriola lalandi:* Implications for Genetic Management in Aquaculture

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Key words: *Seriola lalandi*, cryptic speciation, genetic differentiation, yellowtail, microsatellites

ABSTRACT

On the west coast of the United States, the California yellowtail, *Seriola lalandi*, is considered a great candidate for mariculture due to its high market demand and value. While most yellowtail production relies on capture of wild juveniles and fattening in offshore pens, successful spawning and rearing techniques for yellowtail have been developed at the Hubbs-SeaWorld Research Institute (HSWRI), with the intent to translocate reared individuals to offshore pens to raise to market size. As aquaculture for yellowtail grows here and around the world, the impact of unintentional releases on wild populations has become an increasingly important issue. As such, most future aquaculture projects will likely require a genetic analysis component for the permitting process. This study aims to examine the genetic diversity of yellowtail over both a global scale and more regionally in the California-Mexico region to develop a baseline of the genetic variability in wild populations and to evaluate locally adapted traits for broodstock selection. A total of 260 specimens collected around the Pacific and in the Atlantic and 755 specimens collected in the northeast Pacific were evaluated using 16 nuclear microsatellite markers. Overall, genetic population structure was highly significant at both the global and regional scale (F_{ST} = 0.0858 and 0.0091, respectively). Pairwise comparisons indicated four distinct groups at the global scale: northeast Pacific, northwest Pacific, south Pacific, and South Africa. However, the pairwise results at the regional scale were not as clear but may indicate differences between yellowtail found nearshore versus offshore. Combined phylogenetic analyses using two mitochondrial and four nuclear genes strongly support at least three evolutionarily distinct groups among the global samples, supporting previous taxonomic hypotheses that *S. lalandi* is a complex of three regional species (*S. aureovittata, S. dorsalis, S. lalandi*). By creating the framework for a global genetic diversity monitoring program, substantial progress can be made towards establishing yellowtail as a commercially viable aquaculture species on the west coast.

INTRODUCTION

Seriola species comprise an important component of global aquaculture production, with an estimated annual value of $1.3 billion (FAO 2012). Although aquaculture is seen as a way to reduce fishing pressure on natural populations, culture can also have a significant impact on the health and stability of wild fish assemblages. Transport and production of cultured fish into different geographic locations or into non-local populations creates a threat to wild fish through introduction of non-native genes and/or maladaptive traits into native populations (Hutchings and Fraser 2008; Dann et al. 2010).

Spatial genetic structure has previously been detected among yellowtail sampled from different regions (Nugroho et al. 2001; Miller et al. 2011). In addition to genetic differentiation among populations of *S. lalandi,* factors such as size at reproductive maturity and temporal separation of spawning seasons have recently reignited the question of whether populations of *S. lalandi* may actually be distinct species. Initially, several nominal species names existed in the literature; Valenciennes 1833 described *S. lalandi* (type locality Brazil), and Castelnau 1872 described *S. grandis* from Australia, New Zealand and Chile. *Seriola aureovittata* (Temminck and Schlegal, 1845) was described in Japan, and Gill 1863 described *Seriola dorsalis* from California. Smith-Vaniz (1986, 1990) placed the latter three names (i.e., *S. grandis, S. aureovittata,* and *S. dorsalis*) into synonymy with *S. lalandi* based on a lack of morphometric variation throughout their range and concluded that previous descriptions of distinct species were due solely to the disjunct nature of the populations. Currently, *S. lalandi* Val. 1833 is considered a single species broadly distributed throughout the Indian, Atlantic and Pacific Oceans.

Levels of genetic diversity, migration, and genetic differentiation among the different regions still need

to be resolved for yellowtail. The impact of unintentional releases on wild populations has become an increasingly important issue as aquaculture grows for this species, particularly in light of international trade of hatchery seed. The goals of the present study were to: 1.) conduct a phylogenetic analysis using multiple mitochondrial and nuclear genes to investigate the phylogenetic structure of *S. lalandi sensu lato*, 2.) determine the validity for the current treatment of the yellowtail jack as a single, globally distributed species, and 3.) establish a baseline of existing genetic divergence among populations prior to potential introgression from translocated fish.

METHODS

Specimens of yellowtail were collected from seven locations throughout their range between May 2007 and July 2010. The locations included Japan (JP), California (CA), Baja California Mexico (MX-P or MX-Pacific), the Gulf of California, Mexico (MX-G or MX-Gulf), Chile (CH), New Zealand (NZ), and South Africa (SA) (Fig. 1). For the microsatellite analyses, a total of 288 tissue samples were analyzed: 20 from JP, 58 from CA, 50 from MX-P, 44 from the MX-G, 32 from CH, 34 from NZ, and 50 from SA. A subset of specimens from this sample was sequenced: 10 from JP, 4 from CA, 10 from MX-P, 8 from MX-G, 4 from CH, 2 from NZ, and 4 from SA. Genomic DNA was extracted from tissues using the DNeasy Blood and Tissue Kit (QIAGEN) following the manufacturer's protocol.

For phylogenetic analyses, portions of two mitochondrial genes (cytochrome oxidase I (COI) and control region (CR)) as well as four nuclear genes (enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase (EHHADH), ubiquitin protein ligase E3A (UBE3A), mixed-lineage leukemia-like protein (MLL), recombination activating gene 2 (RAG2)) were amplified by polymerase chain reaction (PCR). Direct sequencing of these regions was conducted using either an Applied Biosystems 3130XL Genetic Analyzer or an Applied Biosystems 3730XL Genetic Analyzer and further aligned and edited using GENEIOUS v4.8.5 (Drummond et al., 2010), SEQUENCHER v4.5 (Gene Codes), and CLUSTALW (Thompson et al., 1994). Phylogenetic trees for *S. lalandi* were built using maximum likelihood (ML) and Bayesian posterior probability analyses using 42 individuals of S. lalandi, with *Seriola dumerili* as an outgroup. Evolutionary model selection was done using JModeltest (Posada, 2008) and phylogenetic analyses were done using MEGA 6.0 (Tamura et al., 2013) and MrBayes v3.2.3 (Ronquist et al. 2012).

Fifteen microsatellite loci developed for *Seriola* (Porta et al. 2009; Renshaw et al. 2007, 2006; Ohara et al. 2005, 2003; Nugroho and Taniguchi 1999) were used to examine population genetic structure in this study. Samples were analyzed on an ABI 3730XL Genetic Analyzer (Applied Biosystems) and fragment data were analyzed using GENEMAPPER 4.0 (Applied Biosystems) and scored visually. Genetic differentiation was calculated using Weir and Cockerham's (1984) F_{ST} estimates in GENETIX 4.05 (Belkir et al. 2004). To assess hierarchical structuring of microsatellite variation among groups, within groups, and within populations, AMOVA analyses (implemented in ARLEQUIN), including both mitochondrial control region sequence data and microsatellite genotypes, were performed. The model-based Bayesian clustering program STRUCTURE v2.3.4 (Pritchard et al. 2000; Hubisz et al. 2009) was used to examine population structure using a maximum likelihood approach and correlation between

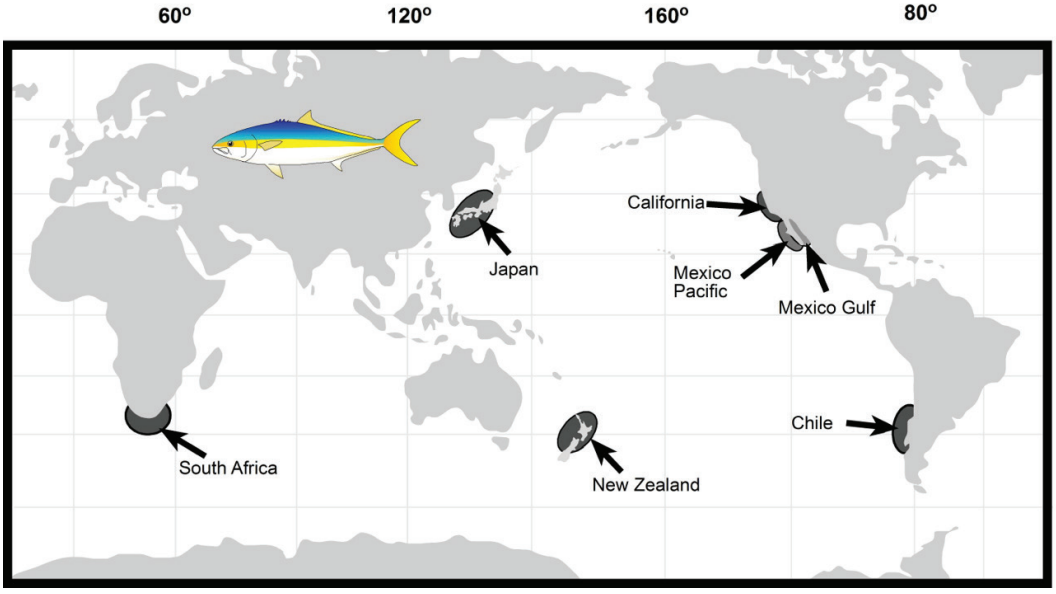

Figure 1. Sample locations of Seriola lalandi, taken from (Martinez-Takeshita et al., in press).

geographic distances (average distance among sampling locations in kilometers) and population structure (F_{ST}) was tested with IBDWS (Jensen et al. 2005).

RESULTS AND DISCUSSION

Spatial genetic structure of yellowtail

Overall genetic differentiation among locations, as revealed by microsatellite data, was highly significant and large (F_{ST} = 0.085; P < 0.001). STRUCTURE results, and pairwise estimates of divergence derived from mtDNA and microsatellite data, support the presence of four significantly differentiated populations in the sampled range corresponding to the N.E. Pacific, N.W. Pacific, S. Pacific, and South Atlantic (Fig. 2).

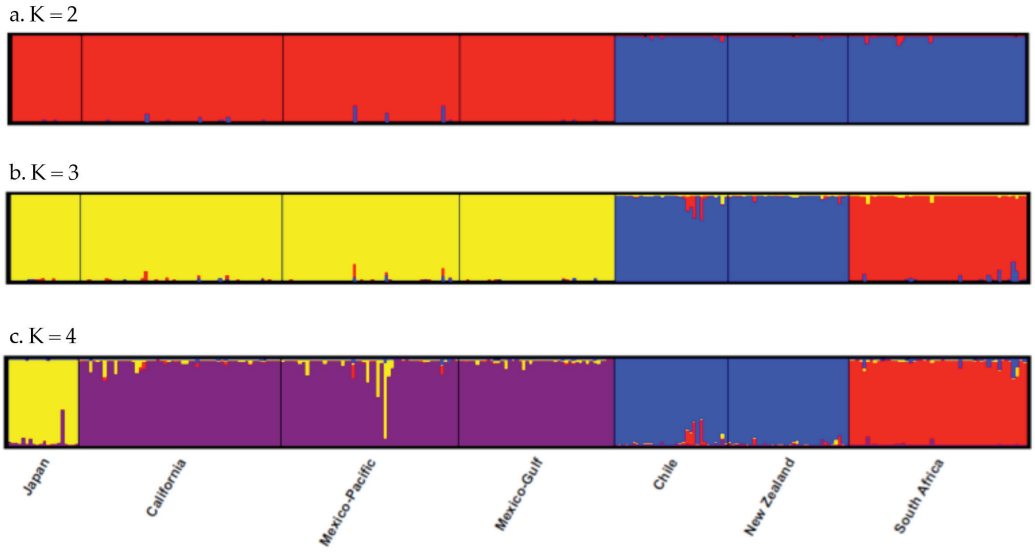

Figure 2. Graphical representations of Bayesian cluster analyses for yellowtail without location as a prior, based on data from 15 microsatellite loci. The three plots show population assignment results for different values of K: (a) K = 2; (b) K = 3; (c) K = 4. Each vertical line represents an individual and its assignment to a particular cluster; solid lines separate the 7 sampled locations. This figure was taken from Purcell et al. (in review).

Genetic structure was especially strong across equatorial regions. For both marker classes, the largest pairwise estimate occurred between the N.E. and S. Pacific. STRUCTURE results showed nearly complete assignment between the northern and southern hemispheres when K = 2. Hemispheric genetic structure may largely be due to thermal restrictions and distinct spawning seasons in each hemisphere. Yellowtail are a temperate and subtropical species found in regions where temperatures range between 18° and 24° C (Fielder and Heasman 2011); equatorial temperatures exceed the thermal range for this species, thereby limiting cross-hemispheric movements (Martinez-Takeshita et al., in press).

Gene flow among N.E. Pacific locations was high relative to the other locations, and pairwise estimates did not detect significant differentiation among these locations. Gene flow between the N.W. and N.E. Pacific appears limited, with significant genetic structure detected between these regions (F_{ST} = 0.0592, 0.0547, 0.0664 with P < 0.001 for CA, MX-P, and MX-G, respectively). In the S. Pacific, gene flow between NZ and CH prevents significant spatial structure from being detected. Strong spatial subdivision was observed between SA and the other Pacific locations (F_{ST} between 0.0775 and 0.1200, P < 0.001 for all comparisons).

Genetic differences among lineages

Phylogenetic and morphological data on the present study of the cosmopolitan yellowtail jack strongly support three clades corresponding to the Northeast Pacific, the Northwest Pacific, and the southern hemisphere (Fig. 3). These findings are consistent with and support the findings from the population genetic analyses based on the mitochondrial sequence data and nuclear microsatellite markers (Purcell et al., in review). Mitochondrial divergence among the three Seriola clades ranged between 4.24% and 4.63%, which corresponds to 2.1 to 2.3 million years of separation among these lineages using the mitochondrial divergence estimate of 2% per million years (Brown et al. 1979; Bowen et al. 2001). In the southern hemisphere, mitochondrial divergence was much lower between the S. Pacific and South Africa (1.19%); by the same calculation, this corresponds to a separation of only 595,000 years.

Nuclear markers were also used to examine divergence among the clades. Estimates of genetic divergence were much lower, 0.15% to 0.31%, however Bayesian posterior and Maximum Likelihood bootstrap values showed strong (93 – 95%) support of the three lineages (Fig. 3). In contrast to the microsatellite data, the nuclear sequences did not reveal additional separation of the South Pacific from the South Atlantic, thus this divergence in the southern hemisphere needs to be investigated in greater depth. There were no fixed sequence differences between specimens collected in the S.

Pacific and South Africa using the nuclear genes, and while the pattern varies slightly between the mitochondrial and nuclear genes, given the low divergence (0.06%) and lack of fixed differences in the nuclear markers, there is inadequate evidence to support these regions as separate species.

Based on the strong phylogenetic structure, it has been demonstrated that three distinct genetic lineages exist within the currently recognized *Seriola lalandi* Val. 1833 species occurring in the NW Pacific, the NE Pacific, and the South Hemisphere (Fig. 4)Pacific and the South Atlantic. We conclude that three cryptic species currently bear the name *Seriola lalandi* Val. 1833. Therefore, we propose the resurrection of the currently available names for these species in each region based on nomenclatural priority: *Seriola lalandi* Valenciennes 1833 in Brazil, South Africa, Australia, New Zealand and Chile, *Seriola aureovittata* Temminck and Schlegel, 1845 in Japan, and *Seriola dorsalis* (Gill, 1863) in California, Pacific Baja, and the Gulf of California, Mexico.

Figure 4

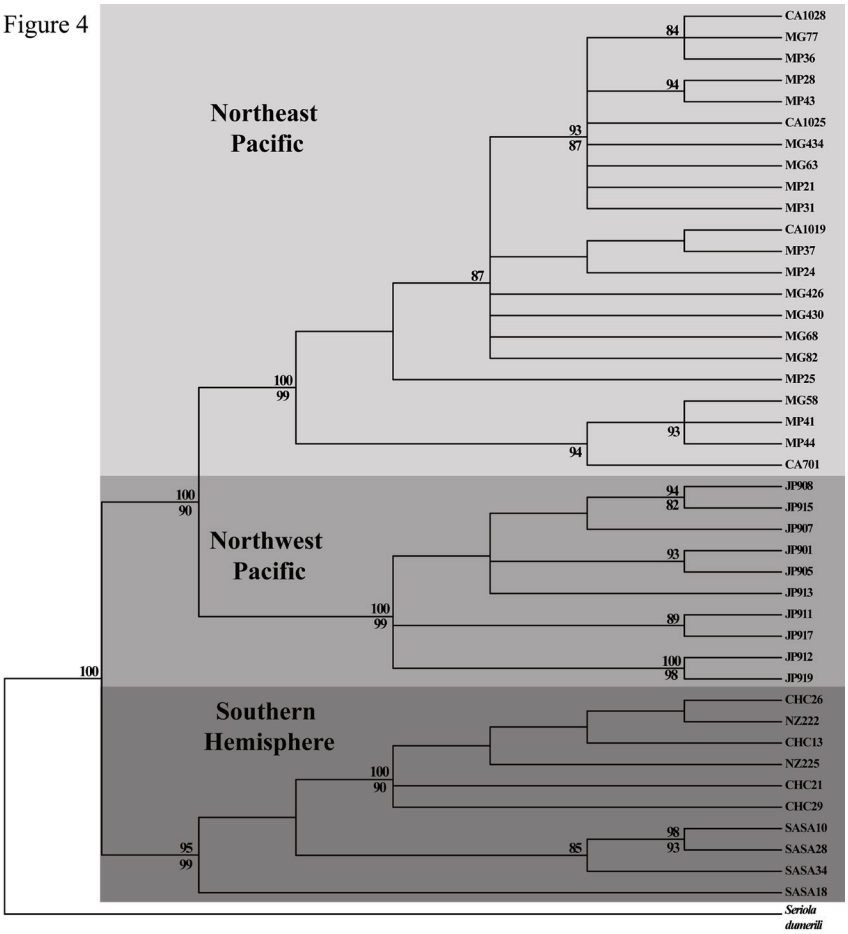

Figure 3. Bayesian derived consensus tree of combined mitochondrial and nuclear DNA sequence data (CR, COI, EHHADH, UBE3A, MLL, RAG2) for Seriola lalandi and rooted with S. dumerili. Specimen designations are JP = Japan; CA = California; MP = Mexico Pacific; MG = Mexico Gulf (Gulf of CA); CH = Chile NZ = New Zealand; SA = South Africa. Values above nodes reflect Bayesian posterior probabilities while values below nodes indicate Maximum Likelihood bootstrap support values, values <80 are not shown. This figure was taken from Martinez-Takeshita et al. (in press).

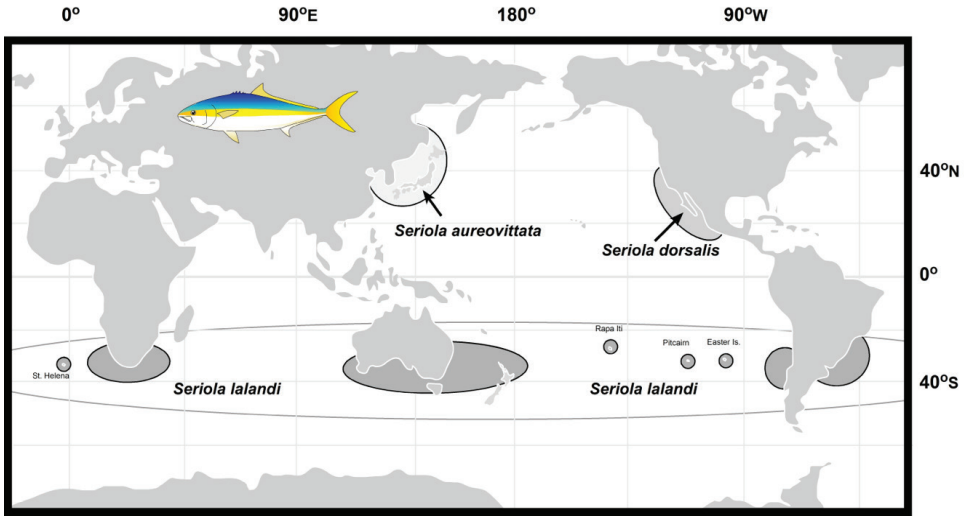

Figure 4. Distributions of proposed species of Seriola formerly referred to as S. lalandi.

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Miller, P.A., A.J. Fitch, M. Gardner, K.S. Hutson, and G. Mair. 2011. Genetic population structure of yellowtail kingfish (*Seriola lalandi*) in temperate Australasian waters inferred from microsatellite markers and mitochondrial DNA. Aquaculture, 319: 328-336.

The authors aimed to determine if there was spatial genetic structure among populations of *S. lalandi* in temperate Australasia. This study was building on research conducted by Nugroho et al. (2001) who found significant variation between the northwest and southwest Pacific, but not within the southwest. A total of 272 specimens were collected from 17 sites in five regions: New South Wales, New Zealand, South Australia, Victoria, and Western Australia. Samples were analyzed using nine nuclear microsatellite markers and a subset of the samples was sequenced at the mitochondrial ND4 region. Both the microsatellite and mitochondrial data indicated that Western Australia was significantly distinct from the other locations. However, no significant genetic structure was detected among the other locations. The authors state that population structure is an important consideration in aquaculture development and should be used to help guide decisions regarding translocation of aquaculture fish among regions.

Nielsen, E.E., J. Hemmer-Hansen, N.A. Poulsen, V. Loeschcke, T. Moen, T. Johansen, C. Mittelholzer, G.-L. Taranger, R. Ogden, and G.R. Carvalho. 2009. Genomic signatures of local directional selection in a high gene flow marine organism; the Atlantic cod (*Gadus morhua*). BMC Evolutionary Biology, 9: 276.

In this study, the authors tested the hypothesis that populations of Atlantic cod show signals of local adaptation to environmental conditions, and therefore show patterns of divergent selection. A genome scan examined variation in 98 gene-associated SNPs to identify outlier loci potentially associated with adaptive population divergence over various geographic scales. A landscape genetic analysis was also used to investigate environmental factors believed to be involved in local adaptation. Results from this study identified eight outlier gene loci that are likely being acted upon by directional selection over a global scale, but regional analyses also revealed seven loci displaying strongly elevated levels of

genetic differentiation. This study is important in considering broodstock selection for aquaculture purposes. Given the very low level of neutral genetic variation (what is measured in most population genetic studies), the number of loci with strong statistical support for selection is surprising. As the authors state, despite being connected by variable levels of gene flow, populations can follow semi-independent adaptive trajectories shaped by their local environments. This is an important consideration in utilizing locally adapted species or populations in aquaculture.

The De Novo Draft Assembly of the Yellowtail, *Seriola dorsalis*, Genome

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Keywords: aquaculture, genomics, broodstock selection, marker assisted selection

ABSTRACT

Seriola species, collectively known as amberjacks, are fish of particular interest to the growing aquaculture industry due to their high value, forming a billion dollar plus component of the sashimi industry. Of these species, the native California *Seriola dorsalis*, is considered a prime candidate for aquaculture development in southern California. In developing aquaculture for this species, methods to improve culture efficiency and effectiveness are of great interest. Genetic resources have been developed and used extensively in agriculture and livestock to improve product quality and quantity, and only more recently have these approaches been applied to select aquaculture species (e.g. rainbow trout, tilapia, catfish, flounder, and Atlantic cod). Previously, technology costs were prohibitive for many species; however accessibility has improved through decreasing sequencing costs and enhanced bioinformatics analyses. With the improved accessibility to this technology, we are working to develop genetic resources for *S. dorsalis* to help improve aquaculture techniques.

In a collaborative effort between the Southwest Fisheries Science Center (SWFSC) and Genome Informatics Facility at Iowa State University, we have created a de novo draft assembly of the genome for *Seriola dorsalis*. DNA sequencing was conducted on juvenile *S. dorsalis* using mate-paired and paired-end reads with the Illumina HiSeq 2500, resulting in approximately 1.2 billion raw reads, for estimated 180X coverage of the 685Mb genome. Two programs were used to assemble the sequencing reads, ALLPATHS-LG (v1.1) and MaSuRCA. MaSuRCA resulted in a greater number of scaffolds (n = 86,357) than ALLPATHS-LG (n = 2,460) and, similarly, more contigs (n = 102,628 and n = 40,683, respectively). However, the longest scaffold size was nearly three times larger using MaSuRCA; 14,669,447 bp versus 5,745,343 bp. The N50 scaffold length was also greater with MaSuRCA (N50 = 2,145,274) than with ALLPATHS-LG (N50 = 900,636), as was the N50 contig length (N50 = 171,508 and 36,582, respectively). Genes were annotated using Maker2. A web portal (Genome Browser) to directly view the genome, gene annotations, gene models, and markers was also created to provide a publically available resource. This study represents the first draft genome for *Seriola dorsalis*. When correctly applied, this genomic approach has the potential to improve broodstock selection through identification of genes underlying complex and/or economically important traits, characterization of variation (both beneficial and detrimental), and marker-assisted selection for this and other *Seriola* species. Investment in the genetic resources for *Seriola* will contribute to making aquaculture practices more economically viable and will help improve domestic seafood production for these valuable finfish.

INTRODUCTION

In the United States, the yellowtail, *Seriola dorsalis*, is a strong candidate for future development of offshore commercial aquaculture in southern California. Although artificial propagation and production from broodstock populations have been successful in yellowtail, production may be further improved by employing the genomic tools and breeding program designs that have already proven successful in other aquaculture species (Dong et al. 2015). In order to develop environmentally friendly and economically sustainable aquaculture, an understanding of the genetic basis of traits that currently limit/enhance development and progress of domestic aquaculture is desirable.

Genomic selection and various genetic techniques are powerful tools for the improvement of plant, livestock, and aquaculture breeding practices (Taylor 2014); they have led to large increases in the rate of genetic improvement in a short amount of time (Goddard and Hayes 2009). In particular, marker assisted selection has the potential to greatly increase the efficiency and effectiveness of culture practices. Compared with traditional breeding practices, marker assisted selection is faster, more reliable, and could help avoid inbreeding depression by not requiring highly inbred family lines to target complex traits (Ozaki et al. 2013). The genomic approaches would also make it easier to identify genes underlying those complex traits, which would not only improve the understanding of these traits but would also be used to benefit culture practices (Goddard and Hayes 2009). Next generation sequencing (NGS) has helped to revolutionize this area of research through decreasing costs and increasing number of research applications (Dunham et al. 2014); this has enabled the development of genetic resources for breeding and culture purposes in a much wider range of species (Huete-Pérez and Quezada 2013).

Only more recently have these genomic approaches been applied to select aquaculture species (e.g. rainbow trout, Atlantic salmon, tilapia, catfish, flounder, Atlantic cod, and the Japanese eel) (Henkel et al. 2012; Ozaki et al. 2012; Terova et al. 2013; Dunham et al. 2014). Genotyping By Sequencing (GBS), Genome-Wide Association Studies (GWAS), and Quantitative Trait Loci (QTL) mapping approaches can be used in aquaculture to identify genetic variation affecting phenotypic traits of interest or other economically relevant qualities; identification of that variation can in turn facilitate efficient implementation in breeding schemes by use of marker assisted selection (Sodeland et al. 2013). These analyses have been used to improve various aspects of aquaculture production and life history traits, such as disease resistance, growth rate, tolerance of environmental stressors, diet/nutrition, reproduction, general health, and other key traits in aquaculture species (Quinn et al. 2012; Cerdá, and Manchado 2013; Huete-Pérez and Quezada 2013; Dunham et al. 2014; Fuji et al. 2014).

We are developing genetic resources for S. dorsalis to help improve aquaculture techniques. In a collaborative effort between the SWFSC, the Genome Informatics Facility at Iowa State University and the Hubbs-SeaWorld Research Institute, we have created a de novo draft assembly of the genome for *Seriola dorsalis*. The genomic tools and resources created in this project will provide a much-needed framework for researchers, hatcheries and producers to begin using marker-assisted selection in *Seriola* culture practices. This framework is indispensable in working to overcome the limitations that currently hinder rapid growth of hatchery and offshore cage production for *Seriola* culture in the continental U.S. and Hawaii. Developing these genetic resources and tools are necessary steps for making broodstock-based aquaculture of *Seriola* sustainable and economically feasible.

METHODS

DNA was extracted from a single juvenile yellowtail *(S. dorsalis)* (approximately 50 days post hatch) reared at Hubbs-SeaWorld Research Institute (HSWRI). The purified DNA from the single fish was sent to Iowa State for sequencing on the Illumina HiSeq 2500. Four lanes were used to sequence the *S. dorsalis* genome: a 150bp paired end lane, a 2,000bp mate pair lane, an 8,000bp mate pair lane, and a 12,000bp mate pair lane. This sequencing generated 1.2 billion raw sequencing reads providing 180X coverage for the genome. Sequence quality was assessed using FastQC (v 0.10.1). Since the quality scores were satisfactory no trimming was performed. To assemble the raw reads for *S. dorsalis*, two assembly programs were used, ALLPATHS-LG (1.1) (Gnerre et al. 2011) and MaSuRCA (2.1) (Zimin et al. 2013; Neale et al. 2014). ALLPATHS-LG (1.1) is an older program but is still considered one of the better programs that specializes in assembling shorter sequencing reads (such as the ones generated by the Illumina HiSeq). MaSuRCA (2.1) is a newer assembly program that specializes in generating longer super-reads from the shorter sequences. The genome was assembled using both programs to determine which assembly method generated a better and more complete de novo draft genome. MaSuRCA assembly pipeline uses QuORUM kmer based error correction to correct raw reads. From these error corrected reads, super reads were generated by extending short reads on either ends. For the average reads size of 106bp, kmer size of 74 was used for graph construction. The super reads along with the filtered long linking reads were then used with the CABOG assembler (Miller et al. 2008) to generate the final assembly. CABOG assembler was configured to use default parameters except for scaffolder error rate (cgwErrorRate) of 0.15 and overlap kmer size of 30. Maker2 (Holt and Yandell 2011) was used for gene annotation. Maker was initially run with just the evidences (using protein/

gene sequences from closely related species). With the initial crude predictions, SNAP (v2013-11-29)(Korf 2004) hmm were generated. GeneMark (v2.3) (self-trained) (Borodovsky and Lomsadze 2011) hmm's were also generated using only the scaffolds. Revised gene predictions were carried out using hmm's from GeneMark and SNAP. These revised gene models were then used to train AUGUSTUS (v2.5.5) (Stanke et al. 2006) and final gene predictions were called using AUGSUTUS, SNAP, GeneMark and FGENESH (v20150830) (Softberry Inc.,) (using Cynoglossus matrix). The genome and the gene models were then loaded as tracks on local, and GBrowse (v2.0) (Stein 2013) was used for visualization.

RESULTS AND DISCUSSION

In the genome assembly, the longest contiguous sequence (a.k.a. contig) MaSuRCA generated was almost three times the length of the longest ALLPATHS contig (Fig 1), and the scaffold was more than twice as large as the ALLPATHS longest scaffold (Fig. 2). For the total number of scaffolds, the MaSuRCA assembly ended up with 700 fewer scaffolds than the ALLPATHS assembly did (Fig. 3). The most widely used measure to describe genome assemblies is the N50 length of scaffolds or contigs, which is essentially a weighted mean, favoring the longer (or more informative) contigs or scaffolds (Baker 2012; Adams 2003).

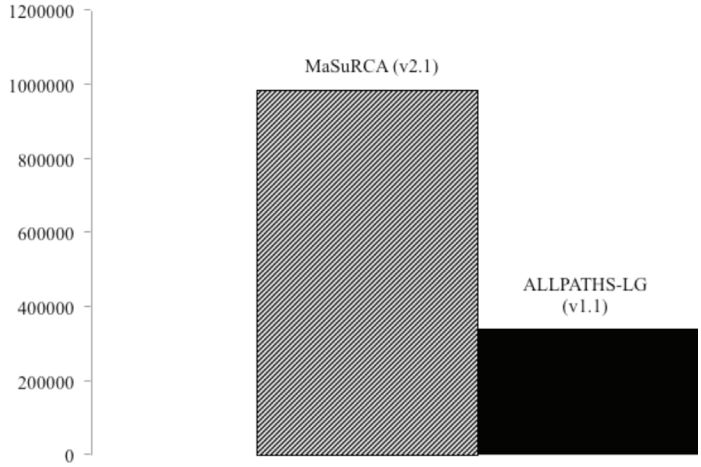

Figure 1: Longest contiguous sequence in the S. dorsalis draft genome using two assembly programs, MaSuRCA (2.1) and ALLPATHS-LG (1.1).

The N50 contig length using MaSuRCA was 141,285bp and for ALLPATHS-LG it was 36,582bp (Fig. 4). Similarly the N50 scaffold length was longer using MaSuRCA than the ALLPATHS-LG assembly program, with N50 scaffold lengths of 1,514,642 and 900,636, respectively (Fig. 5). However, both programs

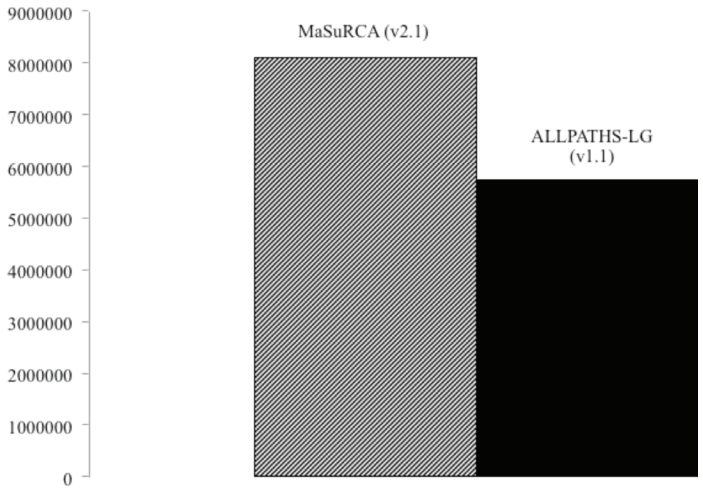

Figure 2: Longest scaffold in the S. dorsalis draft genome using two assembly programs, MaSuRCA (2.1) and ALLPATHS-LG (1.1).

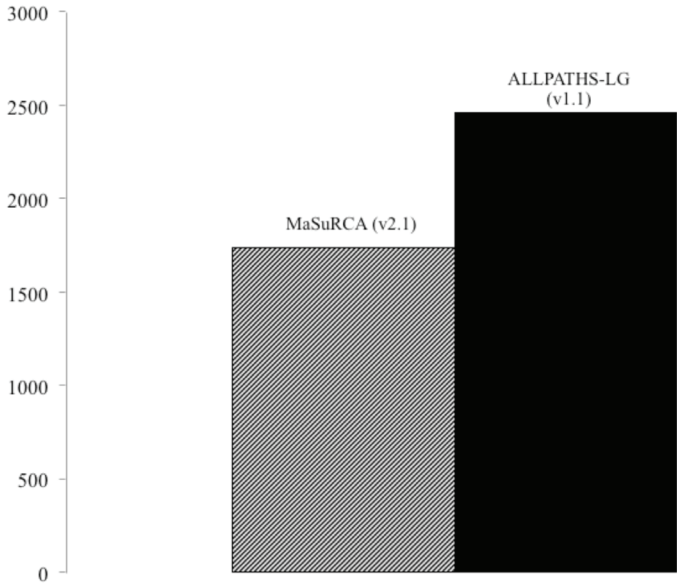

Figure 3: Number of scaffolds in the S. dorsalis draft genome using two assembly programs, MaSuRCA (2.1) and ALLPATHS-LG (1.1).

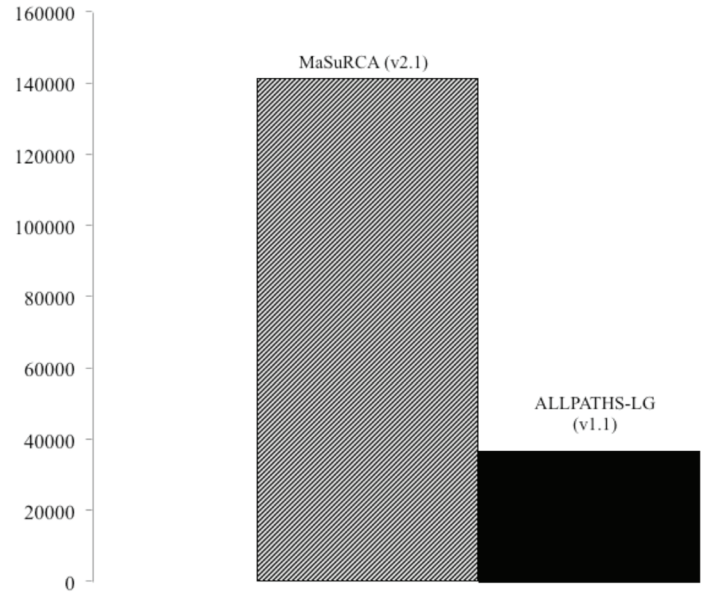

Figure 4: The N50 contig length of the S. dorsalis draft genome using two assembly programs, MaSuRCA (2.1) and ALLPATHS-LG (1.1).

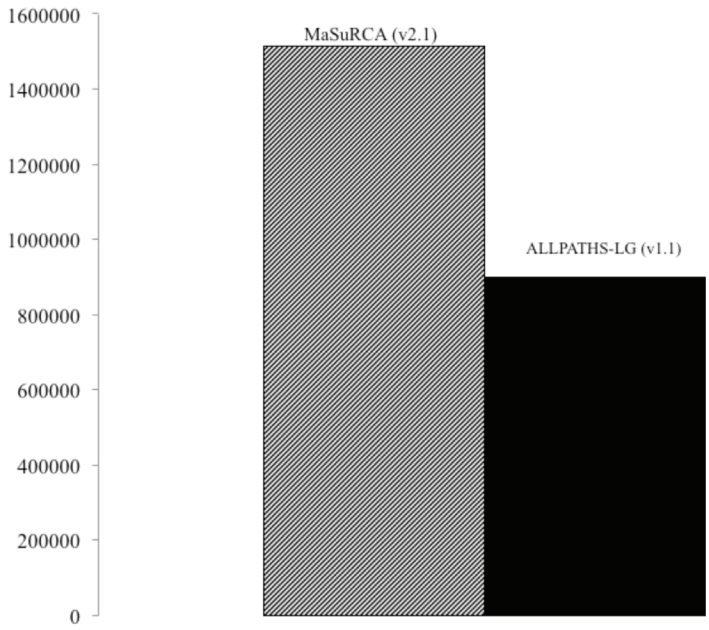

Figure 5: The N50 scaffold length of the S. dorsalis draft genome using two assembly programs, MaSuRCA (2.1) and ALLPATHS-LG (1.1).

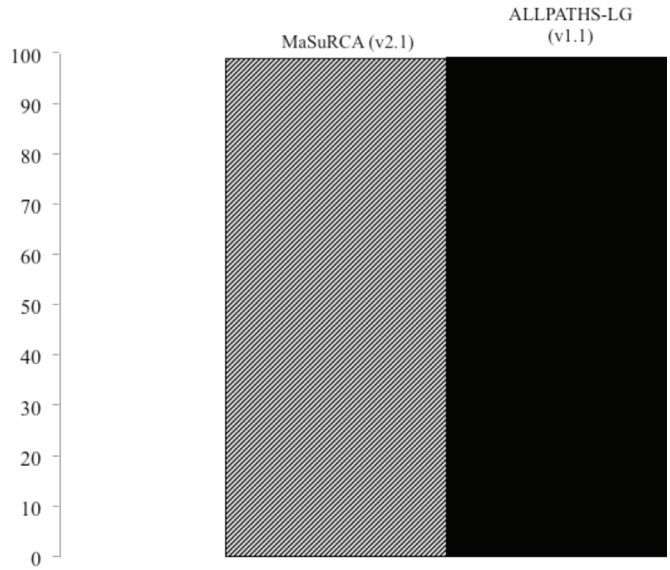

Figure 6: The percent of the S. dorsalis draft genome assembly contained within scaffolded contigs using two assembly programs, MaSuRCA (2.1) and ALLPATHS-LG (1.1).

performed similarly in the placing the assembly into scaffolded contigs, with 98.8% of the assembly placed using MaSuRCA and 99.2% placed using ALLPATHS-LG (Fig. 6).

Another measure of genome assembly quality is determining the CEGMA (Core Eukaryotic Gene Mapping Approach) number for the genome (Parra et al. 2007). This approach determines how many of these 'core genes' can be found at full-length (or nearly full-length) within a single scaffold. For the MaSuRCA assembly, 243/248 genes were found (or 97.98%), and for the ALLPATHS-LG assembly, 240/248 (or 96.77%) of the genes were found. Both are considered high values among draft genome assemblies (http:www.acgt.me/blog/2014/4/8/which-genome-assembler-gives-you-the-best-genome-assembly).

This de novo draft genome assembly resulted in 180x coverage of the small-to-moderately sized 685 Mb genome of *S. dorsalis*. Both the MaSuRCA and ALLPATHS-LG programs worked well for the assembly; it was estimated that 97% and 93% of the genome was captured by these two programs, respectively. After filtering for low quality scaffolds, the current version of this genome has 1,439 scaffolds, with an N50 value of 2,560,911bp. Ninety percent of the total assembled bases are represented by only the largest 410 scaffolds. Overall, the *S. dorsalis* genome assembled remarkably well using the Illumina sequencing data.

To be able to utilize the sequencing data, we created a web-based tool to visualize the genomic, transcriptomic, and genetic data. Sequences are organized by scaffold (Fig. 7) and then can be zoomed on to view individual transcripts, with clickable links to BLAST results and the transcript sequences (Fig. 8). With the additional transcriptomic data (Buonaccorsi

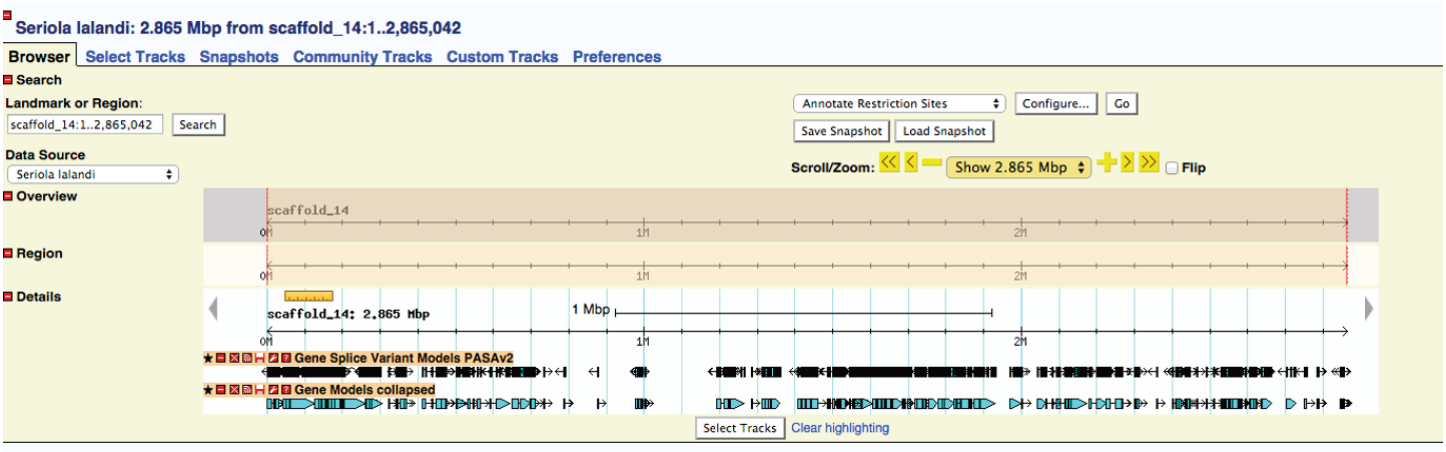

Figure 7: Genome browser for S. dorsalis showing an entire scaffold.

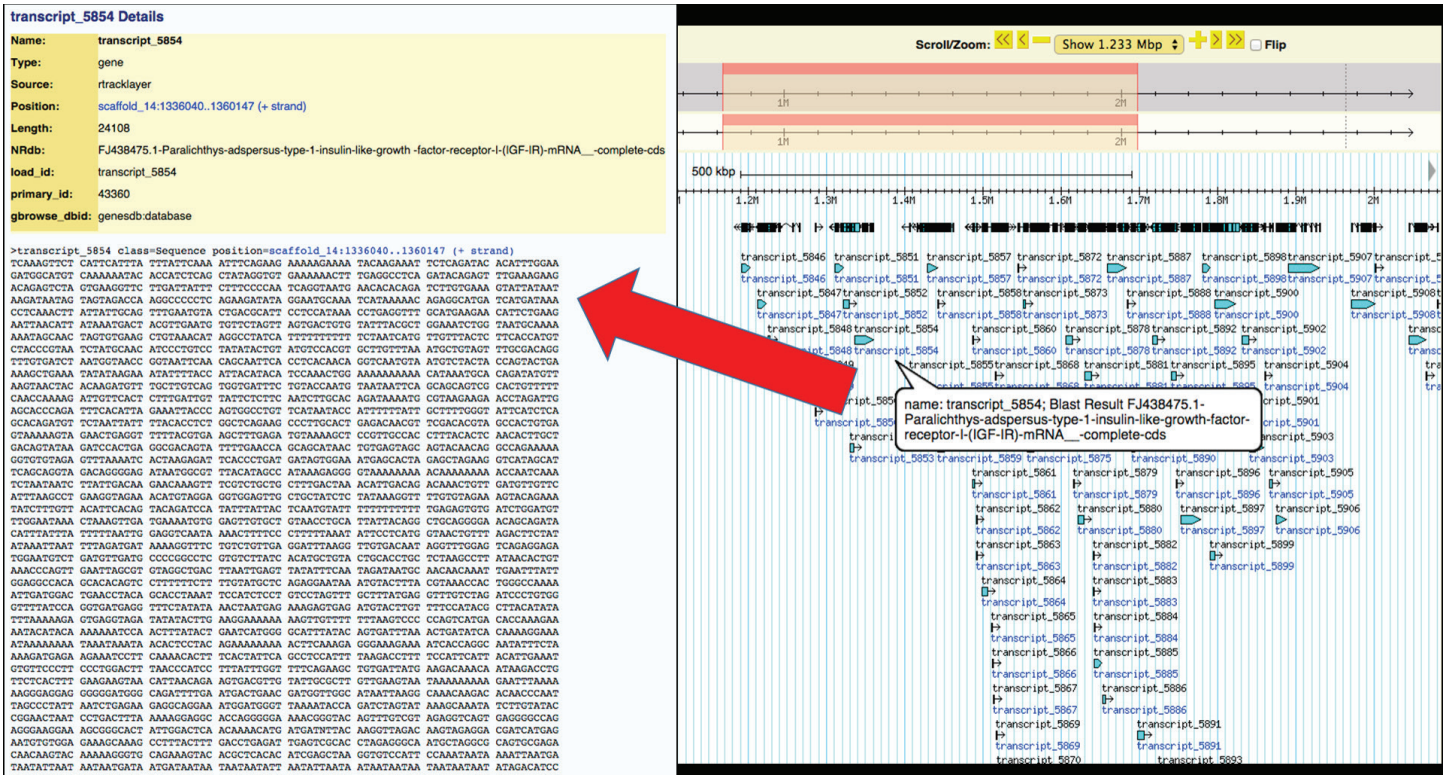

Figure 8: Genome browser for S. dorsalis showing BLAST search results for a transcript and the accompanying sequence.

et al. in press) incorporated into the genome browser, we can also look at gene variants and splicing models in the data.

Utilizing this draft genome assembly, we may be able to use comparative analyses to aid our understanding of *S. dorsalis* genome. In particular, exploring other genomes that have been characterized for traits of interest may greatly accelerate research for those traits in *Seriola*. These analyses will continue to improve as the genomes for additional species are sequenced
(Fig. 9).

In aquaculture, genomic data have been used to identify disease resistance, genes associated with growth rates and resilience to different types of stressors, fillet quality, and genes related to reproduction and general health (Whatmore et al. 2013). Creating a de novo draft assembly for *S. dorsalis* is an important step to looking at these economically important traits. Analyses are ongoing in this project, and while much work is still needed to be able to best utilize these genomic resources, the development of a draft genome for *Seriola* is an enormous first step to helping improve broodstock selection and aquaculture practices for these species.

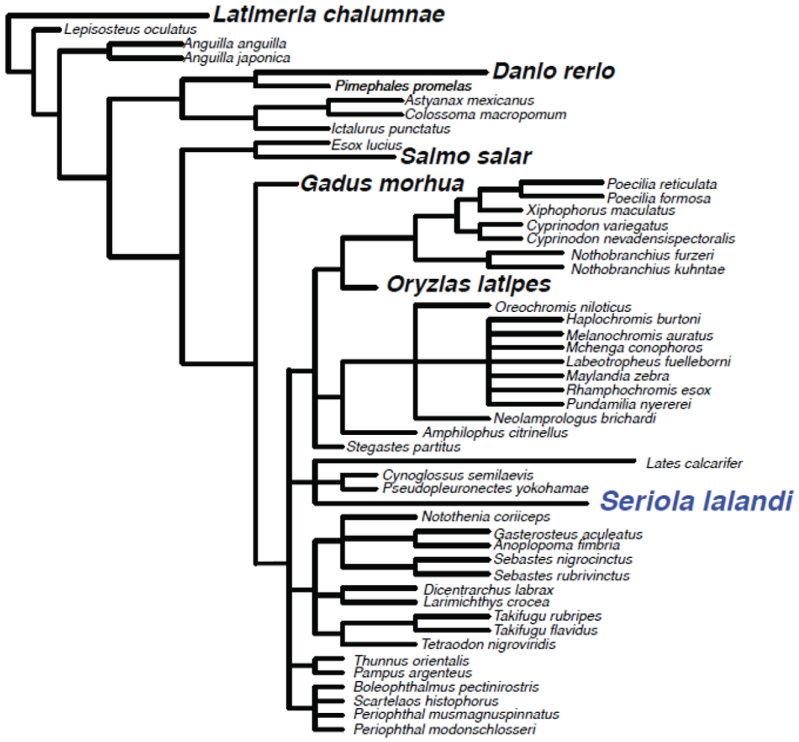

Figure 9: NCBI common tree of teleost genome sequences with S. lalandi (now S. dorsalis) placed within the tree.

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ANNOTATED BIBLIOGRAPHY

Cerdá, J. and M. Manchado. 2013. Advances in genomics for flatfish aquaculture. Genes and Nutrition, 8: 5-17.

In this review, the authors describe the various 'omics' approaches that have been used to investigate different aspects of flatfish biology, and that have been used to develop genomic resources available for flatfish research and aquaculture improvement. The authors go over the next-generation sequencing approaches that have been used in de-novo genomic sequencing and transcriptome sequencing for various flatfish species, along with the advantages and disadvantages of these approaches. In this review, the authors also discuss how the technology is being applied to specifically address aquaculture questions such as diet (e.g., nutrigenomics) and changes in the culturing techniques during the different life-stages (e.g., changes in transcriptome over larval development). This review was of interest as an overview of how these genomic and transcriptomic approaches have been applied toward improving aquaculture for these species, and how we may similarly improve yellowtail culture.

Henkel, C.V., R.P. Dirks, D.L. de Wijze, Y. Minegishi, J. Aoyama, H. Jansen, B. Turner, B. Knudsen, M. Bundgaard, K. Lyneborg Hvam, M. Boetzer, W. Pirovano, F.-A. Weltzien, S. Dufour, K. Tsukamoto, H. Spaink, G. and van den Thillart. 2012. First draft genome sequence of the Japanese eel, *Anguilla japonica*. Gene, 511: 195-201.

In this study, the authors used a de novo assembly protocol to create the first draft genome of the commercially important Japanese eel, Anguilla japonica. Although considerable research has been conducted on this species, including the development of some genetic resources such as a recently developed linkage map, the tools are still far from being able to understand the genomic mechanisms of this organism's biology. The genome of this non-model species was assembled using a whole-genome shotgun sequencing strategy utilizing the Illumina GAIIx or HiSeq 2000. The assembled genome size is 1.15 Gbp, which is contained in 323,776 scaffolds that have an N50 of 52,849 bp, a minimum scaffold size of 200 bp and a maximum scaffold size of 1.14 Mbp. The authors state that development of this genome is an important component to the development of genetic resources for fresh water eels and key in selective breeding of this species for aquaculture purposes. There are many similarities between the Japanese eel and the California yellowtail in terms of aquaculture importance, currently existing genetic resources, and the motives in generating a draft genome.

Whatmore, P., N.H. Nguyen, A. Miller, R. Lamont, D. Powell, T. D'Antignana, E. Bubner, A. Elizur, and W. Knibb. 2013. Genetic parameters for economically important traits in yellowtail kingfish, *Seriola lalandi*. Aquaculture, Vol 400-401: 77 – 84.

The authors report on the genetic properties of muscle fat content, condition score and deformity and their genetic associations with body and carcass traits in yellowtail kingfish (*Seriola lalandi*). A total of 10 microsatellites were used to construct pedigrees for the progeny of 22 full- and half-sib families (eight sires and six dams). Heritability was estimated for body and carcass traits (fillet weight, fillet yield), fat content, condition score and deformity, and genetic relationships among these traits. The estimates of heritability for body and carcass traits were moderate ($h^2 = 0.15$ to 0.30), and fillet fat content showed moderate heritability (0.41 ± 0.26).The estimate for condition score was 0.15 ± 0.11, but the heritability for deformity was close to zero ($h^2 = 0.02$). The genetic correlations between body and carcass (fillet weight and fillet yield) traits were high and positive (0.57 to 0.94). The results suggest that selection for improving body weight, fillet weight and fat content could be effective as evidenced by their moderate heritability. Genomic data may reveal QTL-associated markers for these traits, which could then be used in broodstock selection.

Identification of Quantitative Trait Loci and Marker-Assisted Selection by Using Genomics Information in Yellowtail *(Seriola quinqueradiata)*

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Keywords: yellowtail, *Seriola quinqueradiata*, *Benedenia seriolae*, disease resistance, quantitative trait loci (QTL), marker-assisted selection (MAS)

ABSTRACT

Benedenia infections caused by the monogenean fluke ectoparasite *Benedenia seriolae* seriously impact marine finfish aquaculture. We have discovered the evidence that contributes to detailing the phenotypic resistance to Benedenia disease in yellowtail (*Seriola quinqueradiata*). Two putative quantitative trait loci (QTL) associations, of medium to large effect of with Benedenia disease resistance, were localized to linkage groups Squ2 and Squ20. Finding the QTL region strongly supports the potential for success of marker-assisted selection (MAS) for resistance to Benedenia disease. The aim of this study was to confirm the QTL significant region and compare the susceptibility, depending on the difference of linkage disequilibrium (LD) block for Benedenia disease resistance in yellowtail, for use in marker-assisted selection to increase the rate of genetic improvement for this trait.

Two major QTL regions (BDR-1, BDR-2) were employed for marker-assisted selection in F1 siblings from QTL selection candidates to produce F_2 full-sib families. F_1 siblings were placed in two groups which are according to whether or not they have inherited the QTL significant LD about Benedenia disease resistance. Two crossbreed types of F_2 full- sib families were established by one-on-one crossing from F_1 siblings. The families were putative resistant families (R-families) whose parents were selected for their inheritance of QTL significant LD block, and the other families were putative susceptive families (S-families) whose parents were selected for not having inherited the QTL significant LD block.

In artificially induced infection, we had compared the F_2 R-families (total, n=1200) and F_2 S-families (total, n=1200) in three month old stage. The two different type of QTL significant LD block were reared together and challenged by exposure to *B. seriolae*. F_2 R-family, F_2 S-family and about 120,000 *B. seriolae* hatchlings per fish were placed in each tank. After 10 days, we measured the number of parasites in each fish. We had performed infection experiment six times. All infection experiment showed the same result, the F_2 R-family fish had significantly fewer parasites than the F_2 S-family fish.

In natural induced infection, we had compared the susceptibility of *B. seriolae* with F_2 R family (n=67), F_2 S family (n=40) and F1 progeny from the wild parental fish (n=50). One hundred fifty-seven of the one year old fish were pit tagged in total, and the number of *B. seriolae* was counted eight rounds for one year in each fish. All eight rounds of natural infection were showed the significant results. The F_2 R-family fish had significantly fewer the number of parasites than the F_2 S-family fish.

We employed LD blocks based on QTL significant region for MAS, and confirmed their effects in subsequent F_2 full- sib families. Furthermore, it means QTL significant regions are identified from these wild ancestors to the breeding population. It would be possible to introgress certain properties of the wild population in the breeding population. These results indicate that it is possible to rapidly develop domesticated strains having commercially important traits by MAS in aquaculture.

INTRODUCTION

Construction of genetic linkage map and identification of quantitative trait loci (QTL) are beginning to make an impact on animal breeding, by providing DNA markers linked to QTL affecting phenotypic traits. Such molecular markers can be used in marker-assisted selection (MAS), selection based partly or fully on DNA marker genotypes. Among the traits relevant for MAS, disease resistance traits are of particular interest, since the phenotypic measurement of these traits is often difficult.

MAS involves selection of animals carrying genomic regions that are involved in the expression of traits of interest through molecular markers. With the development and availability of an array of molecular markers and dense genetic linkage map in animals, MAS has become possible for traits both governed by major genes as well as QTL.

The advantages of MAS are obvious as compared with the conventional selective breeding. MAS are especially useful for traits that are difficult to measure, or are expressed the phenotype late in development. Implementation of MAS requires DNA markers that are tightly linked to QTL for traits of interest based on QTL mapping or association studies (Lande and Thompson 1990). Ideally, the DNA markers should be the linkage disequilibrium (LD) block underlying the phenotypic variation. QTL studies in aquaculture species covered a wide range of traits including disease resistance, growth, stress resistance, reproduction and other traits. The results of these studies provide a good starting point to search for QTL within breeding populations. Of the QTL from experimental crosses, only a small number of them have been followed up by confirmation and fine-mapping. The responsible genes have not been identified for detected QTL. However, there are already a few applications of MAS in commercial breeding programs in aquaculture species (Fuji et al. 2007; Moen et al. 2009; Ozaki et al. 2012).

In aquaculture, disease resistance traits are of particular importance. In intensive culture systems, opportunities for avoidance or escape are minimal. Furthermore, interactions between fish and viral, microbial, parasite pathogens that may be harmless under natural conditions often result in disease problems in aquaculture systems because of the added stress from biological, physical and chemical factors (Wedemeyer 1996). In contrast to farm animals, the animal strains used in aquaculture are usually very recent derivatives of wild strains (Duarte et al. 2007), and therefore have had little time to adapt to the new disease pressures.

The yellowtail amberjack (*Seriola quinqueradiata*) is, as its relative the gold-striped amberjack (*S. lalandi*) and the greater amberjack (*S. dumerili*), an important species in marine finfish aquaculture in Japan. Production of cultured species of yellowtail in Japan was approximately 152,800 tons in 2009, which accounts for 59% of marine finfish aquaculture in Japan (MAFF Japan 2009). Yellowtail has been cultured in southern areas of Japan using juveniles caught from natural stock. Although research on disease, nutrition and pond management has supported the development of the yellowtail aquaculture industry, genetic improvement programs leading to improve yellowtail lines are only at the beginning.

Benedenia disease caused by infection by *Benedenia seriolae* is a serious parasitic disease for yellowtail in aquaculture, leading to secondary infection due to viral or bacterial disease. This is because fish rub their bodies against the fish cage to remove the parasite. In certain conditions, the mortality is quite high especially in juvenile fishes. Although the way of removing the parasite is generally to soak the fish in a freshwater bath, this method requires a high cost and is labor intensive. Thus, Benedenia disease is difficult to prevent in marine aquaculture systems. Besides from the point of view of wildlife conservation, yellowtail aquaculture is considered as a hotbed for enhancement of parasite transmission (Ogawa 1996; Hutson et al. 2007). Risk management is an important consideration for the long-term sustainability of the aquaculture industry.

In previous study, we performed QTL analyses using wild F_1 strains of *S. quinqueradiata* to elucidate the genetic evidence of resistance to Benedenia disease. By using the high-density linkage map with microsatellite and SNP markers, we identified two chromosomal regions containing QTL (BDR-1, BDR-2) that were associated with Benedenia disease resistance. Two putative QTL associations, of medium to large effect of with Benedenia disease resistance, were localized to linkage groups Squ2 and Squ20. These two loci were responsible for ranging from 32.9 to 35.5% of the total phenotypic variation (Ozaki et al. 2013). Finding the QTL region strongly supports the potential for success of MAS for this disease.

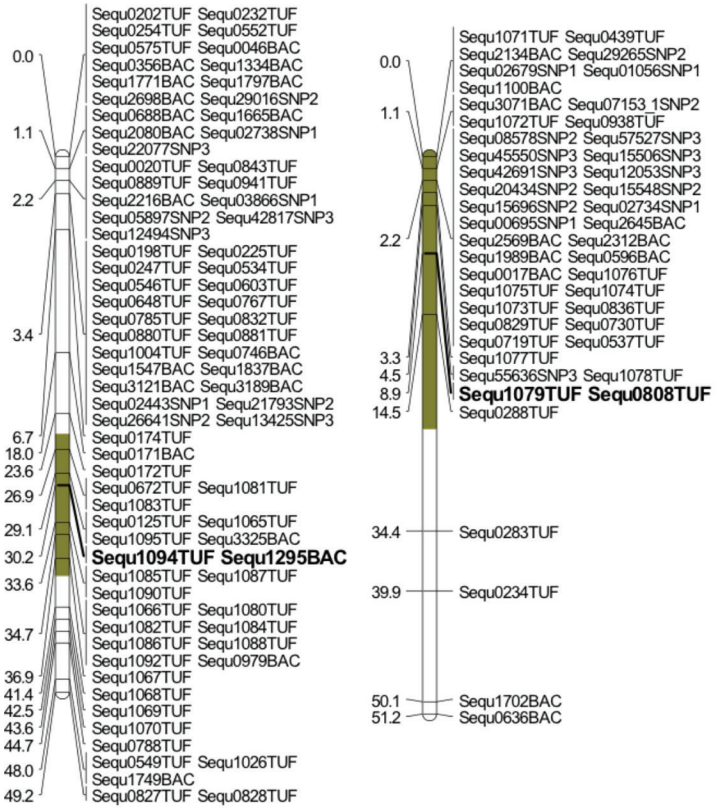
Here, we report on results from a project that used MAS and confirmed their effects in subsequent F_2

full-sib families. These results indicate that it is possible to rapidly develop domesticated strains having commercially important traits by MAS, where investigation of a QTL at the level of an entire breeding population yielded results that are directly applicable for MAS within that population.

MATERIALS AND METHODS

Confidence intervals of QTL region about Benedenia disease resistance

We show the confidence intervals of QTL regions for Benedenia disease resistance from previous study in Figs. 1a and 1b. Two regions of the linkage groups were identified to be significantly associated with Benedenia disease. The QTL (BDR-1) at Squ2F identified by simple interval mapping (SIM) was observed as a high single peak as genome-wide logarithm of the odd score (LOD) significance level (LOD = 4.71). And the other QTL (BDR-2) region of Squ20F linkage group had slightly exceeded the genome-wide LOD significance level (LOD=2.98). Each of the LOD peaks, at locus Sequ1295BAC in Squ2F, and at Sequ0808TUF in Squ20F, can explain the phenotypic variance 21.4 and 14.1% by SIM [10]. When LOD peaks were combined, the two loci could explain the phenotypic variance ranging up to 35.5%. QTL confidence intervals were estimated by 1.8-LOD support interval with 95% confidence interval probability coverage (Manichaikul et al. 2006).



In both confidence intervals of QTL regions, we had confirmed the genotypes within each locus in F_1 selection candidates. In the QTL (BDR-1) region at Squ2F linkage group, we confirmed that a linkage disequilibrium (LD) block was conserved without reorganization of chromosome in meiosis between marker loci Sequ0672TUF and Sequ1067TUF (Fig. 1a). And also in the other QTL (BDR-2) region of Squ20F, we confirmed that an LD block was conserved without recombination between Sequ1071TUF and Sequ0288TUF (Fig. 1b). And we had known the LD block associated with Benedenia disease resistance from wild ancestor within QTL reference families.

Simple sequence repeat analysis to confirm the LD block in QTL regions

Simple sequence repeat (SSR) 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 MgCl2, 0.2 mM dNTP, 1% BSA, 0.025 U of Ex Taq DNA polymerase (TaKaRa Bio, Inc., Shiga, Japan) and 25 ng template DNA. Suitable annealing temperatures for each microsatellite marker were used. PCR was performed on a MJ PTC-100 (Bio-Rad, CA, USA), and the program conditions were 95 °C for 2 min for initial denaturation, followed by 35 cycles of 30 s 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 6% PAGE-PLUS gel (Amresco, 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).

Wild ancestor, F_1 selection candidates and LD-MAS in F_2 full-sib families

The wild ancestor fish of *S. quinqueradiata* were captured in the coastal waters of Goto Fukue Island (Tsushima Strait, Nagasaki Prefecture). One thousand juveniles were kept in a growing fish pen for two years. Two hundred of the three year old fish were pit tagged, and the number of *B. seriolae* counted six times to select the ideal parents. The selected fish

Figure 1. Localization of significant markers for Benedenia disease resistance in linkage group Squ2F and Squ20F. Squ(linkage group)F marker distance in female map: (left) Squ2F, (right) Squ20F. Marker absolute map distances are given in (cM), with 95% confidence probability LOD support interval indicated as a bold line.

were reared until maturity. We prepared F_1 families for QTL analysis (Fig. 2). And we had found two QTL medium to large effects for Benedenia disease resistance in F_1 families (Ozaki et al. 2013).

Two major QTL regions were employed for marker-assisted selection in F_1 selection candidates to produce F_2 full-sib families. F_1 selection candidates were selected in two groups according to whether or not they inherited the LD block for Benedenia disease resistance. Then, two types of F_2 families were established by one-on-one crossing from F_1 selection candidates. Six families were putative resistant families (R-families) whose parents were selected for their inheritance of Benedenia disease resistance LD block, and six families were putative susceptive families (S-families) whose parents were selected for not having inherited the Benedenia disease resistance LD block (Figs. 2, 3).

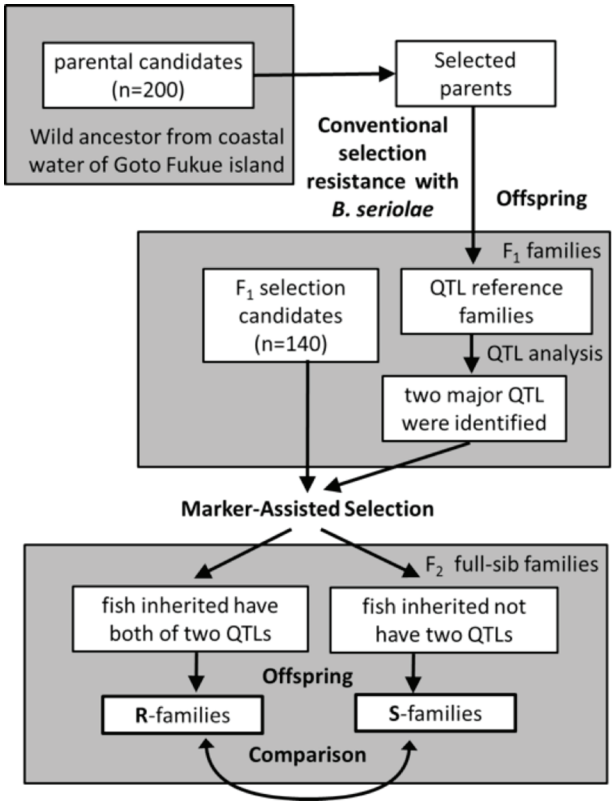


Figure 2. Schema of fish strains. Two major QTL regions were employed for marker-assisted selection in F_1 selection candidates to produce F_2 full-sib families. F_1 selection candidates were selected in two groups according to whether or not they inherited the LD block for Benedenia disease resistance.

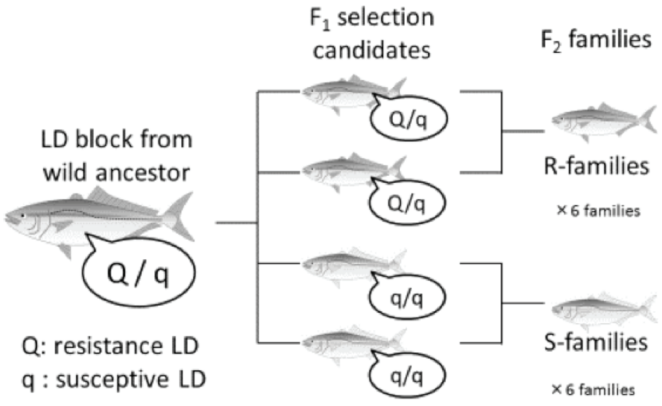


Figure 3. Schema of crossbreeding in MAS. Two types of F_2 families were established by one-on-one crossing from F_1 selection candidates. Six families were putative resistant families (R-families) whose parents were selected for their inheritance of Benedenia disease resistance LD block, and six families were putative susceptive families (S-families) whose parents were selected for not having inherited the Benedenia disease resistance LD block.

Phenotypic measurement and comparison depend on the LD block for Benedenia disease resistance in artificially induced infection

About parasite collection for artificial infection experiment, *B. seriolae* used for artificial infection were collected from adult fish stock in growing pens. Mesh nets were hung in fish pens in which adult fish were parasitized by *B. seriolae*. Eggs of *B. seriolae* stuck to the net and a method to collect only *B. seriolae* was established and confirmed under experimental conditions several times for reproducibility (Nagakura et al. 2010). Collected *B. seriolae* eggs could be induced to hatch by a 15 minute exposure to fluorescent light. Hatched larvae were kept in a shaded tank before infection.

In artificially induced infection, we had compared the F_2 R-families (total, n=1200) and F_2 S-families (total, n=1200) in three month old stage. In preliminary experiments, it was confirmed that the parasite did not exist on the test fish bodies, which were soaked in a freshwater bath for five minutes. And the fish were allowed to recover from freshwater stress for two days before artificial infection. Hatched *B. seriolae* larvae were introduced to experimental fish tanks, and individual fish were exposed to one hundred larvae of *B. seriolae*. One F_2 R family (n=200), one F_2 S family (n=200) and about 40,000 *B. seriolae* hatchlings were mixed up in same tanks (Fig. 4). Water temperature was kept at 25±1 centigrade degrees during the infection experiment for 10 days until the larvae grew to the countable adult stage. Ten days after exposure to *B. seriolae*, each fish was individually dipped in a tank of freshwater to remove the parasites. Thus, in the context of this study we recorded the total number of *B. seriolae* per fish. We prepared set of test water tanks in six times.

Phenotypic measurement and comparison depend on the LD block for Benedenia disease resistance in natural induced infection

We had prepared the families in the same of artificial infection experiment, which have the difference of LD block associated with Benedenia disease resistance. Those families are F_2 R family (n=67) and F_2 S family (n=40). In addition for the control group, we prepared

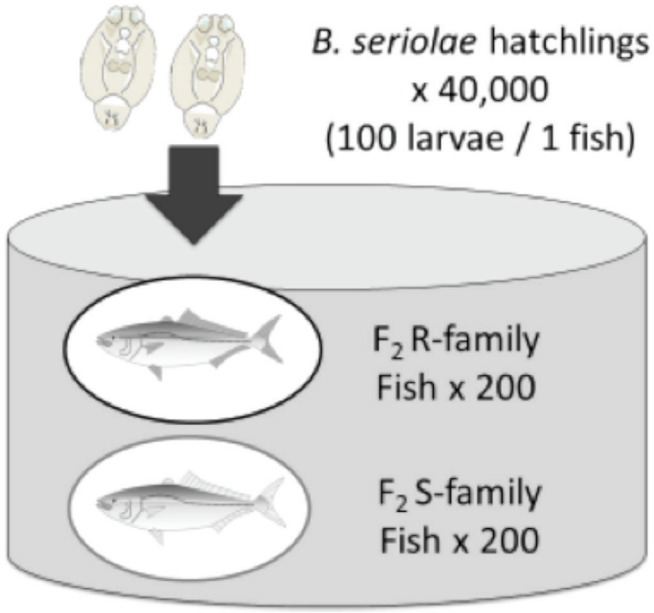


Figure 4. Schema of Artificially induced infection. We compared the F_2 R-families (total n=1200) and F_2 S-families (total n=1200) at the three month old stage.

F_1 progeny (n=50) from wild parental fish. The F_1 progeny were selected random sampling from mixed three families, and mixed three families were sampling same number of progeny from each family crossbred by one on one mating. Total number of 157 fish were prepared with beginning of natural infection experiment. Fish were cultured from July to June for one year in fish cage at the coastal waters of Goto Fukue Island. During the year, fish were grown from 40cm to 70cm.The fish raising methods are same condition as practical mariculture of yellowtail. When we started the natural infection experiment, each fish was injected the pit tag in their body for the tracking of the susceptibility of Benedenia disease in individual. Subsequently, fish are bathed in fresh water to eliminate *B. seriolae* on the body surface. Then natural induced infection had started, all fish were kept in the same fish cage during the experimental period. The first investigation is conducted at 4-8 weeks after starting the natural infection experiment. And we counted the number of *B. seriolae* in each fish, which were stick on the surface of yellowtail skin as susceptibility of Benedenia disease.

The investigation is conducted using the procedure that: (a) put the yellowtail in the bag made of Tetoron Russell Weave Net individually; (b) put the bag into the fresh water tank for 5 minutes to remove the parasites; (c) identify the fish by the pit tag and count the parasites that have appeared on the body surface in each fish; (d) calculate the sum of the number of parasites, which was on the fish the body surface, and parasites in the bag, which were peel off the fish the bag surface; and (e) after the investigation, return the fish to the fish cage to continue maintained. In total number of times, we performed the investigation for eight rounds.

Table 1. Evaluation of phenotypic data, in the tank experiment: artificially induced infection.

		Total number of parasite		The number of parasites/body surface	
		Mean(SD)	p.value	Mean	p.value
Tank1	F_2-R	53.8 ± 17.8	ns	1.01	***
	F_2-S	56.7 ± 18.2	0.16660	1.12	**0.00088**
Tank2	F_2-R	40.0 ± 18.8	***	0.74	***
	F_2-S	55.0 ± 21.4	**1.22E-11**	1.09	**3.91E-16**
Tank3	F_2-R	51.0 ± 17.8	***	0.78	***
	F_2-S	60.0 ± 16.7	**4.27E-07**	0.96	**6.19E-11**
Tank4	F_2-R	35.3 ± 14.4	***	0.56	***
	F_2-S	43.0 ± 13.9	**8.11E-08**	0.66	**3.01E-06**
Tank5	F_2-R	55.7 ± 14.8	***	0.71	***
	F_2-S	68.1 ± 18.8	**1.69E-12**	0.81	**3.15E-07**
Tank6	F_2-R	30.3 ± 13.8	***	0.41	*
	F_2-S	36.8 ± 16.1	**1.26E-05**	0.45	**0.04311**

ns: not significant
p.value< 0.05*
p.value< 0.01**
p.value< 0.001***
The pairwise t test compared the two type of cross using F_2 R-families vs. F_2 S-families in each tank. And the graphs are indicated test water tank in six times.

RESULTS

Evaluation of phenotypic data, in the tank experiment: artificially induced infection.

The pairwise t test compared the two type of cross using F_2 R-families vs. F_2 S-families in each tank. And the graphs are indicated test water tank in six times (Table 1). In infection experiment, the total number of parasite in F_2 R-families are significantly fewer than F_2 S-families except Tank 1.

Evaluation of phenotypic data, in the field experiment: natural induced infection

ANOVA and Dunnett test compared the three type of cross in F_2 -families using F_2 R-families vs. F_2 S-families, and F_2 R-families vs. F_1 progeny from wild parental fish (Table 2). In all of investigation results, the number of parasite in F_2 R-families are significant difference than F_2 S-families. And the investigation days except July 20, Feb. 5, and June 3 in 2012, the

Table 2. Evaluation of phenotypic data, in the field experiment: naturally induced infection.

	Total number of parasite		ANOVA p.value		Dunnett p.value	
		Mean(SD)			P	
July. 20, 2012	F_2-R	19.5±11.6			-	
	F_2-S	30.2±15.6		F_2-R vs. F_2-S	**4.42E-05**	***
	F_1-W	21.4±9.1	**0.002** **	F_2-R vs. F_1-W	0.645	ns
Aug. 10, 2012	F_2-R	31.1±18.5			-	
	F_2-S	49.4±24.8		F_2-R vs. F_2-S	**1.72E-04**	***
	F_1-W	58.3±23.9	**6.23E-09** ***	F_2-R vs. F_1-W	**1.41E-09**	***
Nov. 2, 2012	F_2-R	22.2±13.8			-	
	F_2-S	28.8±14.7		F_2-R vs. F_2-S	**0.045**	*
	F_1-W	31.6±10.6	**5.01E-04** ***	F_2-R vs. F_1-W	**3.93E-04**	***
Nov. 28, 2012	F_2-R	25.7±19.4			-	
	F_2-S	53.1±26.4		F_2-R vs. F_2-S	**1.10E-07**	***
	F_1-W	36.8±20.4	**1.03E-05** ***	F_2-R vs. F_1-W	**0.013**	*
Dec. 27, 2012	F_2-R	36.6±22.5			-	
	F_2-S	88±44.5		F_2-R vs. F_2-S	**1.03E-11**	***
	F_1-W	58.4±29.2	**4.46E-08** ***	F_2-R vs. F_1-W	**4.86E-04**	***
Feb. 5, 2013	F_2-R	17.1±8.9			-	
	F_2-S	30.3±11.7		F_2-R vs. F_2-S	**2.69E-09**	***
	F_1-W	19.7±7.3	**5.90E-06** ***	F_2-R vs. F_1-W	0.251	ns
May.2, 2013	F_2-R	6.5±3.8			-	
	F_2-S	13.4±4.4		F_2-R vs. F_2-S	**5.05E-12**	***
	F_1-W	9.6±4.1	**1.01E-09** ***	F_2-R vs. F_1-W	**1.16E-04**	***
June. 3, 2013	F_2-R	18.1±8.6			-	
	F_2-S	30.7±11.7		F_2-R vs. F_2-S	**3.49E-07**	***
	F_1-W	21.8±11.3	**1.09E-05** ***	F_2-R vs. F_1-W	0.107	ns
Sum	F_2-R	177.1±88			-	
	F_2-S	325.5±121.2		F_2-R vs. F_2-S	**2.77E-10**	***
	F_1-W	257.3±84.7	**3.79E-08** ***	F_2-R vs. F_1-W	**3.08E-05**	***

ns: not significant, p.value< 0.05*, p.value< 0.01**, p.value< 0.001***

ANOVA and Dunnett test compared the three type of cross in F_2 -families using F_2 R-families vs. F_2 S-families, F_2 R-families vs. F_1 progeny from wild parental fish.

total number of parasite in F_2 R-families are fewer than F_1 progeny from wild parental fish. Even in a natural induced infection, it is presume that the number of parasite in F_2 R-families are significant difference than F_2 S-families and F_1 progeny from wild parental fish.

DISCUSSION

We employed LD blocks based on QTL significant region for MAS, and confirmed their effects in subsequent F_2 full-sib families. Furthermore, it means QTL significant regions are identified from these wild ancestor to the breeding population. It would be possible to introgress certain properties of the wild population in the breeding population. These results indicate that it is possible to rapidly develop domesticated strains having commercially important traits by MAS in aquaculture.

MAS will greatly increase the efficiency and effectiveness of breeding compared to traditional breeding programs. The fundamental advantage of MAS compared to conventional phenotypic selection is that it is faster since the early selection could be made without collecting the phenotypic data, and fish will be selected with high reliability, and successive generations will enable avoidance of inbreeding depression. And it is more accurate based on genetic selection instead of phenotypic selection.

In addition, the potential of MAS and marker-assisted introgression (MAI) use for disease risk management of marine aquaculture finfish is considered. Wild aquatic species are not selected and still maintain high genetic diversities. Individuals have high potential for genetic breeding regarding phenotypic variation. Natural populations will be more appropriate to contribute to those genetic resources to find large QTL effects than strain populations, as breeding materials. Therefore, aquatic species have a real possibility that marker-assisted selection could be a success.

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Ozaki A, Yoshida K, Fuji K, Kubota S, Kai W, Aoki JY, Kawabata Y, Suzuki J, Akita K, Koyama T, Nakagawa M, Hotta T, Tsuzaki T, Okamoto N, Araki K, Sakamoto T (2013) 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.

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Ozaki A, Yoshida K, Fuji K, Kubota S, Kai W, Aoki JY, Kawabata Y, Suzuki J, Akita K, Koyama T, Nakagawa M, Hotta T, Tsuzaki T, Okamoto N, Araki K, Sakamoto T (2013) 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 infections caused by the monogenean fluke ectoparasite *Benedenia seriolae* seriously impact marine finfish aquaculture. Genetic variation in host has been inferred to play a significant role in determining the susceptibility to this parasitic disease. To evaluate the genetic basis of Benedenia disease resistance in yellowtail (*Seriola quinqueradiata*), a genome-wide and chromosome-wide linkage analyses were initiated using F_1 yellowtail families (n = 90 per family) based on a high density linkage map with 860 microsatellite and 142 single nucleotide polymorphism (SNP) markers. Two major quantitative trait loci (QTL) regions on linkage groups Squ2 (BDR-1) and Squ20 (BDR-2) were identified. These QTL regions explained 32.9–35.5% of the phenotypic variance. On the other hand, the relationship between QTL for susceptibility to *B. seriolae* and QTL for fish body size were investigated. The QTL related to growth was found on another linkage group (Squ7). As a result, the authors present first genetic evidence that contributes to detailing phenotypic resistance to Benedenia disease, and the results will help resolve the mechanism of resistance to this important parasitic infection of yellowtail.

Fuji K, Koyama T, Kai W, Kubota S, Yoshida K, Ozaki A, Aoki J-y, Kawabata Y, Araki K, Tsuzaki T, Okamoto N, Sakamoto T (2014) Construction of a high-coverage bacterial artificial chromosome library and comprehensive genetic linkage map of yellowtail S*eriola quinqueradiata*. BMC Research Notes 7:200.

Japanese amberjack/yellowtail (*Seriola quinqueradiata*) is a commonly cultured marine fish in Japan. For cost effective fish production, a breeding program that increases commercially important traits is one of the major solutions. In selective breeding, information of genetic markers is useful and sufficient to identify individuals carrying advantageous traits but if the aim is to determine the genetic basis of the trait, large insert genomic DNA libraries are essential. In this study, toward prospective understanding of genetic basis of several economically important traits, the authors constructed a high-coverage bacterial artificial chromosome (BAC) library, obtained sequences from the BAC-end, and first comprehensive female and male linkage maps of yellowtail using Simple Sequence Repeat (SSR) markers developed from the BAC-end sequences and a yellowtail genomic library. The total insert length of the BAC library the authors constructed here was estimated to be approximately 11 Gb and hence 16-times larger than the yellowtail genome. Sequencing of the BAC-ends showed a low fraction of repetitive sequences comparable to that in Tetraodon and fugu. A total of 837 SSR markers developed here were distributed among 24 linkage groups spanning 1,026.70 and 1,057.83 cM with an average interval of 4.96 and 4.32 cM in female and male map respectively without any segregation distortion. Oxford grids suggested conserved synteny between yellowtail and stickleback. In addition to characteristics of yellowtail genome such as low repetitive sequences and conserved synteny with stickleback, the genomic and genetic resources constructed and revealed here will be powerful tools for the yellowtail breeding program and also for studies regarding the genetic basis of traits.

Aoki J-y, Kai W, Kawabata Y, Ozaki A, Yoshida K, Tsuzaki T, Fuji K, Koyama T, Sakamoto T, Araki K (2014) Construction of a radiation hybrid panel and the first yellowtail (*Seriola quinqueradiata*) radiation hybrid map using a nanofluidic dynamic array. BMC Genomics 15:165.

Yellowtail (*Seriola quinqueradiata*) are an economically important species in Japan. However, there are currently no methods for captive breeding and early rearing for yellowtail. Thus, the commercial cultivation of this species is reliant upon the capture of wild immature fish. Given this, there is a need to develop captive breeding techniques to reduce pressure on wild stocks and facilitate the sustainable development of yellowtail aquaculture. the authors constructed a whole genome radiation hybrid (RH) panel for yellowtail gene mapping and developed a framework physical map using a nanofluidic dynamic array to use SNPs (single nucleotide polymorphisms) in ESTs (expressed sequence tags) for the DNA-assisted breeding of yellowtail. Clonal RH cell lines were obtained after ionizing radiation; specifically, 78, 64, 129, 55, 42, and 53 clones were isolated after treatment with 3,000, 4,000, 5,000, 6,000, 8,000, or 10,000 rads, respectively. A total of 421 hybrid cell lines were obtained by fusion with mouse B78 cells.

Ninety-four microsatellite markers used in the genetic linkage map were genotyped using the 421 hybrid cell lines. Based upon marker retention and genome coverage, the authors selected 93 hybrid cell lines to form an RH panel. Importantly, the authors performed the first genotyping of yellowtail markers in an RH panel using a nanofluidic dynamic array (Fluidigm, CA, USA). Using this, 580 markers containing ESTs and SNPs were mapped in the yellowtail RH map. The authors successfully developed the first yellowtail RH panel to facilitate the localization of markers. This high-density physical map will serve as a useful tool for the identification of genes related to important breeding traits using genetic structural information, such as conserved synteny. Moreover, in a comparison of 30 sequences in the RH group 1 (SQ1), yellowtail appeared to be evolutionarily closer to medaka and the green-spotted pufferfish than to zebrafish. The authors suggest that synteny analysis may be potentially useful as a tool to investigate chromosomal evolution by comparison with model fish.

Examining Host-Pathogen Interactions at Mucosal Surfaces Reveals Novel Molecular Targets for Columnaris Disease Intervention

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Keywords: columnaris disease, RNA-seq, transcriptome, mucosal health, rhamnose-binding lectin

ABSTRACT

Columnaris disease, caused by the bacterial pathogen *Flavobacterium columnare*, is a major problem globally and leads to tremendous losses of freshwater fish, particularly in intensively farmed aquaculture species. Despite its widespread importance, our understanding of *F. columnare* infectious processes remains limited. Specifically, little is known regarding the mechanisms controlling pathogen adhesion and replication on host mucosal surfaces. Utilizing next-generation sequencing-based RNA-seq, we profiled the transcriptome in columnaris disease resistant and susceptible families of channel catfish to examine transcriptional differences in mucosal barriers (e.g, skin and gill) before pathogen exposure and at early timepoints following a columnaris challenge. The results revealed a consistent pattern of basal immune polarization between resistant and susceptible fish prior to challenge, including key differences in expression of genes linked to mucin abundance and composition, lysozyme levels, and a rhamnose-binding lectin, a potential receptor for *F. columnare*. Next, we profiled the transcriptome of mucosal tissues of channel catfish under conditions of routine feeding versus short-term feed deprivation. Surprisingly, we found numerous shared mucosal gene expression signatures between resistant/fed fish and susceptible/fasted fish. The position of these molecular actors on ectopic mucosal surfaces could make them accessible for use as prognostic indicators of disease fitness and highly amenable to modulation through dietary or topical prophylactic or therapeutant approaches.

INTRODUCTION

In contrast to their terrestrial agricultural counterparts, farmed fish species rely more heavily on mucosal barriers as they are continuously interacting with an aquatic milieu teeming with microbes. Unlike classical immune centers, such as the spleen or anterior kidney, the accessibility of mucosal surfaces through immersion/dip treatments or dietary changes allows for tailored chemical and nutritional strategies to maximize mucosal and, therefore, organismal health (Beck and Peatman 2015). Indeed, many areas of intense research in aquaculture over the last decade have hinged upon a better understanding of mucosal health.

However, studies on immunity in farmed fish species, as a whole, have either been comparative in nature, seeking to verify the existence of immune components known in mammals, or oriented around artificial disease challenges, with little or no examination of underlying mechanisms.

Opportunely, an array of new molecular tools has become available in recent years, which is rapidly evolving this area of study. The dramatic decline in sequencing costs brought about by a shift to massively parallel sequencing platforms has far-reaching consequences for the study of immunity in aquaculture species. Transcriptome sequencing and gene expression profiling (RNA-seq) offer a rapid approach to characterizing actors in mucosal tissues and cell types and their changes following disease challenge, environmental perturbation, nutritional change, or a combination thereof.

In the present series of studies, we sought to better understand the molecular immune actors that are present within mucosal immune compartments, both at rest in naïve healthy channel catfish and after experimental infection. We first compared the global gene expression in the gill and skin isolated from two different families of channel catfish with known differential sensitivities (resistant versus susceptible) to *Flavobacterium columnare*, the causative agent of columnaris disease. Our analysis revealed early mechanisms of pathogen adhesion and invasion and uncovered exciting and unanticipated associations between nutrition and immunity. This led us to next compare the transcriptomes of mucosal tissues

derived from channel catfish that were subjected to a period of short-term feed deprivation as compared to a well-fed treatment group. These studies bring to light new links connecting mucosal immunity, disease susceptibility, and nutritional status (Sun et al. 2012; Beck et al. 2012; Liu et al. 2013; Peatman et al. 2013; Li et al. 2014).

MATERIALS AND METHODS

Gene expression profiling

We utilized Illumina-based RNA-seq to profile global transcriptional changes in the gill and skin of columnaris disease resistant and susceptible channel catfish before and after a columnaris disease challenge and the gill and skin of channel catfish fed daily versus fish deprived of feed for 7 days (d). Columnaris challenges were conducted using the "Ultra Low-Flow System" described by Mitchell and Farmer (2010), which allows for a natural progression of the disease after challenge in a flow-through environment. Samples were collected at 0 h, 1 h, 2 h, 4 h, 8 h, and 24 h post-challenge. Pooled biological replicates (consisting of equal amounts of tissue from replicate fish) were collected from each experimental group and stored until RNA extraction. For the feeding study, juvenile channel catfish were randomly distributed (30 per tank) among three 600 L tanks. Fish were then subjected to one of three treatments. Fish in treatment group 1 were fed to satiation three times daily with a standard catfish ration for 7 d. In treatment 2, fish were withheld feed for 7 d. In treatment 3, fish were withheld feed for 7 d, subsequently fed to satiation, and at 4 h following the introduction of food, fish were sacrificed. All fish were sacrificed on day 7, and portions of the gill and skin were isolated from equivalent locations on each fish and stored until RNA extraction and sequencing. Sequencing (Illumina HiSeq 100bp paired-end sequencing) was performed at the HudsonAlpha Institute for Biotechnology (Huntsville, Alabama). RNA-seq library preparation and sequencing was carried out as previously described (Li et al. 2012; Sun et al. 2012). Before assembly, raw reads were trimmed by removing adaptor sequences and ambiguous nucleotides. Reads with quality scores less than 20 and length below 30 bp were all trimmed. The resulting high-quality sequences were used in the subsequent assembly (Miller et al., 2010). The de novo assembly was performed by de Brujin graph assembler ABySS (version 1.3.2) (Simpson et al., 2009). Briefly, the clean reads were first hashed according to a predefined k-mer length, the 'k-mers'. After capturing overlaps of length k-1 between these k-mers, the short reads were assembled into contigs. The k-mer size was set from 50 to 96, assemblies from all k-mers were merged into one assembly by TransABySS. In order to reduce redundancy, the assembly results from different assemblers were passed to CD-Hit version 4.5.4 (Li and Godzik, 2006) and CAP3 (Huang and Madan, 1999) for multiple alignments and consensus building after trimming contigs less than 200 bp. The threshold was set as identity equal to 1 in CD-Hit, the minimal overlap length and identity equal to 100 bp and 99% in CAP3. The high quality reads from each sample were mapped onto the TransABySS reference assembly using CLC Genomics Workbench software. During mapping, at least 95% of the bases were required to align to the reference and a maximum of two mismatches were allowed. The total mapped reads number for each transcript was determined and then normalized to detect RPKM (Reads Per Kilobase of exon model per Million mapped reads). The proportions-based test was used to identify the differently expressed genes between resistant and susceptible families with corrected p-value < 0.05 (Kal et al., 1999). After scaling normalization of the RPKM values, fold changes were calculated. Analysis was performed using the RNA-seq module and the expression analysis module in CLC Genomics Workbench (Robinson and Oshlack, 2010)2010. Transcripts with absolute fold change values of larger than 1.5 and a false discovery rate of less than 0.05 were included in downstream analyses as differentially expressed genes. We utilized qPCR for validation of RNA-seq results and for examining putative biomarker expression in additional individual fish following published protocols (Li et al. 2012).

Modulating rhamnose-binding lectin levels in vivo

While numerous changes in gene expression were observed, sequencing data revealed that a rhamnose-binding lectin (RBL) was both highly and exclusively expressed in the susceptible family of catfish and in fasted fish. To better understand the significance of this lectin family on columnaris disease pathogenesis, we blocked RBL with supraphysiological doses of exogenous ligand. Briefly, a generic population of channel catfish fingerlings were stocked at a rate of 30 per tank. In triplicate tanks, the native ligand for RBL, the sugar L-rhamnose, was added to tanks at rates of 0 mM, 20 mM, and 40 mM. Fish were exposed to the sugars for 15 minutes prior to bacterial challenge. At the same timepoint, all challenged tanks received the same dose of *F. columnare* cells from the same preparative stock. For each sugar, a single tank was exposed to the 40 mM concentration but not challenged with the bacterium as a negative control.

As an additional negative control, a single tank of unchallenged fish containing no sugar was used. Fish were monitored daily and any dead fish were promptly removed.

RESULTS AND DISCUSSION

Previously, global transcriptome profiling of the channel catfish gill revealed a rhamnose-binding lectin (RBL) whose expression was induced greater than 100-fold soon after *F. columnare* experimental infection (Sun et al. 2012). Here, RBL expression was found to be highly expressed in susceptible fish before challenge and strongly upregulated early after challenge, suggesting RBL expression is positively correlated with host susceptibility to an *F. columnare* infection (Fig. 1A). Saturation of the RBL with the ligand L-rhamnose lowered its expression levels (not shown) and decreased *F. columnare* mortality in a dosage-dependent manner in a subsequent challenge infection (Fig. 1B). Taken together, these results suggest that host expressed rhamnose-binding lectins are mediating *F. columnare* binding to the surface mucosa. We next asked if changes in feeding, previously tied to *F. columnare* susceptibility (Klesius et al. 1999), could impact RBL expression. Indeed, a period of 7 d fasting upregulated RBL expression in the catfish gill greater than 120-fold (Fig. 1C). Expression of RBL was exquisitely sensitive to feeding status as RBL levels were strongly downregulated within 4 h of feeding in fasted fish (Fig. 1C).

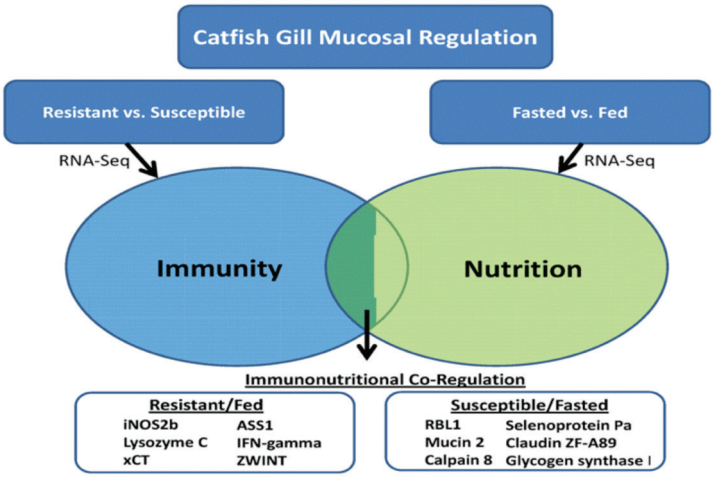

Figure 2. Connecting host susceptibility with nutritional status, evidence for immune-nutritional co-regulation. Shared patterns of gene expression are evident in columnaris resistant fish and well-fed fish. Similarly, fish susceptible to columnaris disease share many transcriptional hallmarks with fish subjected to short-term feed deprivation. Some example genes are listed here.

This striking co-regulation of a mucosal lectin by both infection and nutritional status led us to a broader examination of the RNA-seq data, which revealed numerous shared host gene signatures in fasted vs. fed and *F. columnare* resistant vs. susceptible catfish. Our results indicate that, beyond RBL, critical components of the innate immune response governing host susceptibility to *F. columnare* were perturbed by short-term feed deprivation (Fig. 2; Beck et al. 2012; Peatman et al. 2013; Liu et al. 2013; Li et al. 2014a). Both F. columnare susceptible and fasted (for 7 days) channel catfish featured altered arginine metabolism pathways, critical for production of inducible nitric oxide synthase (iNOS) and its effector nitric oxide (NO). iNOS expression, which we found to be extremely abundant in the fed catfish gill, was down-regulated greater than 17-fold following fasting. We also found evidence of immuno-nutritional co-regulation of lysozyme

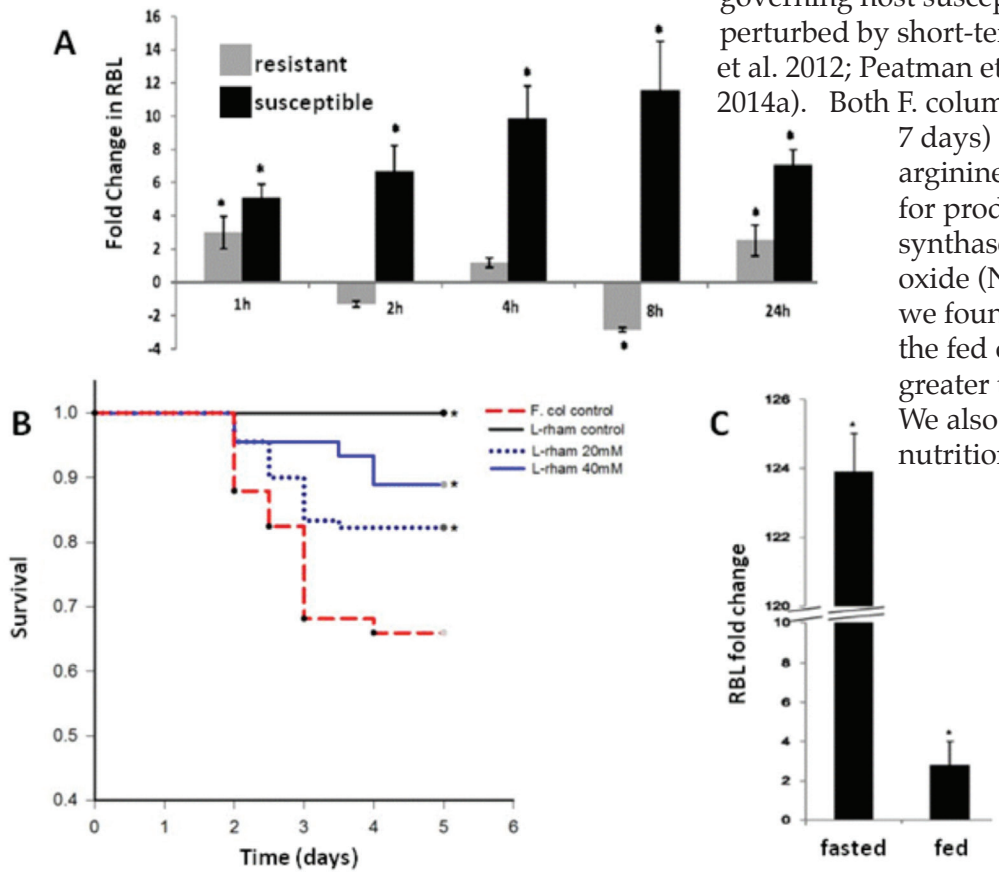

Figure 1. Foe and famine: striking co-regulation of rhamnose-binding lectin expression by infection and nutritional status. A.) Fish from a columnaris disease susceptible family strongly upregulate RBL expression after pathogen encounter. B.) Saturation of RBL receptors with ligand (L-rhamnose) protects fish from columnaris disease in a dose-dependent manner. C.) RBL expression in the gill is markedly upregulated after short-term feed deprivation in channel catfish.

levels, MHC class I/II profiles, mucin secretion, and the chemokine repertoire (Liu et al. 2013; Li et al. 2014a).

We highlight these studies as an illustration of how sequencing approaches can reveal key mucosal players and pathways missed by relying too heavily on mammalian immune paradigms. To bring these RNA-seq studies from the level of interesting observation to application, we are currently studying the impact of rhamnose on both host susceptibility and pathogen virulence, formulating diets to experimentally modulate goblet cell formation and mucin secretion, and pursuing RBL knockout fish lines.

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The authors examined the role of a family of carbohydrate binding molecules termed rhamnose-binding lectins (RBL) in columnaris disease. After a laboratory challenge with *Flavobacterium columnare*, the authors monitored the levels of RBL in the gill of two different families of channel catfish; one family

was previously shown to be susceptible to columnaris disease, while the other was completely resistant. Unexpectedly, the authors found that RBL expression in the gill was strongly upregulated in the columnaris-susceptible family of channel catfish but not the resistant. To better determine if RBLs were influencing columnaris pathogenesis, fish were pretreated with the native RBL ligands L-rhamnose and D-galactose, and investigators found that these sugars protected channel catfish against columnaris disease, likely through competition with *F. columnare* for binding on host RBL. The research team also examined the role of nutritional status on RBL regulation and found that RBL expression was upregulated in fish fasted for 7 d (as compared to fish fed to satiation daily), yet expression levels returned to those of satiated fish within 4 h after re-feeding. Collectively, these findings position RBL as an important determinant of columnaris disease outcomes and reveal new aspects linking innate immunity to feed availability.

Peatman, E., Li, C., Peterson, B.C., Straus, D.L., Farmer, B.D., Beck, B.H. 2013. Basal polarization of the mucosal compartment in *Flavobacterium columnare* susceptible and resistant channel catfish *Ictalurus punctatus*. Molecular Immunology. 56(4):317-327.

Despite recent progress in *Flavobacterium columnare* research, little was known about how host molecular and cellular components modulated rates of adhesion, tissue invasion, and ultimately, mortality. Here, the authors profiled gene expression differences in gill between two channel catfish families (*Ictalurus punctatus*) differing in their susceptibility to F. columnare at both basal (in naïve fish), and at early timepoints post-infection. Between group comparisons revealed over 1,700 unique genes differentially expressed greater than 1.5-fold at one or more timepoints. The authors focused on the analysis of basal differential expression between resistant and susceptible catfish as these genes could potentially reveal molecular actors modulating the differential susceptibility to infection noted between the two families. A number of critical innate immune effectors, including iNOS2b, lysozyme C, IL-8, and TNF-alpha were constitutively higher in resistant catfish gill, while susceptible fish showed high expression levels of secreted mucin forms, a rhamnose-binding lectin previously linked to susceptibility, and mucosal immune factors such as CD103 and IL-17. Taken together, the immune and mucin profiles obtained by RNA-seq suggest a basal polarization in the gill mucosa, with susceptible fish possessing a putative mucosecretory, tolerogenic phenotype which may predispose them to *F. columnare* infection.

Liu, L.G., Li, C., Su, B., Beck, B.H., Peatman, E. 2013. Short-term feed deprivation alters immune status of surface mucosa in channel catfish (*Ictalurus punctatus*). PLoS One. 8(9):e74581. doi:10.1371/journal.pone.0074581.t004.

Short-term feed deprivation is a frequent occurrence in farmed fish species and results from seasonal changes, production strategies, or as a means to combat certain disease outbreaks. In channel catfish, periods of fasting have been linked to increases in the susceptibility of fish to columnaris disease. The authors examined changes at the transcriptional level in the gill and skin (the target tissues of columnaris) in fish that were subjected to a 7 day period of fasting. In comparison to fish fed daily, fasted fish showed changes in the levels of over 1,500 genes. Strikingly, the results showed a marked downregulation of innate immune actors involved in the host defense to bacterial pathogens. These changes are predicted to impact the delicate recognition/tolerance balance for commensal and pathogenic bacteria on the skin and gill. The highlighted transcriptional profiles reveal exciting new interrelationships between nutrition, mucosal integrity, and immunity in teleost fish.

Genetics and Breeding of Highly Fecund Marine Species

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Keywords: selection, inbreeding depression, sweepstakes reproductive success, crossbreeding, heterosis

ABSTRACT

Aquaculture genetics and breeding is dominated by a focus on selection, largely as a result of its transformative success in improving salmon and rainbow trout. However, application of selection to highly fecund marine species faces challenges, which are not found with low fecundity animals, including freshwater and anadromous fish—higher likelihood of random genetic drift, owing to sweepstakes reproductive success, higher genetic loads, and most important, larger non-additive components of genetic variance in yield-related traits.

Crossbreeding offers an alternative strategy for improving highly fecund species with these characteristics. For example, the Pacific oyster shows dramatic heterosis (hybrid vigor) for yield and can be improved through crossbreeding of inbred lines, as demonstrated in corn, rice, and other major crops. In addition to improving yield, crossbreeding provides a means for improving triploid oysters and the tetraploid lines used to produce triploid seed. Unfortunately, heterosis has been little investigated in other highly fecund aquatic species, including marine fishes.

The Pacific oyster also shows strong inbreeding depression for yield characteristics, particularly mortality of early life stages. High early mortality (type-III survivorship) is a consequence of strong natural selection against deleterious mutations that are likely generated as a by-product of high fecundity. Selection for early life-history traits or for survival in the face of diseases or environmental challenges must account for this strong background selection. Finally, unequal reproductive contributions by broodstock (sweepstakes reproductive success) reduce effective population sizes and increase genetic drift in closed aquaculture populations, which then have increased risk of losing genetic diversity and fitness, owing to inbreeding depression. Crossbreeding programs, because they are based on controlled, systematic inbreeding, can prevent inbreeding depression from reaching farmed populations. Progress in crossbreeding can be accelerated, moreover, by application of genomic science to a global, mechanistic understanding of physiological and metabolic processes underlying production traits.

INTRODUCTION

The characteristics of farmed plants and animals have traditionally been improved by two fundamentally different methods, selection and crossbreeding. Selection is based on additive genetic variance in the trait or traits to be improved. When a large proportion of the variance in a trait is caused by additive genetic variance, the trait has high heritability, and the "breeding values" of individuals can be assessed by phenotypic information from progeny or relatives. Alleles with favorable influence on the trait in question are passed through the gametes of individuals with high breeding value, improving that trait in the next generation (Fig. 1A). Selection has been extraordinarily successful in improving livestock species and is arguably the single most important factor responsible for the remarkable growth of salmonid aquaculture over the last 45 years (Gjedrem 2010, 2012).

Crossbreeding, on the other hand, is based on non-additive genetic variation in the traits or traits to be improved (Fig. 1B). Non-additive genetic variation results from an interaction between alleles at the same locus (dominance) or between alleles at different loci (epistasis), such that the performance of a particular genotype cannot be reliably predicted from the average performance of its parents. The classic example of non-additive variation is hybrid vigor in corn, rice, and other crops (Crow 1998; Birchler et al. 2010). High-yielding corn hybrids are produced by crossing elite inbred lines that have been selected for their special combining ability after testing millions of combinations. These inbred lines are typically derived from the same species, which is naturally

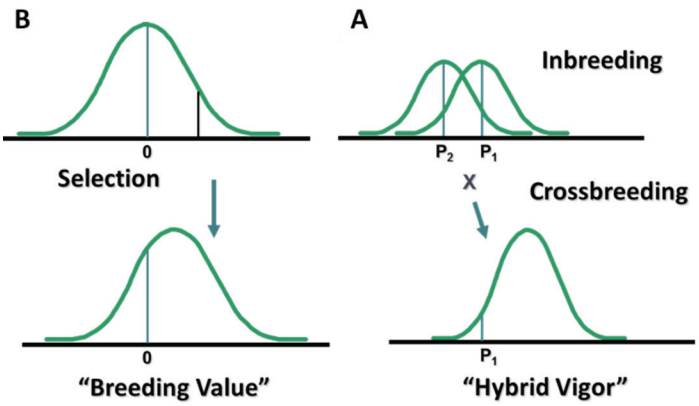

Figure 1. Bell-shaped curves represent the relative frequencies of trait values, say for yield, in large breeding populations. Yield, on the horizontal axis, is standardized to have a mean of zero and a standard deviation of 1.0; yields to the right of the zero are above-average, those to the left, below average. Most individuals are close to the average and very few families have extremely high or extremely low yields. (A) The process of selective breeding begins, in the upper left figure, by taking parents of the next generation from the upper end of the yield-distribution (i.e. to the right of the black vertical line). To the extent that these selected parents pass genes to their offspring that cause those offspring to have high yield, we say that the parents have high "breeding value." The distribution of yield is shifted to the right in the next generation (lower left figure), so that the offspring generation has a higher average yield than its parents. In this way, steady improvement can be achieved. Note also that genetic diversity is reduced, as a consequence of having been propagated by only a select fraction of the population at each generation. (B) Crossbreeding is indicated when the highest yield is achieved by hybrid offspring of two inbred parent lines. This is the phenomenon of "hybrid vigor," which has dramatically improved the yields of the major agricultural crops over the last half-century. Note that hybrid vigor is not strictly heritable, i.e. the highest performing individuals in the upper end of the lower right distribution cannot pass on high yield to their offspring because performance depends on a combination of genes that is re-shuffled during sexual reproduction. Superior hybrids must be produced each generation by repeating the same cross of inbred parent lines. Note also that the distribution of yield in the hybrid population has a smaller standard deviation because the individuals are genetically uniform.

outcrossing; thus, crossbreeding is distinguished from interspecific hybridization. While crossbreeding is mostly applied to crops, it is also used in combination with selection in cattle, swine, and poultry breeding programs. Crossbreeding may be an important means for improving production of highly fecund marine aquaculture species. Here, the Pacific oyster *Crassostrea gigas* provides a case study for illustrating this point of view.

The great fecundity of marine fish and shellfish distinguishes them from most terrestrial animals and even from many freshwater and anadromous fishes (Palumbi and Hedgecock 2005). High fecundity is generally coupled in marine species with high early mortality (type-III survivorship) and delayed maturation (adult longevity), forming what Winemiller (2005) calls the "periodic" life history. This periodic life history is adaptive to the vagaries of the marine environment, especially the oceanographic variability that impacts early development and recruitment. At the same time, however, the periodic life history may impact domestication and improvement for aquaculture in two ways, (1) by making possible large variance in reproductive success, increasing genetic drift, and (2) by increasing the number and potentially the initial frequencies of new mutations, most of which are deleterious, contributing to a large genetic load and a high potential for inbreeding depression.

Genetics and breeding of the Pacific oyster *Crassostrea gigas*

Selection for disease resistance in different oyster species has been successful (Haskins and Ford 1979; Dégremont et al. 2015a, b), largely because the natural selective pressure on diseases is quite severe. Family-based selection has also been successful in the Pacific oyster for improving resistance (and susceptibility) to summer mortality (Dégremont et al. 2010) and for increasing yield (Langdon et al. 2003; Kube et al. 2011). More recently, implementation of family-based selection programs, utilizing an animal model, economic weighting of traits, and index selection on multiple traits (Kube et al. 2011; Small et al. 2016; Langdon et al. 2016, submitted), has demonstrated the power of selection to improve the performance and to reduce the costs of producing farmed oysters. Again, the demonstrated response to selection in these cases is compelling evidence of substantial additive genetic variance in the underlying traits.

Non-additive genetic variance is also important in oysters, however, as evidenced by yield heterosis (hybrid vigor) in the Pacific oyster that is as dramatic as that in maize (Crow 1998), even in crosses among inbred lines derived from the same wild population (Hedgecock et al. 1995; Hedgecock and Davis 2007; Fig. 2). Yield heterosis is associated with equally dramatic inbreeding depression (Evans et al. 2004), attributed to a remarkably large load of deleterious recessive and partially dominant mutations (Bierne et al. 1998; Launey and Hedgecock 2001; Plough and Hedgecock 2011; Plough 2012; Plough et al. 2016). Inbreeding depression (ID) is the proportional reduction in fitness (w) or traits related to fitness, such as viability or growth, of inbred relative to random-bred individuals: : $ID = 1 - (w_{inbred} / w_{random\ bred})$. Evans et al. (2004) found a 25% reduction in yield for oysters with an average inbreeding of ~0.2 (slightly less than the expected inbreeding level of progeny from a brother-sister mating). Hedgecock and Davis (2007) obtained ID = 0.64 for seed weight in inbred lines. Most of the inbreeding depression in oysters is expressed as early mortality, which is

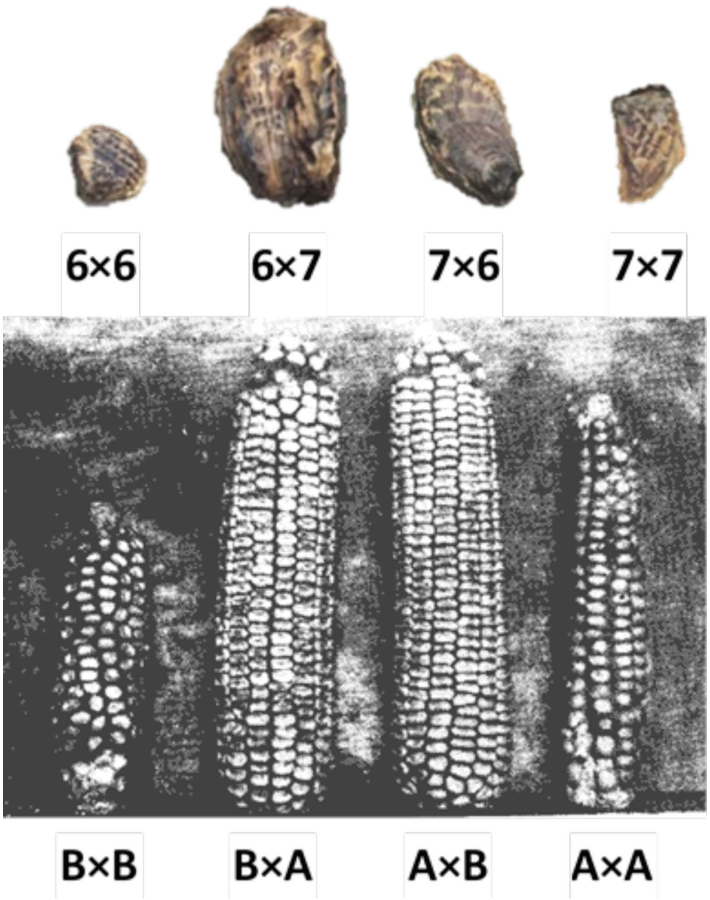

Figure 2. Crossing inbred lines of oysters and of corn produces reciprocal hybrid oysters that are, in both cases, much higher-yielding than their inbred parent lines (top). Bottom photograph of hybrid corn reproduced from original by G.H. Schull in 1908.

substantial, as much as 99% of fertilized eggs (Launey and Hedgecock 2001; Plough and Hedgecock 2011). We have estimated that an average wild oyster carries a minimum of about 15 lethal mutations (compared 3 or 4 in humans or fruit flies).

A large mutational load in oysters was predicted by G. C. Williams (1975), in the Elm-Oyster model for the advantages of sexual reproduction in species with high fecundity and high early mortality. Since high fecundity and high early mortality are the dominant life history features among marine fish (Winemiller 2005) and invertebrates (Thorson 1950), considerable scope for genetic improvement likely lies in crossbreeding of inbred lines.

Challenges for selection of marine species

While selection has clearly proven effective in improving production traits of oysters, sustaining these genetic gains over longer time periods poses several challenges. First, competition for food among filter-feeding oysters within a rearing unit can be much more severe than competition for food among livestock. It is very difficult to monitor the feed intake of individuals and very easy for some individuals to have greater access to food than others. Such competition can cause an amplification in the variance of size among individuals within a cage, potentially obscuring their inherent "breeding value." To some extent, this competition can be ameliorated through vigilant husbandry, in which animals are periodically redistributed within rearing units. Still, measures of individual performance are bound to be much less reliable indicators of breeding value in oysters than in livestock, because of close communal rearing and competition for food. For this reason, many programs select among oyster families based on average yield per production unit, rather than the performance of individuals.

Second, individual oysters are worth much, much less than prize livestock sires. Prize bulls can sell for hundreds of thousands of dollars, for example. Moreover, despite their reputation for fecundity, a male oyster can have a smaller lifetime reproductive capacity than a prize bull, whose artificially cryopreserved sperm can be periodically harvested over his lifetime and used repeatedly to inseminate thousands of cows over time. The record in this regard is 400,000 cows inseminated by a single bull. Since gametes are stripped from mature oysters, a single oyster will generally be used once, to fertilize tens of millions of eggs, of which only about 1% will survive.

The value of prized livestock sires comes from their demonstrated breeding value, as established through progeny testing. Strip spawning precludes progeny testing. Routine cryopreservation of oyster sperm has been demonstrated (Dong et al. 2007) and would make progeny tests possible, but this technology has not been widely adopted by industry. Breeding values can be established from information on the performance of relatives, and selected populations propagated by use of related sires and dams.

Second, inbreeding depression is severe in oysters and likely other highly fecund marine species and may eliminate or even reverse genetic gains from selection, if inbreeding is not held in check. Selection inevitably results in inbreeding, since only a small part of the population is used for reproduction, and individuals in small populations eventually become highly related. Improvement, then, requires a balance between the response to selection and the increase in inbreeding depression over time. There are very few data on long-term selection programs in oysters. Selection for MSX resistance of eastern oysters,

initiated by Dr. Hal Haskin in Delaware, led ultimately to such a decline in growth rate that the resistant stocks were not commercially useful. On the other hand, a well-designed family-based selection program can keep inbreeding at about 1% per generation (Kube et al. 2011).

Finally, another challenge to the long-term efficacy of selective breeding is high variance in the reproductive success of oysters and other highly fecund marine species (Hedgecock and Pudovkin 2011). Variance in offspring numbers inevitably causes a reduction in genetic diversity, by reducing the effective size of the population, Ne, compared to the actual size of the population, N. For example, Boudry et al. (2002) obtained sperm from five male oysters and used it to fertilize eggs from five female oysters, thereby producing 25 families in a full, factorial cross. In this case, N = 5 males + 5 females = 10. In one experiment, Boudry et al. (2002) made 25, separate fertilizations and combined equal numbers of embryos from each family at 3 h post-fertilization. After 90 days, they used genetic markers to determine the proportional contribution of each family to the mix, which were far from equal, although most of the 25 families had some representation. Still, the effective size of the offspring population was 6.2, substantially lower than 10. In a second experiment, Boudry and colleagues pooled equal numbers of eggs and equal amounts of sperm in a mixed fertilization, mimicking the mass spawns that are typical of commercial shellfish hatcheries. Again, after 90 days, the proportional contributions of each family to the mix were highly skewed, making the effective size of the offspring population only 3.2, instead of 10. Similar results were obtained by Matson (2010). Mass spawns can greatly decrease population sizes, causing a loss of genetic diversity and leading inevitably to inbreeding. This can be ameliorated by performing controlled pair-crosses and keeping families separate at all life stages or by use of molecular markers to reconstruct the parentage of individuals in a cohort.

Although risks from these challenges to the long-term sustainability of selective breeding can be managed, crossbreeding offers an alternative method for improvement that eliminates several of these challenges. Crossbreeding also allows substantial non-additive variance to be used.

Challenges and opportunities for crossbreeding of marine species

Like corn, oysters show hybrid vigor in experimental crosses (Fig. 2; Hedgecock et al. 1995; Hedgecock and Davis 2007). As noted, the life-history and genetic parallels between oysters and plants were first noted by G. C. Williams (1975) in his Elm-Oyster Model. Both oysters and corn are naturally outcrossing species, so the phenomenon of hybrid vigor is not observable in wild populations but can only be seen, first, by making inbred parent lines artificially and, then, by making crosses between inbred parents. The resulting hybrids almost always yield more than their inbred parents but, on average, may be no different than the average wild genotype.

There are two reasons why crossbreeding does not simply restore the average wild type, however. First, in a crossbreeding program, one is constantly selecting among lines for those with superior combining ability. Our preliminary data suggest that a doubling of oyster yield could be achieved with current "elite" inbred lines (Fig. 3). A doubling of yield in corn took ~30 years in the double-cross era (from ~1930 to ~1960; see Fig. 1 in Crow 1998). Second, a hybrid population is genetically uniform, rather than heterogeneous, like a random-mating wild population, so that the crop is uniformly high-yielding (suggested by the reduced variance in Fig. 1B). Currently, shellfish farmers incur substantial costs in grading product for the size-dependent, live, half-shell market.

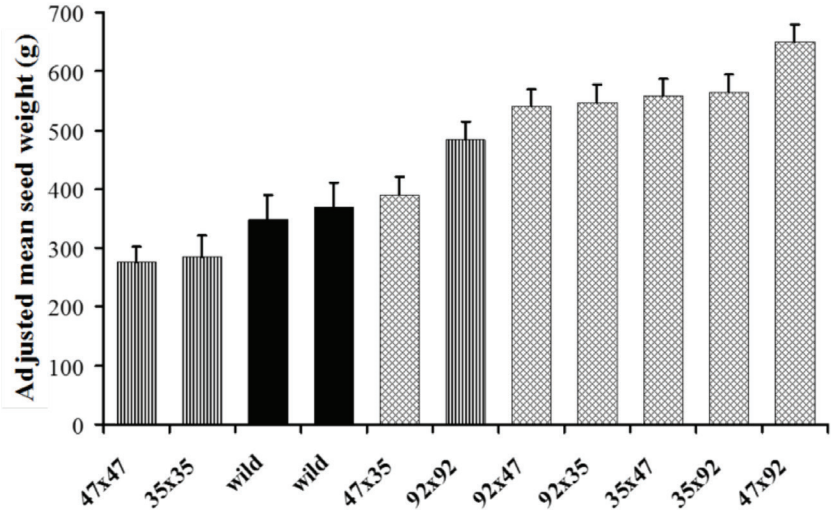

Figure 3. Hybrids produced by a factorial cross of males and females from a set of superior inbred lines. The best hybrids show twice the yield of oysters produced from wild parents. Note also that reciprocal hybrids are often different (e.g. 47×92 > 92×47) and that one inbred line performs better than the two wild families.

Progress in crossbreeding can now be accelerated through the use of genomic biotechnologies based on fundamental understanding of the mechanisms of hybrid vigor (Applebaum et al. 2014). The publication in 2012 of a genome sequence for the Pacific oyster (Zhang et al. 2012) provided resources for fundamental research that are uncommon for marine aquaculture species. When combined with experimental approaches that provide well-controlled biological contrasts for physiological analyses, these genomic resources allow powerful insights into biological mechanisms.

One example of an experimental approach, which is greatly enhanced by having a genome, is Quantitative-Trait Locus (QTL) mapping. A QTL map for live-weight at 10 months of age in one F2 Pacific oyster family shows five, major, significant peaks, suggesting that variance in growth is mainly controlled by five regions of the genome (Fig. 4). Interestingly, preliminary estimates of genotypic values at each of these QTL contradict the two oldest theories of heterosis, the dominance and overdominance hypotheses (see Crow 1998; Birchler et al. 2010), since the heterozygotes are like the inferior inbred genotype at each QTL. With the genome sequence in hand, we can begin to examine the several hundred genes in the vicinity of each peak (out of a total of 28,027; Zhang et al. 2012) for their potential role in regulating growth physiology (Applebaum et al. 2014). Such fundamental knowledge could lead to the development of biomarkers that could be used to predict which cross will produce the best hybrids early in the life cycle, saving much effort in progeny testing.

Finally, crossbreeding offers a path to the improvement of triploid oysters (Piferrer et al. 2009) and ultimately the tetraploid broodstocks that are used to generate triploids for production (Allen 2011). First, it should be noted that, in plants, heterosis is "progressive" in polyploids, i.e. it increases with the diversity of genomes in polyploids (Birchler et al. 2010); for yield or hardiness of tetraploid plants, ABCD > AABB > AAAB. Previously, tetraploid oysters were made from triploid females (Guo and Allen 1994; McCombie et al. 2005), which were derived from chemical inhibition of the second meiotic division of a fertilized egg. If the female used for chemical induction of triploidy were a hybrid (AB) and the fertilizing sperm were provided by a male from an unrelated but well-combining inbred line (CC), then the triploid progeny would be a mix of AAC, BBC, and ABC, depending on the distance of genes from the centromere on each chromosome. It would be difficult to optimize the heterosis in such a mix and to transfer it to the tetraploid level. Recently, however, a new method for making tetraploids directly from diploid parents has opened up a promising new path for inducing heterosis in tetraploid stocks (Benabdelmouna and Ledu 2015).

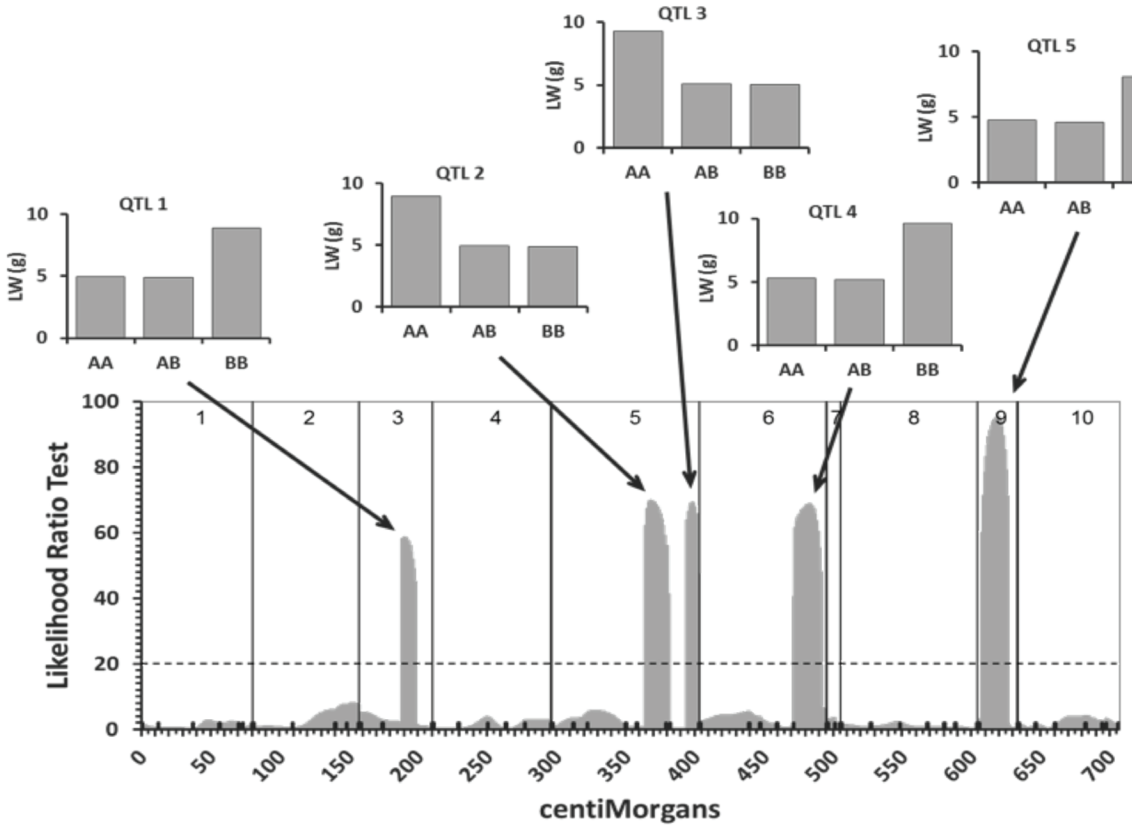

Figure 4. A Quantitative Trait Locus (QTL) map for live weight at 10 months of age in an F2 family of the Pacific oyster. The x-axis of the lower panel represents the linkage map of the oyster, in centiMorgans, across the 10 chromosomes; the black tick marks show the locations of the 53 genetic markers for which each of 376 individuals was typed. The Y-axis gives a measure of the statistical association between individual genotypes at each position of the genome and individual live weight; the dotted line is a threshold for significance. Estimates of genotype live weights at the five major QTL peaks shows that the heterozygote is in all cases like the smaller homozygote, a result that fits neither the dominance nor the overdominance theories of hybrid.

CONCLUSIONS

1. Marine fish and shellfish can undoubtedly be improved by selective breeding. Because broodstock are still being obtained from natural populations in many cases, traits of importance in production are likely to have substantial additive genetic variance and will respond to selection.
2. The periodic life history of oysters and other marine animals presents a set of unique challenges to selection, such as defining the units of selection, determining breeding values in the face of competition in communal rearing, inbreeding depression, and high variance in reproductive success, leading to small effective population sizes.
3. Oysters and perhaps other highly fecund marine fish and shellfish can also be improved by crossbreeding, which can capture substantial, non-additive, genetic variance in yield and other traits. Hybrid vigor has been documented in the Pacific oyster, but there are few studies to date on inbreeding and crossbreeding of other bivalve molluscs and marine fish. Crossbreeding also offers a path to improvement of polyploid varieties, which are useful in production and in preventing reproductive interactions between farmed and wild populations.
4. There are no major technical impediments to domesticating and improving farmed marine fish and shellfish, but disciplined breeding programs are needed to conserve natural biodiversity and make marine aquaculture sustainable in the long run.
5. Genomic tools are helping to accelerate improvement, by revealing mechanisms underlying heterosis and other traits, which may, in turn, lead to the development of biomarkers for identifying superior combining ability early in the life cycle.

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This paper describes the research necessary for revealing the physiological and metabolic mechanisms underlying resilience in the face of global climate and ocean change. The Pacific oyster is a model system, because it has well developed genetic and genomic resources for fundamental and applied research into complex phenotypes, such as survival, growth, sex determination, and disease and stress resistance.

Hedgecock D. and J. P. Davis. 2007. Heterosis for yield and crossbreeding of the Pacific oyster *Crassostrea gigas*. Aquaculture 272S1:S17–S29.

This paper provides definitive evidence, from diallel or factorial crosses among inbred lines of the Pacific oyster, that heterosis (hybrid vigor) for yield is pervasive. This same phenomenon underlies genetic improvement programs for yields of corn, rice, and other major crops. Additive genetic effects on yield, the basis for selection, exist but generally account for smaller proportions of yield variance than non-additive genetic components. Non-additive genetic effects include special combining abilities and reciprocal effects, which include maternal effects and non-maternal, nuclear-cytoplasmic interaction effects. These findings strongly suggest that crossbreeding should be used to improve the yield of farmed Pacific oysters and, by implication, the yield of many highly fecund marine fish and shellfish.

Hedgecock D. and A. I. Pudovkin. 2011. Sweepstakes reproductive success in highly fecund marine fish and shellfish: A review and commentary. Bull. Mar. Sci. 87: 971-1002.

Sweepstakes reproductive success, whereby a fraction of the spawning population contributes the vast majority of recruits, occurs in natural populations of highly fecund marine metazoan animals. The same process occurs readily in hatchery-propagated populations used for supplementation or aquaculture. Typical practices in aquatic hatcheries may rapidly degrade the genetic diversity of hatchery-propagated populations, to the long-term detriment of these resources.

Plough L. V. and D. Hedgecock. 2011. QTL analysis of stage-specific inbreeding depression in the Pacific oyster *Crassostrea gigas*. Genetics 189: 1473-1486.

Pacific oysters show inbreeding depression for survival and growth. In this study, we show that a substantial proportion of early mortality, usually greater than 90%, can be attributed to selection against deleterious mutations. Each oyster carries about 12 lethal mutations. Half of the genotype-dependent mortality occurs during metamorphosis. This genetic load on early mortality has implications for breeding efforts that focus on early life-history stages.

Zhang, G., X. Fang, X. Guo, L. Li, R. Luo, F. Xu, P. Yang, L. Zhang, X. Wang, H. Qi, Z. Xiong, H. Que, Y. Xie, P. W. H. Holland, J. Paps, Y. Zhu, F. Wu, Y. Chen, J. Wang, C. Peng, J. Meng, L. Yang, J. Liu, B. Wen, N. Zhang, Z. Huang, Q. Zhu, Y. Feng, A. Mount, D. Hedgecock, Z. Xu, Y. Liu, T. Domazet-Lošo, Y. Du, X. Sun, S. Zhang, B. Liu, P. Cheng, X. Jiang, J. Li, D. Fan, W. Wang, W. Fu, T. Wang, B. Wang, J. Zhang, Z. Peng, Y. Li, N. Li, J. Wang, M. Chen, Y. He, F. Tan, X. Song, Q. Zheng, R. Huang, H. Yang, X. Du, L. Chen, M. Yang, P. M. Gaffney, S. Wang, L. Luo, Z. She, Y. Ming, W. Huang, S. Zhang, B. Huang, Y. Zhang, T. Qu, P. Ni, G. Miao, J. Wang, Q. Wang, C. E. W. Steinberg, H. Wang, N. Li, L. Qian, G. Zhang, X. Liu, Y. Li, Y. Yin, and J. Wang. 2012. The oyster genome reveals stress adaptation and complexity of shell formation. Nature 490:49-52

This paper reports completion of a draft genome-sequence for the Pacific oyster, which has a very high level of sequence polymorphism and a high redundancy of genes for resistance to environmental stress.

Assessment of Fertilization Ability of Cryopreserved Sperm in Fish Using Interspecific Hybridization

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ABSTRACT

Fertilization ability of cryopreserved sperm of the yellowtail *Seriola quinqueradiata* (YT) in interspecific hybridization was examined to increase opportunities for assessment of the sperm. At first, we examined the appropriate concentration of dimethyl sulfoxide (DMSO) ranging from 1 to 20% with artificial seminal plasma. The activity of frozen-thawed sperm was evaluated after 24 h. A frozen-thawed sperm of 10% DMSO was shown in the highest sperm activity (73.3 ± 3.3%; mean ± SE) and corresponded to more than 80% of the activity of the pre-freeze sperm (83.3 ± 6.7%). To assess the fertilization capacity of the frozen-thawed sperm of YT, which is not easy to collect high quality eggs frequently, we examined artificial fertilization using longtooth grouper *Epinephelus bruneus* (LG) eggs; (1) LG x LG (female x male) and (2) LG x YT. The fertilized eggs of LG x YT were developed and hatched as similar with those of LG x LG. The rates of fertilization, embryogenesis and hatching in LG x YT were 39.5, 2.5 and 0.8%, whereas those in LG x LG were 51.5, 20.9 and 19.9%, respectively. Although the rates of embryogenesis and hatching in LG x YT was lower rates than those in LG x LG, the rate of fertilization in LG x YT was comparatively high and corresponded to approximately 75% of it in LG x LG. Relative DNA contents of 29 individuals in LG x YT were confirmed to be diploid and shown in the intermediate value between both species. That is, the cryopreserved sperm has a fertilization capacity, suggested that it can be assessed its fertilization capacity by using a rate of fertilization even in the case of interspecific hybridization.

INTRODUCTION

Yellowtail *Seriola quinqueradiata* is one of the most important target species for aquaculture in Japan, and corresponds to about 60% of the aquaculture production of marine fish. However, most of the seedling supply of yellowtail for commercial aquaculture depends on wild fish (Morita et al. 2012). The development of techniques for artificial seedling production of yellowtail has recently been initiated at Fisheries Research Agency (FRA) or a few Japanese fish farms, but the numbers are insufficient for the contributions to aquaculture. To activate the aquaculture industry of yellowtail, FRA has initiated the control of spawning season and the breeding program to develop yellowtail strains with commercially desirable traits (e.g. disease resistant and fast growth strains).

Because yellowtail is generally reared in sea cages and requires several years to sexual maturation, there is a risk of loss of parental fish through infectious disease, red tide problems or accidents in rearing (Morita et al. 2012). Therefore, it is important to establish the preservation and restoration method for the developed strain. So far, cryopreservation of sperm (reviewed in Suquet et al. 2000; Cabrita et al. 2010) and germ cells (Lee et al. 2013) have been more or less successful although cryopreservation of fish embryos and mature oocytes have not yet (Mazur et al. 2008), as result of their large size, high yolk content and low membrane permeability (Hagedorn et al. 1996, 1998). Especially, cryopreservation of fish sperm has been developed (Holtz 1993; Babiak et al. 1997; Dreanno et al. 1997; Chen et al. 2004) and widely used to preserve sperm in many fish species (reviewed in Suquet et al. 2000; Cabrita et al. 2010). However, there is no report on cryopreservation of yellowtail sperm. We have tried to develop it, but the viewpoint was limited to the kinds of extenders or cryoprotectants, rather than the appropriate concentration of cryoprotectant.

In the present study, to develop the cryopreservation of yellowtail sperm, we examined the appropriate concentration of dimethyl sulfoxide, which is used as an effective cryoprotectant. Then, to evaluate the fertilization capacity of frozen-thawed yellowtail sperm, we tried to apply an interspecific hybridization using longtooth grouper, as the eggs are easily collectable, at Kamiura Laboratory in FRA. In contrast, yellowtail eggs are not easily collected, even

in the spawning season. Indeed, there is a risk of the evaluation for a fertilization capacity using interspecific hybridization because it sometimes results in a fatality (Morishima et al. 2002). However, if our challenge was successful, this kind of method would be expected as a highly versatile technology for other fish species.

MATERIALS AND METHODS

Dilution and freezing of sperm

Each sperm from three males of yellowtail (YT) was diluted 1:9 in artificial seminal plasma (ASP, Fujinami et al. 2003) containing 1, 5, 10, 15 and 20% dimethyl sulfoxide (DMSO, Sigma-Aldrich Inc., St. Louis, MO). The diluted sperm was loaded into 0.5 ml straw tube (Fujihira Industry Co., Ltd., Saitama, Japan) and enclosed by straw powder (Fujihira Industry Co., Ltd., Saitama, Japan). The straw tubes filled with diluted sperm were placed on dry ice plate for 10 min, and then plunged into liquid nitrogen. After storage for 24 h in liquid nitrogen, the frozen sperm was thawed in a beaker at 20°C for 30 s for its activity observation.

Evaluation of fertilization capacity

After two months storage in liquid nitrogen, the cryopreserved sperm of YT was thawed in a beaker at 20°C for 30 s for a fertilization test. Fertilization test was performed using frozen-thawed YT sperm and fresh longtooth grouper (LG) sperm (diluted 1:9 in ASP) with LG eggs. Five g of eggs (about 10,000 eggs) was fertilized with each sperm (approximately 1.0 x 10^9 sperms) and gently mixed before activation with 500 ml seawater. The floating eggs were transferred into net cages and incubated at 20°C. The rate of fertilization, embryogenesis and hatching was evaluated at 2-4 cells, embryo and hatched larval stage, respectively.

Sampling and ploidy determination

To determine the ploidy of LG x YT larvae, a total of 71 individuals (30 individuals in LG x LG, 29 in LG x YT and 12 in YT x YT) were sampled. Note that hatched larvae in YT x YT were collected at a different schedule. All individuals were subjected to flow cytometry for relative DNA content of whole body cells in order to determine their ploidy status. Wheile the YT x YT individual adjusts a value of 70 using the flow cytometer PA (Partec GmbH, Germany), the LG x LG individual exhibits a value of approximately 100 due to the difference of relative DNA content. Therefore, the LG x YT should be theoretically their intermediate values of approximately 85. All samples were measured for relative DNA content by excitation in the near ultraviolet (UV) range from a mercury lamp of the flow cytometer PA (Partec GmbH, Germany), after DAPI (4',6-deamidino-2-phenylindole) staining with a preparation kit for animal cells provided by the manufacturer of the flow cytometer (Partec GmbH, Germany).

RESULTS AND DISCUSSIONS

To determine the appropriate concentration of DMSO in YT sperm, the diluted sperms were cryopreserved in 1-20% DMSO concentrations with artificial seminal plasma. According to the observation after 24 hours, the rate of sperm activity was significantly different among each DMSO concentration (Kruscal-Wallis test; $F_{2,68}=1291.876$, $P < 0.001$, Scheffe's post hoc test, $P < 0.05$). The sperm frozen with 10% DMSO showed the highest sperm activity as compared with those with other DMSO concentrations (Fig. 1), and retained about 80% of the pre-freeze sperm activity. On the other hands, sperms frozen with 1 and 5% DMSO showed the aggregation of sperm after thawing, and their activities were very low (less than 3%, Fig. 1). The cryopreservation of sperm in some marine fish species has been available on 10-20% DMSO concentration (e.g. Wayman et al. 1997; Chen et al. 2004; see also reviewed in Suquet et al. 2000; Cabrita et al. 2010). Similar concentration was observed in the yellowtail sperm.

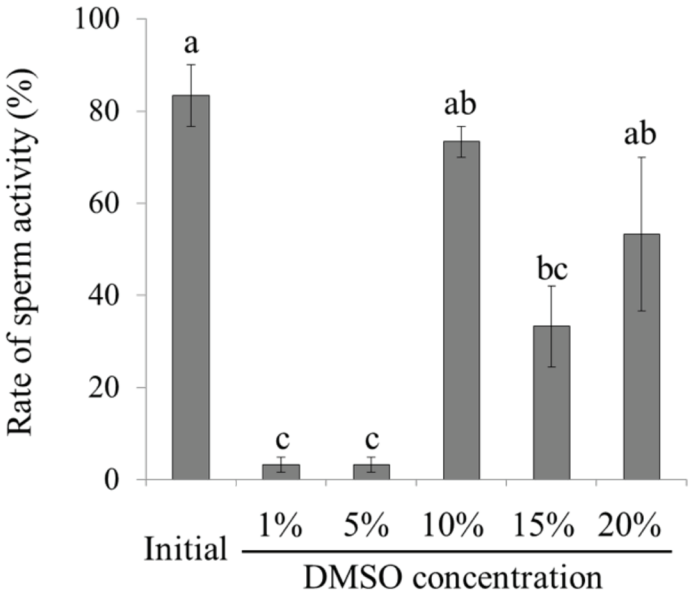

Figure 1. Comparisons of the rate of sperm activity in the yellowtail sperm frozen with different DMSO concentrations. Symbols with the same letter are not significantly different at P < 0.05 (Scheffe's post hoc test).

The rate of fertilization, embryogenesis and hatching in LG x LG were 51.5, 20.9 and 19.9%, whereas those in LG x YT were 39.5, 2.5 and 0.8%, respectively. Note that those in YT x YT examined at different schedule were 67.7, 51.0 and 49.9% for the same lot of

cryopreserved sperm and 83.9, 75.5 and 74.1% for fresh YT sperm, respectively (Y. Shimada, unpublished data). LG x YT showed the lower rates of embryogenesis and hatching than LG x LG, but the rate of fertilization in LG x YT was comparatively high. In addition, we found a morphological characteristic of LG x YT was the presence of more pigment cells like a melanophore than LG x LG (Fig. 2). However, all LG x YT larvae were observed by malformation (Fig. 2) and died within 12 h after hatching. This means that LG x YT was a fatality although it could be fertilized and hatched.

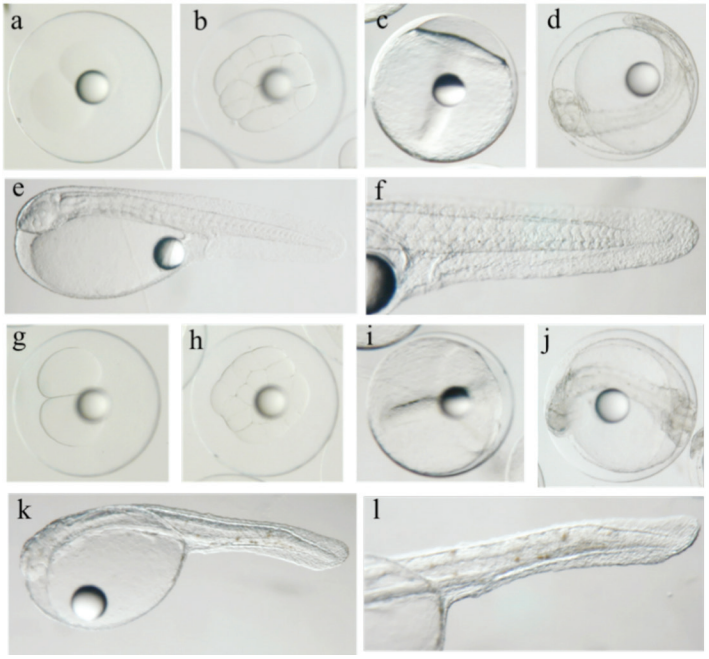

Figure 2. Developmental stages of LG x LG (a-f) and LG x YT (g-l); 2-cell (a and g), 16-cell (b and h), gastrulation (c and i), embryo (d and j), just hatched larva (e and k) and magnified photograph (f and l).

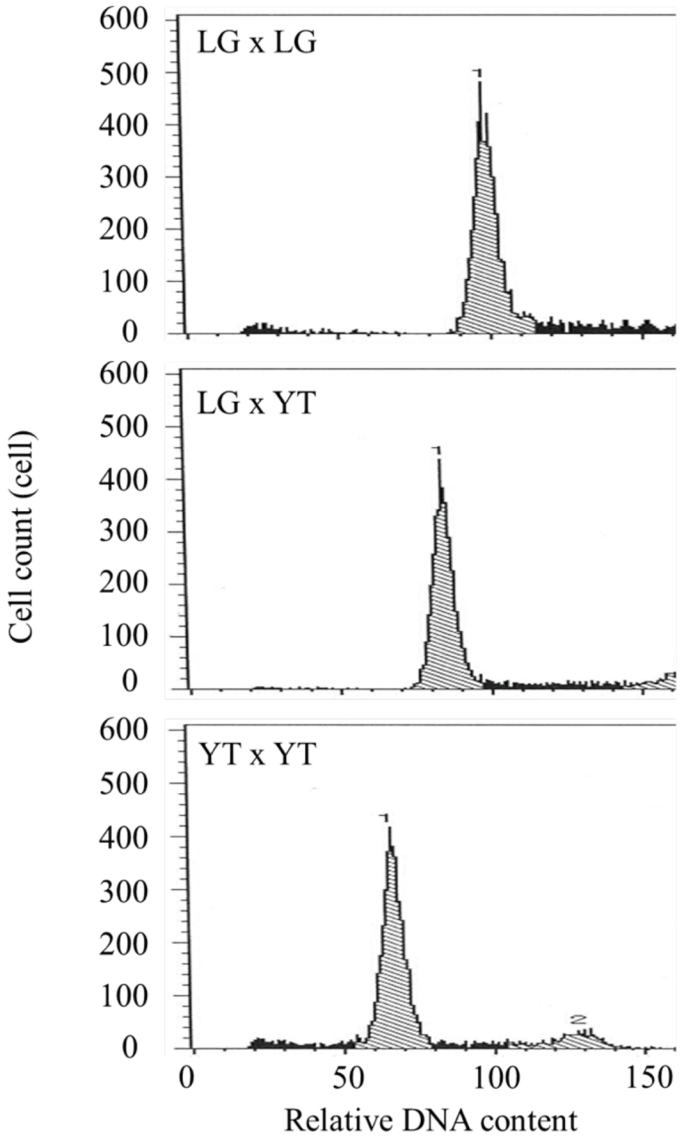

Figure 3. Example of relative DNA content in LG x LG, LG x YT and YT x YT.

To confirm that LG x YT is a hybrid fish, we performed the ploidy analysis using flow cytometry. We showed examples of relative DNA content of YT x YT, LG x YT and LG x LG (Fig. 3), which were 66.7 ± 0.4 (mean ± SE), 98.2 ± 0.4 and 82.8 ± 0.3, respectively (Fig. 4). The relative DNA content of each fish was significantly difference (one-way ANOVA; $F_{2,68}$=1291.876, $P < 0.0001$). We found that relative DNA content of LG x YT was the intermediate value between two species (Scheffe's post hoc test; $P < 0.0001$, Fig. 4). If a haploid individual was observed in LG x YT, it is thought to be the outcome of gynogenesis (embryogenesis initiated by only maternal chromosomes without syngamy), parthenogenesis (all female reproduction in the absence of sperm) or androgenesis (embryogenesis initiated by only paternal chromosomes without syngamy; Liu et al. 2001; Morishima et al. 2002). No haploid individuals have been observed in all sampled LG x YT individuals. Therefore, these results demonstrate that LG x YT is a hybrid fish. That is, we could develop the cryopreserved sperm of YT with a fertilization capacity. We also showed that it is possible to assess the fertilization capacity of YT cryopreserved sperm by rate of fertilization, even in interspecific hybridization.

In future studies, we aim to develop the large scale of cryopreserved sperm to apply to yellowtail hatcheries. In addition, we might be able to evaluate fertilization capacity of sperm from several fish species in one female if we found a marine fish species with more easily collected eggs.

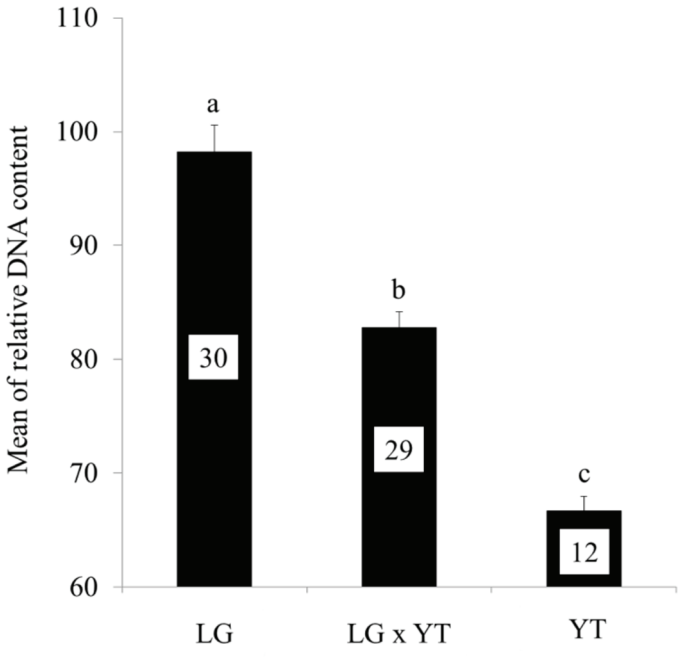

Figure 4. Mean of relative DNA contents in LG x LG, LG x YT and YT x YT. Symbols with the same letter are not significantly different at P < 0.0001 (Scheffe's post hoc test). Numbers in bar show sample size.

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M. Suquet, C. Dreanno, C. Fauvel, J. Cosson, and R. Billard. 2000. Cryopreservation of Sperm in Marine Fish. Aquac. Res. 31:231-243.

Since 1953, cryopreservation of sperm in fish has been performed on about 30 marine species. The paper reviews the techniques used and the results published in these species. The quality of frozen-thawed semen was evaluated using previously standardized biotests (i.e. fertilization capacity). Of the investigated cryoprotectants, dimethyl sulphoxide (DMSO) generally leads to the best results. Cooling rates range from 8 °C to 99 °C min-1; the thawing rate is generally high. Compared with freshwater species, a high percentage of sperm survives in cryopreservation. Therefore, the cryopreservation of marine fish sperm is suited for application in aquaculture.

T. Morita, N. Kumakura, K. Morishima, T. Mitsuboshi, M. Ishida, T. Hara, S. Kudo, M. Miwa, S. Ihara, K. Higuchi, Y. Takeuchi, and G. Yoshizaki. 2012. Production of Donor-Derived Offspring by Allogeneic Transplantation of Spermatogonia in the Yellowtail (*Seriola quinqueradiata*). Biol. Reprod. 86: 1-11.

Authors established a method for the allogeneic transplantation of yellowtail spermatogonia and the production of donor-derived offspring. Donor cells (i.e. type A spermatogonia) were collected from immature (10-month-old) yellowtail males, labeled with PKH26 fluorescent dye, and transferred into the peritoneal cavities of 8-day-old larvae. Fluorescence observation at 28 days post-transplantation revealed that PKH26-labeled cells were incorporated into recipients' gonads. To assess whether donor-derived spermatogonia could differentiate into functional gametes in the allogeneic recipient gonads, gametes collected from nine male and four female adult recipients were fertilized with wild-type eggs and sperm. Analysis of microsatellite DNA markers confirmed that some of the first filial (F) offspring were derived from donor fish, with the average contribution of donor-derived F_1 offspring being 66% and the maximum reaching 99%. These findings confirmed that authors' method was effective for transplanting yellowtail spermatogonia into allogeneic larvae to produce donor-derived offspring.

S. Lee, Y. Iwasaki, S. Shikina, and G. Yoshizaki. 2013. Generation of Functional Eggs and Sperm from Cryopreserved whole testes. Proc. Nat. Acad. Sci. 110: 1640-1645.

The conservation of endangered fish is of critical importance. Cryobanking could provide an effective backup measure for use in conjunction with the conservation of natural populations; however, methodology for cryopreservation of fish eggs and embryos has not yet been developed. Authors established a methodology capable of deriving functional eggs and sperm from frozen type A spermatogonia (ASGs). Whole testes taken from rainbow trout were slowly frozen in a cryomedium, and the viability of ASGs within these testes did not decrease over a 728-d freezing period. Frozen-thawed ASGs that were intraperitoneally transplanted into sterile triploid hatchlings migrated toward, and were incorporated into recipient genital ridges. Transplantability of ASGs did not decrease after as much as 939-d of cryopreservation. Approximately half of triploid recipients produced functional eggs or sperm derived from the frozen ASGs and displayed high fecundity. Fertilization of resultant gametes resulted in the successful production of normal, frozen ASG-derived offspring. Feasibility and simplicity of this methodology will call for an immediate application for real conservation of endangered wild salmonids.

Genetic and Physiological Studies of Health and Fitness in Aquaculture-Reared California Yellowtail, *Seriola dorsalis*

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Keywords: *Seriola*, aquaculture, respiration, parentage analysis, swim tunnel, deformity

ABSTRACT

The California yellowtail (*S. dorsalis*) is a likely candidate for the future development and expansion of offshore commercial aquaculture in the southern California region. However, rearing methods for this and other *Seriola* species are still unreliable, resulting in highly variable survival rates and prevalence of physical malformations. The causes of, and solutions for, the variability and abnormalities in the offspring remain largely unresolved and are likely associated with several factors, such as nutritional imbalances (in the larvae, juveniles, and/or broodstock), culture conditions (e.g. light, temperature, salinity), and genetic background of the parents. To better understand the connection between individual brood fish and abnormal offspring, and to gain insight into possible causes of growth variation and deformities, we conducted genetic analyses of juvenile fish exhibiting deformities and extreme size variation. Juvenile *Seriola dorsalis* (43 to 50 days post hatching) were collected from three production runs at Hubbs-Sea World Research Institute (HSWRI) in the summer/fall of 2012. Three different types of juvenile fish were collected: 1) small and large fish (representing juveniles with different growth rates, 2) deformed fish (this included various skeletal or soft tissue abnormalities), and 3) randomly sampled fish (not sorted). Genetic samples from the 21 broodstock individuals were also collected. Genetic markers (16 nuclear microsatellites already optimized for this species) were used to evaluate parent-progeny relationships to test whether observed physical traits (e.g., size differences, various types of deformities) are associated with pedigree. In addition, sustained swimming capacity and oxygen consumption rates were measured for randomly sampled juvenile yellowtail (15-20 cm fork length) as additional fitness measures and compared to the performance of wild caught juveniles. Analyses are ongoing, but genetic analyses of juveniles sampled from HSWRI show significant differences in parental contribution between offspring with deformities, growth variation, and the randomly sampled offspring. Aquaculture-reared yellowtail also showed significantly lower maximum sustainable swimming speeds and higher oxygen consumption rates than wild caught individuals, indicating reduced aerobic fitness in farm-raised fish. Understanding the genetic and physiological processes contributing to yellowtail seed stock quality is of considerable value to hatchery and aquaculture facilities rearing this species. Information gained in this study can be used to inform hatchery decisions and potentially facilitate the culture of yellowtail at a larger and more cost-effective scale.

INTRODUCTION

Producing high quality and healthy fish larvae is key to facilitating successful aquaculture ventures, but with the small size and tremendous changes that occur between the hatching and juvenile stages, this aspect of the culture process is very difficult to control. On the west coast of the United States, the native California yellowtail, *Seriola dorsalis* (formerly *S. lalandi*), is considered a serious aquaculture candidate due to its high market demand and value. *Seriola* species are cultured globally, and collectively the *Seriola* production is valued at over one billion U.S. dollars annually (FAO 2012). Hatchery production of these species is rapidly growing but has been hindered by a propensity for deformities and growth heterogeneity developed during larval and early juvenile stages that limit production capacity and efficiency. Reportedly more common in reared than wild fish (Fraser and de Nys 2005), malformations in the larval and juvenile stages directly impact commercial aquaculture by reducing the market value of fish and requiring additional labor for manual sorting to remove these individuals (Nagano et al. 2007; Karahan et al. 2013). In addition

to market value and labor costs, deformities can also lead to diminished growth rates, poor feed conversion rates, and reduced survival (Fraser and de Nys 2005).

These deformities have been reported for a variety of aquaculture species including *Seriola* species (Cobcroft et al. 2004; Roo et al. 2010). Deformity rates for *S. dorsalis* at HSWRI have ranged from 25-40% depending on the cohort (Stuart and Drawbridge 2012), and mass culture in *S. rivoliana* has also been bottlenecked by unreliable production of juveniles as the result of poor spawns and low hatchery survival (Roo et al. 2010). Cranial malformations are common and include jaw deformities, incomplete or malformed opercula, and misshapen skulls. Other types of deformities include shortened (or perch-like) bodies and skeletal abnormalities. Additional internal deformities have been detected in several older juvenile yellowtail (4-6 months) reared at the Southwest Fisheries Science Center (SWFSC), including vertebral compression, vertebral fusion, and extra-ossification of vertebrae in the caudal region.

Extreme variation in larval/juvenile growth rates is another key consideration in production. Variable growth rates lower the survival of smaller larvae/juveniles as a result of cannibalistic predation and physical trauma from larger fish but also increase mortality in larger fish from choking on their smaller cohort members. Growth heterogeneity among larvae is already apparent a few days post-hatching and becomes more pronounced over time. Without size grading, growth heterogeneity results in larvae and early juveniles of the same age being at different developmental stages, often requiring different feeds and culture conditions.

Many factors influence the health, fitness, and survival of larval/juvenile fish; these factors include non-genetic or genetic parental effects on larvae, environmental conditions, and any number of culture methods (Trippel et al. 2005; Brown et al. 2011; Nuñez et al. 2011). In an effort to improve California yellowtail culture, we are investigating several factors that may be responsible for variable physical fitness in the larval and juvenile stages. Spawning dynamics of the broodstock population, and parentage analyses of juvenile fish with physical abnormalities and growth heterogeneity were examined using molecular microsatellite markers. Relative aerobic fitness between cultured and wild juveniles was also investigated using key physiological measures.

METHODS

Spawning dynamics and parentage analyses

Whole juvenile fish (F1 generation) between 43 and 50 days post hatching (dph) were sampled during three production runs at HSWRI in June, August, and September of 2012. Samples of these fish included a randomly sampled group, juveniles with extreme size variation (very small or large), and juveniles with different categories of deformities. Size of the fish varied by production run. Small fish averaged 20.3 (+/- 1.9) mm, 29.5 (+/-1.4) mm, and 21.3 (+/- 1.5) mm, while large fish averaged 36.3 (+/- 2.9) mm, 53.6 (+/-4.2) mm, and 49.5 (+/- 5.1) mm in June (43dph), August (50 dph), and September (43 dph) production runs, respectively. Approximately 1,450 juvenile fish were sampled and included in the analyses. A total of 986 juveniles were examined for variability in the production runs, with 225, 176, and 585 fish sampled in June, August, and September, respectively. For the size comparisons, 191 large fish and 222 small fish were compared to a randomly collected sample of 245 fish. A total of 195 juveniles were identified as having deformities; these deformities included: head deformities (n=3), incomplete operculum (n=125), jaw deformities (n=44), skeletal deformities (n=13), and other non-specified malformations (n=10). The parentages of the juveniles with deformities were also compared to a randomly collected sample of 142 juvenile fish. The broodstock fish were also sampled for genetics during yearly health checks using small, non-lethal fin clips.

Genomic DNA was extracted from the samples using Chelex® resin according to the manufacturer's protocol. The broodstock population was genotyped using 15 microsatellite markers developed for Seriola (Porta et al. 2009; Renshaw et al. 2007, 2006; Ohara et al. 2005, 2003; Nugroho and Taniguchi 1999). Juvenile yellowtail were genotyped with the same 15 microsatellites or a subset of nine microsatellites following parentage analysis optimization. Samples were analyzed on an ABI 3730XL Genetic Analyzer (Applied Biosystems) and fragment data were analyzed using GENEMAPPER 4.0 (Applied Biosystems) and scored visually.

Swim tunnel experimentation

Once large enough (15-20 cm FL), the health and aerobic fitness of aquaculture-reared yellowtail were assessed in comparison to individuals of the same size collected from the wild by examining maximum sustainable swimming speed and oxygen consumption rates at varying swim speeds. This was done using a variable speed, 5.4 l swim tunnel

respirometer (Loligo Systems, Tjele Denmark) with a 30x7.5x7.5 cm working section. Fish were placed in the swim tunnel and allowed to swim steadily at a preferred, low, sustainable speed (typically about 20-25 cm s^{-1}) with a continuous inflow of filtered seawater for at least 1 hour prior to experimentation.

The maximum sustainable swimming speed (critical swimming speed, U_{crit}) was determined by increasing the velocity of the swim tunnel water by 4-10 cm s^{-1} steps every 30 minutes until the fish could no longer maintain steady swimming (defined as no longer being able to stay off the screen at the back of the working section). U_{crit} was determined by the equation:

$$U_{crit} = U_i + [(T_i / T_{ii}) U_{ii})] \quad (1)$$

where U_i is the highest swimming speed maintained for the full 30 minutes, T_i is time swimming at the highest velocity before fatigue, T_{ii} is time step length of each swimming speed (30 min), and U_{ii} is the velocity step (4-10 cm s^{-1}). Swim tunnel water velocity was corrected for solid blocking effects of the fish according to Bell and Terhune (1970).

Oxygen consumption rates (M_{O2}) were determined for each fish at each swimming speed using a fiber optic oxygen sensor connected to a Fibox 3 fiber optic oxygen transmitter (PreSens Precision Sensing GmbH, Regensburg Germany). Shortly (1-2 min) after the each step increase in swimming speed, the respirometer was temporarily closed to the inflow and outflow of fresh seawater and the rate of oxygen consumption was observed until the oxygen level of the swim tunnel water reached approximately 80% saturation, at which point the system was flushed (to return the oxygen saturation level to near 100%) and then M_{O2} measurements were repeated. Typically, 2-3 measurements could be made during each 30 min velocity step. These rates were averaged and counted as a single consumption rate for each fish at each swimming speed. Regression lines were determined for M_{O2} vs. swimming speed and used to extrapolate to a swimming speed of 0 cm s^{-1} to determine resting metabolic rate. Metabolic rates for all fish were adjusted to a temperature of 18°C for comparison using $Q_{10} = 2$.

Wild-caught fish tested for U_{crit} and metabolic rate in comparison to aquaculture-reared individuals were captured by hook and line from local waters (<20 km from shore) typically associated with drifting kelp. Upon transfer to the Southwest Fisheries Science Center these fish were housed in identical tanks and were fed the same diet as aquaculture-reared individuals. Wild caught fish were held 2-4 months in captivity before swim tunnel experimentation.

RESULTS AND DISCUSSION

Spawning dynamics and parentage analyses

To help make the other analyses more informative, we identified and/or confirmed the sexes of the broodstock population. We used biparentally inherited microsatellites to identify the two parents, and then used maternally inherited mitochondrial sequences to identify the maternal parent. This could be done for any broodstock fish for which we had sampled at least one of their offspring. With this information, we were able to look at spawning dynamics in the broodstock population. Among the juveniles sampled across the three production runs, fewer females than males participated in the spawning events (Fig. 1). The contribution of males also appeared to be more evenly distributed among the broodstock fish, while certain female broodstock individuals accounted for most of the production in the three sampled runs.

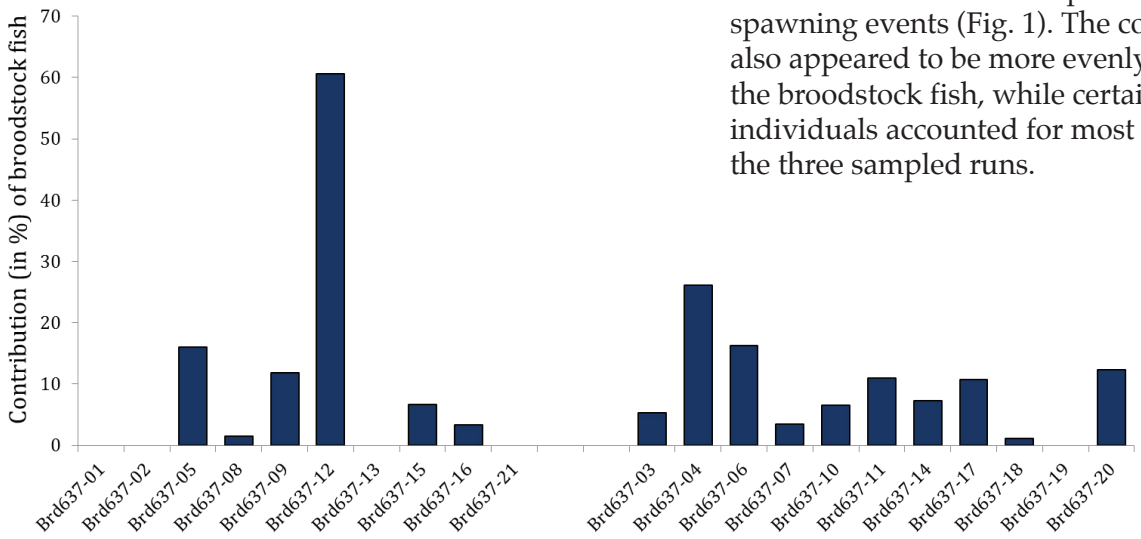

Figure 1. Contributions (in percent) of individual broodstock fish to juvenile offspring collected from three production runs (n=986). Female broodstock are on the left of the figure, males are on the right.

When parental contribution was examined for each of the three production runs, it appears that only two females contributed to each event, with one contributing more dominantly (Fig. 2). Again, male contribution to offspring is spread out among a greater number of fish, although contribution is not even among the males.

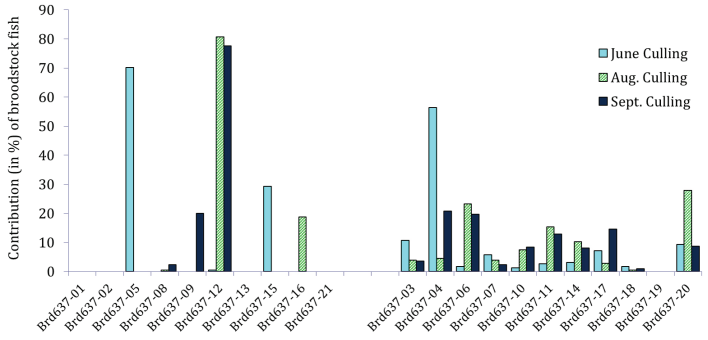

Figure 2. Contributions (in percent) of individual broodstock fish to juvenile offspring collected for three different production runs: June (n=225), August (n=176), and September (n=585). Female broodstock are on the left of the figure, males are on the right.

Individual broodstock fish contribution to offspring exhibiting size heterogeneity was also investigated using parentage analyses. For each sex, broodstock contribution to large or small offspring was compared to a randomly sampled collection of juveniles. Certain female broodstock fish, for example, did significantly

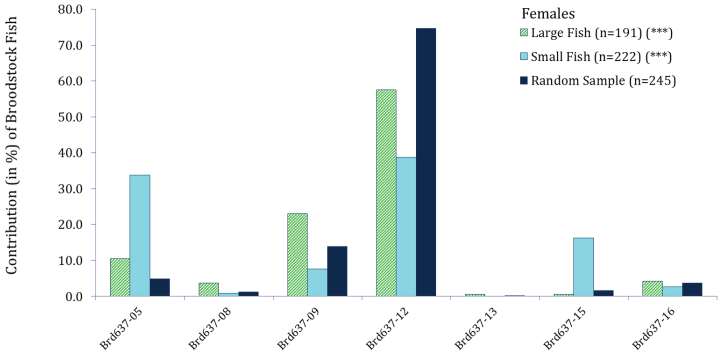

Figure 3. Contribution (in percent) of female broodstock to large (n=191) and small-bodied (n=222) offspring compared with a random sample of juveniles (n=245).

(Chi-square $P < 0.001$) disproportionally contribute to small (e.g., Brd637-05 and Brd637), and to large offspring (e.g., Brd637-09) (Chi-square $P < 0.001$) (Fig. 3). Contributions of individual broodstock fish to juvenile offspring exhibiting various deformities were also examined. Again, particular parents did appear to disproportionately contribute to various deformities. Male parental contribution was significantly different (Chi-square $P < 0.001$) for all deformities compared to the random juvenile sample.

For example, certain males (e.g., Brd637-20 and Brd637-04) largely contributed to head deformities and skeletal, jaw, and operculum deformities, respectively (Fig. 4). However, the female parental contributions to deformities under the incomplete opercula category were not found to be significantly different from the random sample (data not shown).

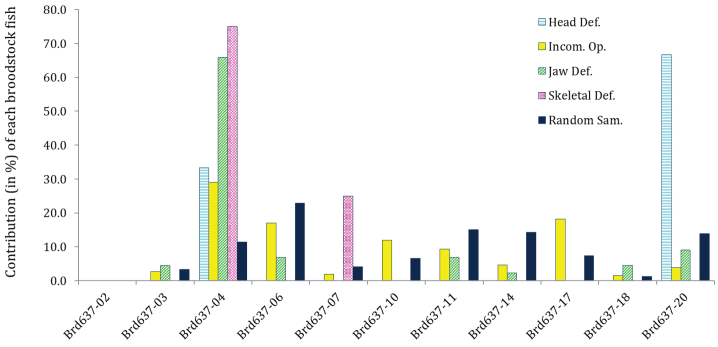

Figure 4. Contributions (in percent) of male broodstock fish to juvenile offspring with various deformities (head deformities (n=3), incomplete operculum (n=125), jaw deformities (n=44), skeletal deformities (n=13)) in comparison to a random sample of juveniles (n=142). Male parental contribution was significantly different (Chi-square $P < 0.001$) for all deformities compared to the random juvenile sample.

We also examined if parental broodstock pairs contributing disproportionately to offspring deformity pattern was detected when examining parental broodstock pairs. Certain broodstock pairs produced more deformed offspring (all deformities combined together) than others (Fig. 5) (no statistical test performed). While certain males may disproportionately contribute to deformed offspring (e.g., Brd637-04), they appear to do worse when paired with particular females (e.g., Brd637-04 with Brd637-05, and Brd637-04 with Brd637-12, shown below) (Fig. 5).

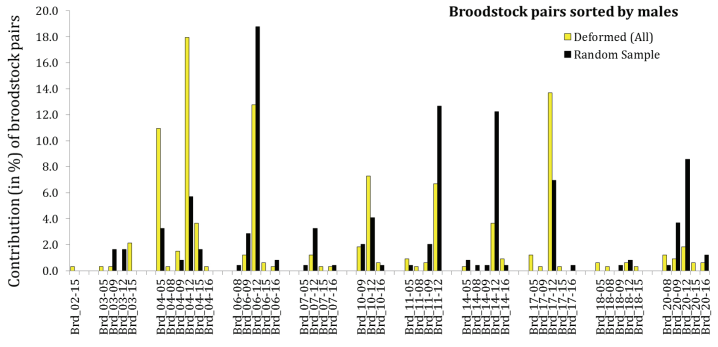

Figure 5. Contributions (in percent) of parental broodstock pairs to juvenile offspring with deformities (all deformities combined) (n=195) compared to a random sample of juveniles (n=142). The broodstock pairs listed here are sorted by individual male fish.

For a broadcast spawning species, such as yellowtail, genetic analyses are required to understand parental contribution to positive and negative traits in F1 populations used for commercial growout and can provide important insight into optimizing the composition of the broodstock population. However, it is important to note that although genetic tools provide useful metrics to examine physical fitness, it does not necessarily mean that there is a genetic component to this fitness. Any number of environmental factors or physical factors in the fish (e.g., age, health, nutritional status, and the genetic makeup of the individual) can influence the growth heterogeneity and deformities.

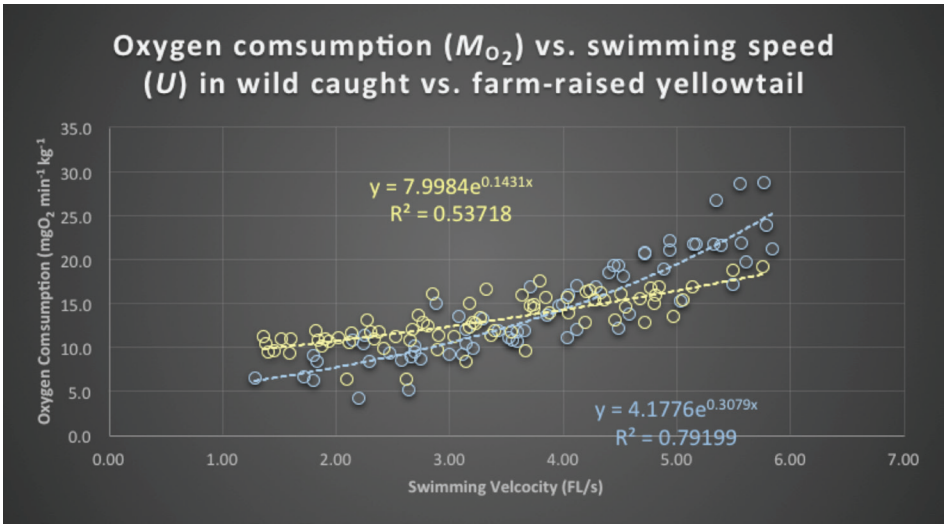

Figure 7: Oxygen consumption (M_{O2}) at varying swimming speeds (U) in 10 aquaculture-reared (yellow) and seven wild caught (blue) juvenile yellowtail, Seriola dorsalis, adjusted to 18°C using Q_{10} = 2. Swimming speeds are standardized in fork lengths per second.

Swim tunnel experimentation

To examine the fitness of juveniles without apparent deformities, physiological measures of maximum sustainable swimming speed and oxygen consumption were obtained in comparison to wild-caught juvenile yellowtail (Fig. 6). The mean maximum sustainable swimming speed for 10 aquaculture-reared yellowtail (17.3 ± 1.04 cm FL) was 82.06 ± 8.62 cm s^{-1} (4.90 ± 0.69 FL s^{-1}), which was not significantly slower than that determined for seven wild caught individuals of similar size (17.7 ± 1.11 cm, 95.16 ± 14.21 cm s^{-1}, 5.36 ± 0.63 FL s^{-1}). The relationships between M_{O2} and swimming speed for all aquaculture-reared and wild caught yellowtail are shown in Figure 7. Extrapolation to a swimming speed of 0 cm s^{-1} suggests that the resting metabolic rate of aquaculture-reared yellowtail (8.00 mgO_2 min^{-1} kg^{-1}) is much higher than that of wild caught fish (4.18 mgO_2 min^{-1} kg^{-1}). These higher metabolic costs indicate that aquaculture-reared fish likely have higher feed and water quality requirements. Thus, the physiological conditioning of young juveniles in aquaculture (e.g., raising fish under constant flow regimes to force continuous swimming) could potentially be used to increase juvenile fitness and potentially reduce production costs.

A

B

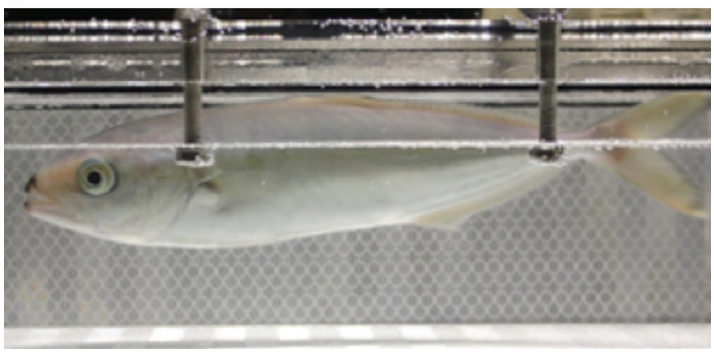

Figure 6: Representative images of aquaculture-reared (A) and wild caught (B) yellowtail swimming in the swim tunnel respirometer. Note the morphological differences in the fish.

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Nuñez, J., D. Castro, C. Fernández, R. Dugué, F. Chu-Koo, F. Duponchelle, C. García, and J.-F. Renno. 2011. Hatching rate and larval growth variations in *Pseudoplatystoma punctifer:* maternal and paternal effects. Aquaculture Research, 42: 764-775.

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Trippel, E.A., G. Kraus, and F.W. Köster. 2005. Maternal and paternal influences on early life history traits and processes of Baltic cod *Gadus morphua*.

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Brown, E.J., M. Bruce, S. Pether, and N.A. Herbert. 2011. Do swimming fish always grow fast? Investigating the magnitude and physiological basis of exercise-induced growth in juvenile New Zealand yellowtail kingfish, *Seriola lalandi.* Fish Physiol. Biochem., 37: 327-336.

The focus of this study was to investigate the effect of exercise on growth of cultured New Zealand yellowtail kingfish, *Seriola lalandi* at different water temperatures. The authors also examined potential sources of physiological efficiency to additionally improve exercise-induced growth. Two growth trials that exposed fish to various exercise levels (determined by current speed) were kept at 14.9° and 21.1°C. Lengths and weights were measured for each fish, along with oxygen consumption rates (later converted to metabolic rates) using a respirometer. Results of these trials showed that long term exercise yielded a 10% increase in growth but only in low flows (0.75 BL s-1) and at a water temperature of 21.1°C. Experiments using a swim flume respirometer indicated that exercise training had no effect on metabolic scope or critical swimming speeds but it did improve swimming efficiency. This study was of interest in working to improve fitness for cultured *Seriola lalandi*.

Nuñez, J., D. Castro, C. Fernández, R. Dugué, F. Chu-Koo, F. Duponchelle, C. García, and J.-F. Renno. 2011. Hatching rate and larval growth variations in *Pseudoplatystoma punctifer:* maternal and paternal effects. Aquaculture Research, 42: 764-775.

The authors evaluated paternal and maternal effects on hatching and growth on initial stages and during an early dry feed adaptation period in *P. punctifer* larvae. *P. punctifer* are a commercially important catfish species from South America that are of particular importance for aquaculture production. It was hypothesized that parental effects may influence larval heterogeneity, which is responsible for high levels of cannibalism and therefore mortality in the culture setting. Artificial reproduction methods were used to set up factorial breeding crosses in this study, for a total of nine families of full siblings. Results showed that parentage significantly influences growth and dry feed adaptation capabilities during the first 26 days post fertilization. This study highlights the importance of parentage and/or parentage-interactions on the fitness and survival of

larval offspring.

Trippel, E.A., G. Kraus, and F.W. Köster. 2005. Maternal and paternal influences on early life history traits and processes of Baltic cod *Gadus morhua*.

The roles of each gender in various life-history traits and overall fitness of their larval offspring has received increasing attention in aquaculture research. While maternal effects have been more commonly studied, the effects of males are not well-documented. The authors investigated the effects of both broodstock parents and bi-parental interaction on fertilization rate, hatching rate, larval traits, yolk utilization, and growth of Baltic cod during the non-feeding larval phase using a crossing design. They found that parental influences varied in their importance during different embryogenesis stages. Females had the greatest effects during early and late embryonic development, and male-female interaction was most important during mid-embryonic stages. Maternal effects influenced several traits and processes, however, paternal effects were only seen in paternal-maternal interactions. This research is relevant in our parentage studies of growth variation and deformities in the California yellowtail.

Demands for Infertility of Cultured Marine Fishes and Study of UV Irradiation at Developmental Stages In Japanese Flounder

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Keywords: infertility, ultraviolet, vasa, Japanese flounder

ABSTRACT

Various kinds of marine fish species are produced in Japan. There has been increasing needs for the technique to sterilize farmed fish, because the sterilization of cultured fish could protect the improved strains, prevent undesired gonadal development that often result in the deterioration of meat quality, and prevent possible negative genetic impact on wild populations. In this way, sterilization of farmed fish is beneficial not only to the aquaculture industry but also for the environmental conservation. However, the applicable techniques for sterilization have not been established in cultured marine fish.

Germ cells play an important role in transmitting genomic information to the next generation. It is known that germ cells develop into sperm in males and eggs in females and this differentiation begins during early embryonic development and concludes in adulthood with gamete differentiation. In this study we investigate using ultraviolet (UV) irradiation on fertilized egg of Japanese flounder, *Paralichthys olivaceus* to block germ cell differentiation.

We examined the effects of irradiation on the hatching rate of fertilized eggs. The UV was applied by a UV cross linker. Hatched larvae were reared until 55 days after hatching (dah), and the juveniles were embedded in paraffin, sectioned, stained using hematoxylin-eosin, for gonadal observations using light microscopy. We also examined the expressions of both *vasa* and *sycp3* (germ cell marker) mRNA in gonads using RT-PCR to indicate the presence of germ cells in the gonads.

The hatching rate decreased as the intensity of UV irradiation increased. The microscopic observation of the gonads revealed the abnormal gonads in the UV-irradiated group. The expression of *vasa* and *sycp3* mRNA was observed in the gonads of the control group indicating the presence of germ cells. On the other hand, the expression of *vasa* and *sycp3* mRNA was not detected in the gonad area of some of the UV irradiated larvae. These results indicate that germ cells may be deleted by UV irradiation of fertilized eggs resulting in flounder that are infertile.

INTRODUCTION

Germ cells play an important role transmitting genomic information to the next generation. It is known that germ cells develop into sperm in males and eggs in females and this differentiation begins during early embryonic development and concludes in adulthood with gamete differentiation. It is known that germ cells develop into sperm in males and eggs in females and this differentiation begins during during early embryonic development and concludes in adulthood with gamete differentiation. These germ cells are derived from primordial germ cells (PGCs) that segregate from the somatic cell lineage during early embryogenesis of organisms Saffman and Lasko 1999). PGCs proliferate and migrate to the developing gonad and become germline stem cells (Lin 1997). When an organism reaches sexual maturity these germ cells follow a unique pathway of spermatogenesis or oogenesis and differentiate into sperm or eggs respectively (Saffman and Lasko 1999).

UV and X-ray irradiation is known to damage DNA damage, arrest cell cycles and cause apoptosis (Iwamoto et al. 1999). Irradiation with X-rays can effectively reduce the number of endogenous PGCs in chickens (Nakamura et al. 2012). Moreover, ultrasound can reduce the total number of epididymal sperm by depleting spermatocytes and spermatids from testes (Tsuruta 2012). Methods to use irradiation to remove germ cells have been reported in experimental animals such as rats and chickens, however, these methods have not been reported in cultured fish.

The Japanese flounder (*Paralichthys olivaceus*) is a teleost fish that has been cultured worldwide. In the Japanese flounder germ cell meiosis marker *sycp3* cDNA has been isolated and the mRNA is expressed in the undifferentiated gonad. In early developmental stages such as during embryonic cleavage the germ cell marker *sycp3* mRNA expression has not been investigated because *sycp3* mRNA expression is initiated around 50~60 dah during sexual differentiation.. Therefore, in this study we investigated expression of the germ cell marker *vasa* mRNA in larvae hatching from flounder embryos treated with UV irradiation prior to hatching.

MATERIALS AND METHODS

Animals

Fertilized eggs were obtained from natural mating and spawning of Japanese flounder reared at 16 °C.

UV irradiation

The UV was applied by a UV cross linker (Ultra-Violet Products, Germany). The intensity of UV ranged from 0 to 200 mJ (millijoule) and the timing of irradiation were set at 4, 12, 28, and 52 hours post-fertilization. Hatched larvae were reared until 55 dah.

Histological analysis

Japanese flounder juveniles at 55 dah were fixed in Bouin's solution at 4 °C overnight, dehydrated in graded ethanol, embedded in paraffin, and serially sectioned at 5 μm thickness.

The sections were used for hematoxylin-eosin staining, immunochemical staining or in situ hybridization. The immunochemical staining and *in situ* hybridization were performed as described previously (Yamaguchi et al. 2007).

RT-PCR analysis

One microgram total RNA extracted from the juveniles using ISOGEN (Nippon gene, Tokyo, Japan) was reverse-transcribed using RNA PCR Kit (Applied Biosystems) at 42 °C for 30 min. The RT-PCR was performed as described previously (Yamaguchi et al. 2007).

RESULTS AND DISCUSSION

We examined the effects of UV irradiation of fertilized eggs on the hatching rate. The intensity of UV ranged from 0 to 200 mJ (Joule) and the timing of irradiation were set at 4, 12, 28, 52 hours post-fertilization. The percent survival of all groups is lowered in proportion to the intensity of the ultraviolet irradiation. Survival in the UV irradiated treatment at 4hpf was higher than the other groups (Fig. 1). These results indicate that the 4 hours post-fertilization 4cell stage embryo may have a relative resistance to UV irradiation.

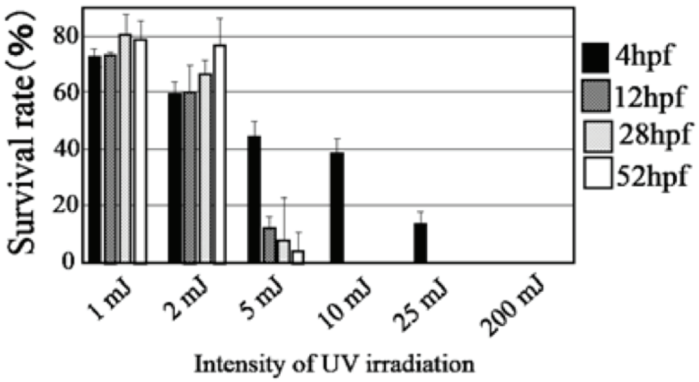

Figure 1. The survival rate of the UV irradiated embryos. The vertical axis is for percent survival, and the horizontal axis is the intensity of UV irradiation. Solid bar indicates the UV irradiated group at 4 hours after fertilization. Striped bar indicates UV irradiated group at 12 hours after fertilization, Dotted bar indicates UV irradiated group at 28 hours after fertilization, and white bar indicates UV irradiated group at 52 hours after fertilization.

To investigate effects of UV irradiation on the flounder's gonad the UV irradiated larvae were reared until 55 dah when the gonads are still sexually undifferentiated (Kitano et al. 1999). The juvenile gonads were sectioned, hematoxylin-eosin stained, and observed using light microscopy. This experiment resulted in two normal gonads in the control group (Fig. 2A), one normal gonad in the 4hpf 10mJ UV irradiated group (Fig. 2B) and no normal gonads could be observed in the 52hpf 5mJ group (Fig. 2C). The UV irradiation treatment during developmental stages induced abnormal gonad development in Japanese flounder.

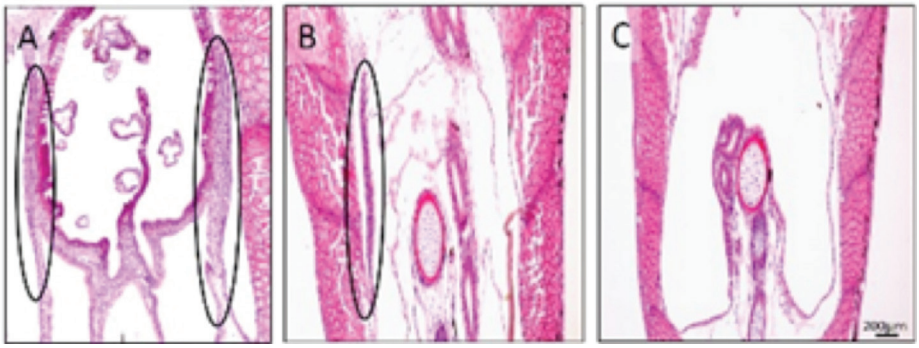

Figure 2. Effect of UV irradiation on the flounder's gonad. A: control (no UV irradiation). B: 10mJ UV irradiation at 4hpf. C: 5mJ UV irradiation at 52hpf. The circle indicates the gonad.

Immunochemical staining or *in situ* hybridization were used to investigate whether *vasa* gene and *sycp3* genes were expressed in the germ cells. *Vasa* protein and mRNA signals were found to be localized in the germ cells. As a result, the *vasa* or *sycp3* were used as a marker for germ cells in the gonad (Fig. 3A, 3B, and 3C). The *vasa* mRNA has been demonstrated to be a germ cell marker in many fish species, such as zebrafish (Olsen et al. 1997), tilapia (Kobayashi et al. 2000), and medaka (Shinomiya et al. 2000). Thus, the *vasa* gene is highly conserved and studied between vertebrate fish. *SYCP3* encodes a component of the synaptonemal complex, which is marker of meiotic prophase (Di Cario et al. 2000). It has been reported that *sycp3* is involved in gonadal differentiation during the sexual differentiation period Japanese flounder (Yamaguchi et al. 2012). To investigate the presence of germ cells in gonad tissue of UV irradiated flounder, we performed RT-PCR for germ cell specific marker *vasa* and *sycp3* mRNA. UV irradiated larvae were reared until 55 dah. Then the gonads were isolated, and RT-PCR analysis was performed. In the control group, the expression of germ cell specific markers *vasa* and *sycp3* mRNA were detected in all individuals. Whereas, in the UV irradiated group the expression of *vasa* and *sycp3* mRNA were not detected (Fig. 3D). These results indicate that germ cells may be deleted by UV irradiation of fertilized eggs.

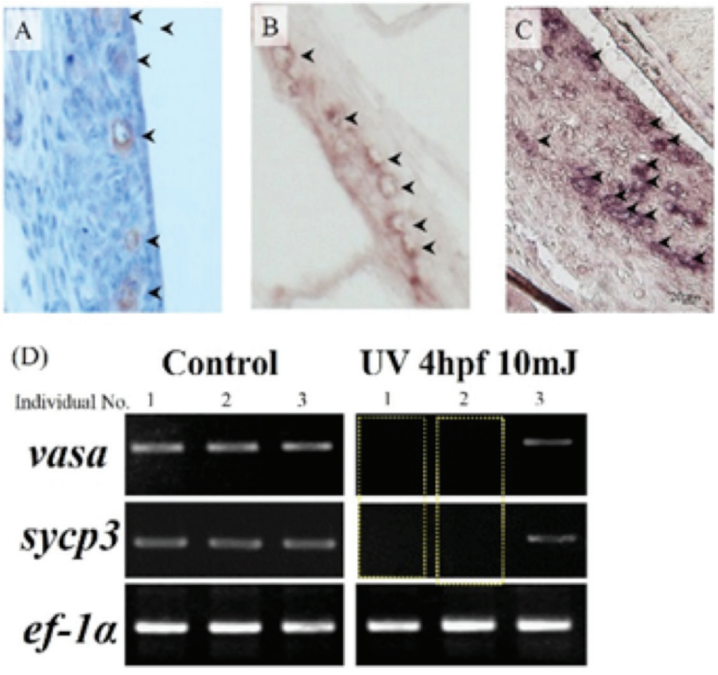

Figure 3. Expression pattern of the vasa or sycp3 gene in UV irradiated Japanese flounder. Immunochemical staining of vasa protein in juvenile flounder at 55dah (A). In situ hybridization analysis of vasa mRNA (B) or sycp3 (C) mRNA in juvenile flounder at 55dah. Arrowheads indicate vasa or sycp3 signal. (D) RT-PCR for germ cell specific marker vasa and sycp3 mRNA. The ef-1a (elongation factor 1 alpha) is internal control.

To investigate the rate of abnormality of gonads in UV irradiated flounders embryos were irradiated with the intensity of 10mJ at the 4-cell stage. The larvae were reared for 55 days after hatching for observation using light microscopy. The samples were Bouin's fixed, sectioned, hematoxylin-eosin stained, and gonads were observed. All larvae in the control group had two normal gonads (Fig. 4A), whereas 20% of larvae in the 4hpf 10mJ UV irradiated group had one normal gonad, and 65% of larvae had no normal gonads (Fig. 4B). This experiment conformed that UV irradiation at developmental stages induced abnormal gonad development in Japanese flounder.

In conclusion, in the most optimum treatment (UV irradiation 4hpf 10mJ), normal gonad development was been in 65% of the flounders. Moreover, the expression of vasa mRNA is not confirmed in UV irradiated flounders. These results indicate the possibility that germ cells were destroyed by UV irradiation and that that UV irradiation can be used to induce sterilization in flounder.

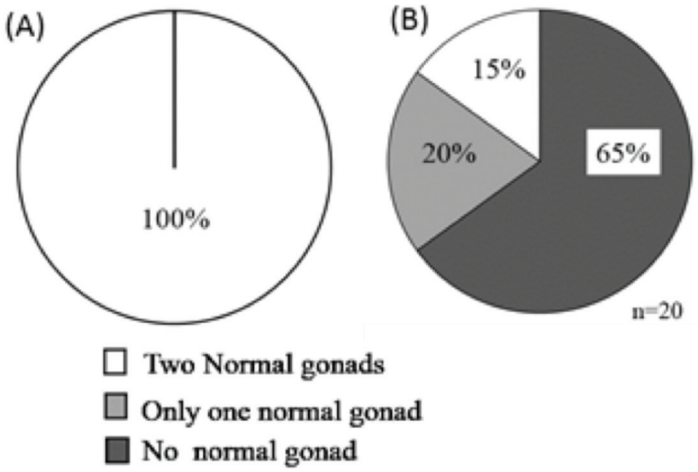

Figure 4. The rate of abnormality of gonads in UV irradiated flounders. UV irradiation intensity of 10mJ at the 4-cell stage. (A) Control receiving no radiation. (B) Gonadal status of 20 larvae at 55 dah observed with light microscopy.

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The Japanese flounder (*Paralichthys olivaceus*) is a teleost fish that has an XX/XY sex determination system. However, the XX flounder can be sex-reversed to phenotypic males by rearing the larvae at high or low water temperatures. Thus, sex in the flounder is determined by genotype plus temperature effects. Therefore, the flounder provides an excellent model to study the molecular mechanisms underlying temperature-dependent sex determination. We previously showed that cortisol, the major glucocorticoid produced by the interrenal cells in teleosts, causes female-to-male sex reversal by directly suppressing mRNA expression of ovary-type aromatase (cyp19a1), a steroidogenic enzyme responsible for the conversion of androgens to estrogens in the gonads. In the present study, we found that exposure to high temperature during gonadal sex differentiation upregulates the mRNA expression of retinoid-degrading enzyme (cyp26b1) concomitantly with masculinization of XX gonads and delays meiotic initiation of germ cells. We also found that cortisol induces cyp26b1 mRNA expression and suppresses specific meiotic marker synaptonemal complex protein 3 (sycp3) mRNA expression in gonads during the sexual differentiation. In conclusion, cyp26b1 expression in gonads is in male-specific manner during gonadal sex differentiation and delays meiosis onset not only in mouse, chick and amphibian, but also in a teleost with TSD. Therefore, CYP26B1 appears to regulate germ cell fate by controlling retinoic acid signaling in a manner common among vertebrates.

Yamaguchi T., N. Yoshinaga, T. Yazawa, K. Gen, and T. Kitano. 2010. Cortisol is involved in temperature-dependent sex determination in the Japanese flounder. Endocrinology, Endocrinology 151:3900–3908.

The Japanese flounder (*Paralichthys olivaceus*) is a teleost fish with an XX/XY sex determination system. However, XX flounder can be induced to develop into either phenotypic females or males, by rearing at 18 or 27 °C, respectively, during the sex differentiation period. Cytochrome P450 aromatase, the product of the gene cyp19, is expressed in various tissues and plays an important physiological role in the regulation of estrogen biosynthesis. Recently, it has been reported that, in nonmammalian vertebrates, the expression of cyp19 mRNA in the ovary is very much higher than in the testis during the period of gonadal sex differentiation. In this sutudy, we demonstrated that cortisol causes female-to-male sex reversal by directly suppressing cyp19a1 mRNA expression via interference with cAMP-mediated activation and that metyrapone (an inhibitor of cortisol synthesis) inhibits 27 °C-induced masculinization of XX flounder. Moreover, cortisol concentrations in 27 °C-reared juveniles were significantly higher than in 18°C-reared fishes during sexual differentiation. In conclusion, this study has presented the evidence of involvement of cortisol in TSD. Cortisol induced the masculinization of XX fish by directly suppressing cyp19a1 mRNA expression via interference with the cAMP-mediated activation. These findings provide new insights into to the elucidation of molecular mechanisms underlying environmental sex determination, including TSD.

RNAseq Analysis of Early Larval Development in *Seriola lalandi* with Emphasis on Differential Development of Digestive System in Fast and Slow Growing Groups

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Key words: *Seriola lalandi*, aquaculture, genomics, RNAseq, growth

ABSTRACT

Seriola lalandi is considered a prime candidate for aquaculture development in southern California. In the U.S., hatchery production of both *S. lalandi* and *S. rivoliana* is rapidly growing but has been hindered by a propensity for deformities and growth heterogeneity developed during larval and early juvenile stages that limit the production capacity and efficiency. The causes of and solutions for the growth variation and high deformity rates remain unresolved. Our aim here is to characterize development of physiological systems important to aquaculture and understand differences in gene expression between size classes that correlate with size heterogeneity. We performed an RNAseq experiment to characterize differences in gene expression between fast and slow growing larval *S. lalandi* at 2, 7 and 17 days post hatch (dph). Each developmental stage was represented by three biological replicates, and each biological replicate was comprised of mRNAs drawn from a pool of 10 individuals. An average of 60 million reads per replicate were obtained from Illumina HiSeq sequencing and subjected to differential gene expression (DGE) analysis. Genes of related function were sorted into clusters, and those that were found at high frequency in the DGE set were identified. Here, we focus on patterns of development related to the digestive system, highlighting progressions with larval stage and variation between size classes. Fish in the smaller size class developed more slowly, with heavier reliance on amylase and lipases. By 17dph, faster growing fish displayed both upregulated alkaline and acidic proteinase metabolic systems.

INTRODUCTION

Harvest rates of global capture fisheries have plateaued with most fisheries operating at maximum or declining harvest levels (FAO 2012). However, as capture fisheries have plateaued, the demand for seafood and fishery products continues to increase. To satisfy this demand, aquaculture production has become increasingly important, making it one of the fastest-growing animal food-producing sectors, growing at an average annual rate of 8.8 percent since the 1980s (FAO 2012). Globally, aquaculture value exceeds US $119 billion (FAO 2012) and contributes to almost half of the fish consumed (Terova et al. 2013). Despite this explosive growth, aquaculture development in the U.S. has lagged behind other countries and is currently ranked 13th in aquaculture production (Langan 2008; FAO 2012).

Seriola species (*S. dumerili, S. lalandi, S. rivoliana, S. quinqueradiata*), collectively known as amberjacks, are fish of particular interest to the growing aquaculture industry due to their high value, forming a billion dollar plus component of the sashimi industry. In many locations, one or more of these species comprise a large percentage of the total marine finfish in culture. In Japan, for example, *Seriola* spp. account for greater than 60% of the total mariculture program (Aoki et al. 2014). Culture of these species has traditionally relied heavily on harvesting and growout of wild juveniles, which can be unpredictable in supply and puts excessive pressure on natural populations (Ohara et al. 2005; Ozaki et al. 2013; Aoki et al. 2014).

In the U.S., two *Seriola* species are currently in commercial culture. The yellowtail, *Seriola lalandi*, is a pelagic species that is globally distributed in tropical and sub-tropical waters (Kolkovski and Sakakura 2004). This species is commercially cultured in Japan, Australia, New Zealand, Chile, and even in the Netherlands using recirculating aquaculture systems

(Abbink et al. 2012; Aguilera et al. 2013; Blanco Garcia et al. 2014). In the northeast Pacific, the range of *S. lalandi* extends from southern Washington State to Mazatlán, Mexico, and the species is seasonally abundant in southern California, with spawning reported from Pt. Conception, California, south around the Baja California peninsula (Sumida et al. 1985, Stuart et al. 2013). This native species is considered a prime candidate for aquaculture development in southern California. For over 10 years, Hubbs-SeaWorld Research Institute (HSWRI) has been experimentally culturing *S. lalandi* and has had good success spawning and rearing this species. Since 2007, they have been developing more intensive culture techniques, with the aim of transferring the technology to the commercial sector in order to help establish a marine fish farming industry in the U.S. (Rotman et al. 2013). Currently, HSWRI is the only *S. lalandi* hatchery in the U.S. (Rotman et al. 2013); it is also one of a few hatcheries world-wide practicing closed life-cycle production and not relying on wild-caught seed stock. As noted above, the related *S. quinqueradiata* now provides 60% of Japanese aquaculture production (152,000 tons in 2009; Ozaki et al. 2013), but growth in production is limited in part by reliance on the capture of wild juveniles due to difficulties in reliable hatchery production of juveniles.

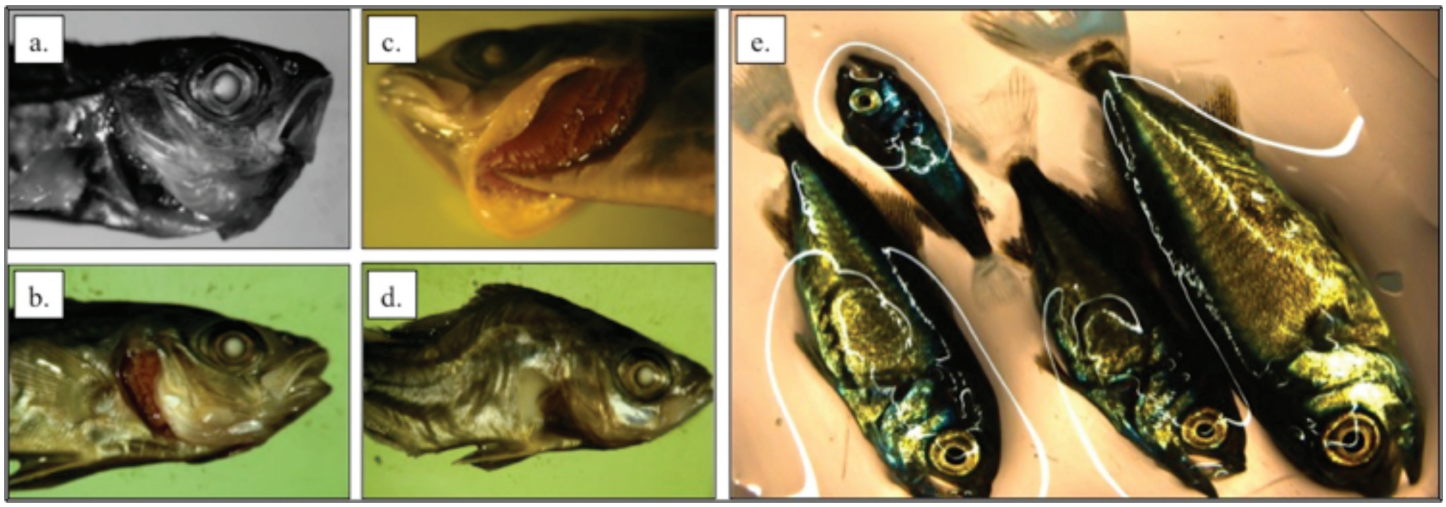

Figure 1: Deformities and growth heterogeneity in cultured S. lalandi juveniles: a.) jaw deformity, b.) incomplete operculum, c.) malformed operculum, d.) skeletal deformity, e.) size variation at 31 days post hatching.

In the U.S., hatchery production of both *S. lalandi* and *S. rivoliana* is rapidly growing but has been hindered by a propensity for deformities and growth heterogeneity developed during larval and early juvenile stages that limit the production capacity and efficiency (Fig. 1). Variable growth rates lower the survival of smaller larvae/juveniles as a result of cannibalistic predation and physical trauma from larger fish but also increase mortality in larger fish from choking on their smaller cohort members (Fig. 1e). Growth heterogeneity among larvae is apparent at only a few days post-hatching, becoming more pronounced over time. Without size grading, growth heterogeneity results in larvae and early juveniles of the same age being at different developmental stages, often requiring different feed items and culture conditions.

Despite exhaustive efforts by multiple groups, there has been limited success in overcoming many of these difficulties, which have limited the growth of this industry. The causes of and solutions for the growth variation and high deformity rates remain unresolved (Stuart and Drawbridge 2012). Some deformities and growth variation are believed to be linked to nutritional deficiencies (in the larvae and/or broodstock), culture conditions (e.g. light, temperature, salinity) and various environmental stressors. However, the high variability in deformities and growth among spawns despite identical culturing conditions suggests that there may be a strong effect of parental genetic background (Roo et al. 2010). Studies examining parentage assignment of juvenile *S. lalandi* at the Southwest Fisheries Science Center (SWFSC) have shown connections between particular parents and deformities in offspring, indicating a potential genetic component to this variability (unpublished data).

Discovering methods to reduce deformities and size variation in offspring is critical to *Seriola* culture. Here, we aimed to identify and study molecular changes underlying the physiological variation in development of digestive system between fast and slow growing groups of larval fish using RNAseq analysis. We obtained samples of fast and slow growing larval *S. lalandi*, sequenced whole larvae mRNA pools, obtained gene counts, analyzed over-arching changes

in gene expression, and focused on characterizing changes in specific digestive enzymes as markers of development and relative fitness among groups.

MATERIALS AND METHODS

RNA was extracted from whole fast and slow growing larval *S. lalandi*. The slow-growing fish were 4.1 to 4.7mm (2dph), 4.7 to 5.2mm (7dph), and 5.4 to 6.4mm (17dph) in length. The fast-growing fish were 4.8 to 5.2mm (2dph), 6.0 to 6.3mm (7dph), and 8.7 to 10.5mm (17dph). Fish in the fast growing 17dph group were visually assessed as in the post-flexion developmental stage. Eighteen cDNA libraries were sequenced using a total of 600 million 50 bp paired end reads at Iowa State University with Illumina HiSeq. The libraries included three replicates for small and large fish at 2, 7, and 17 days post-hatch. Sequence adaptors and low quality ends were removed with Trimmomatic (Bolger 2014). Trimmed reads were aligned to a draft genome sequence (Purcell et al. in press) using GSNAP (Wu and Nacu 2010). Reads that mapped uniquely to the genome were utilized for downstream analysis of differential expression. Raw count data for each gene model was obtained using the HTSeq program along with a GFF file that was generated using Trinity (Haas et al. 2009) and PASA (Haas et al. 2008). QuasiSeq in R (Lund 2012) was used to normalization and differential gene expression analysis. Read counts were normalized for library size using upper quartile normalization methodology. Multiple time points were modeled as a fixed-effect independent variable to increase statistical power. All models assumed a negative binomial distribution of read counts. False discovery rate (FDR) correction was performed using the q-value method (Storey 2003). Open reading frames from assembled transcripts were identified using BlastP against the Danio rerio genome.
DAVID (Huang et al 2009) was used to cluster differentially expressed genes by their function and test for overrepresented gene ontology terms related to digestion in particular. To investigate overexpressed categories in more detail, expression patterns for differentially expressed genes related to digestion in other species were also examined. Tableau (Seattle, WA) was used to visualize trends in S. lalandi gene expression related to digestion.

RESULTS AND DISCUSSION

Count data were obtained for a total of 47,964 different assembled transcripts. A total of approximately 20,000 transcripts had significant protein blast hits to the non-redundant (NR) NCBI database, and 13,000 hit proteins within the Danio proteome. While many more transcripts were identified as differentially expressed using NR than Danio identifiers (Fig. 2), Danio identifiers facilitated analysis of gene clustering and overrepresentation using DAVID. Seven clusters of differentially expressed genes related to digestion were found to be overrepresented in frequency relative to the genomic

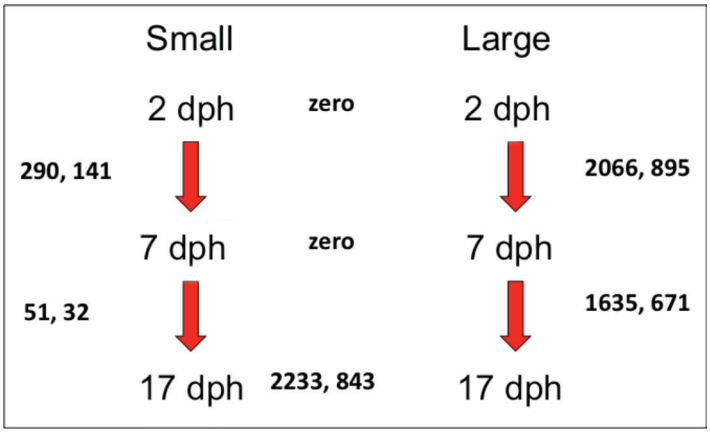

Figure 2. Number of differentially expressed genes among all comparison groups with alternative NR or Danio rerio identifiers. Nodes identify comparison groups for DGE comparisons: large or small fish and days post hatch (2, 7, or 17 dph), and vertices are labelled with number of differentially expressed genes (NR, Danio rerio).

background in pairwise comparisons of fish from different stages and sizes (Table 1). Overrepresented clusters related to alkaline peptidases and their inhibitors, as well as carbohydrate metabolism.

Protease patterns were similar to those found in other species (Zambonino-Infante et al. 2008; Zambonino-Infante and Cahu 2010), with a faster developmental progression in fish from the larger group. The primary alkaline peptidases, trypsin, chymotrypsin, and elastase, were high from the start of the experiment and increased faster in larger fish (Table 2). Peptidase inhibitors showed a similar pattern, with expression increasing faster in larger fish. These are necessary to inactivate and control peptidases to protect native tissue. Proteases characteristic of a mature stomach were seen only in individuals from the large group. Acidic digestion was detected with concurrent dramatic increases in pepsinogen and pepsin transcripts, along with transcripts for hydrogen chloride pump that is necessary to activate pepsinogen.

Table 1. Seriola lalandi RNAseq overrepresented differentially expressed gene clusters related to digestion. Experimental column labels identify comparison groups for DGE comparisons: large (Lg) or small (Sm) fish and days post hatch (2, 7, or 17 dph).

Cluster	Lg 2dph vs 7dph	Lg 7dph vs 17dph	Sm 2dph vs 7dp	17dph Sm vs Lg
Peptidase	42	39	12	
Serine proteases, trypsin family	10	16		9
Carbohydrate catabolic process	11			15
Trypsin/chymotryp	18	20		21
Peptidase inhibitor	12	12		12
Cysteine-type endopeptidase inhibitor activity, c..		4		6
Cysteine protease	7			

Lipases were present early in both groups and showed varying patterns over time and between groups. Pancreatic bile salt activated lipase (BAL) is the major digestive neutral lipase in teleosts for the digestion of fat, secreted from the vertebrate pancreas into the intestine (Saele et al. 2010). Transcripts for BAL and phospholipases (not shown) showed no consistent trends, with both pronounced increases and decreases depending on the transcript (Table 2). Hepatic lipase, however, showed dramatic increases in large 17 dph fish only. This enzyme is a major lipase involved in phospholipid and triglyceride metabolism (Connelly 1999), and here likely indicates a more developed liver in the faster progressing group.

Amylase is a major metabolic enzyme for digestion of starch. Its expression has been well studied and is characterized by high levels early, followed by a decreasing trend (Douglas et al 2000; Zambonino-Infante et al. 2010). Given the consistency among species, these changes have been labelled "genetically programmed" (Zambonino-Infante et al. 2010). Larger fish were further in their progression, with lower levels overall and faster decreases. Nonetheless, there was evidence of a higher general carbohydrate metabolism in larger fish (data not shown), so they may be obtaining carbohydrates from alternative sources, such as chitin from Artemia in their feeding program.

Overall, developmental patterns were similar to those seen in other marine fish. Fish in larger group showed stronger, earlier increases in many protein digestive enzymes. Larger fish showed less reliance on starch, and presumably more advanced protease, lipase, and carbohydrate profiles. This study demonstrates utility of the RNAseq method in tracking changes expected in development and discovering new patterns in enzyme systems related to growth. Arrested development of slow-growing fish digestive tract indicates that a single food is not likely to be efficiently digested by both fast and slow growing larvae. Until extreme growth rate heterogeneity can be prevented, separation of fish from different growth stages and tailored feeding is warranted until all fish are post-flexion in developmental stage. Future analysis will involve looking for shifts in relevant regulatory gene expression levels for "master control genes" and mutations in regulatory genes between groups.

Table 2. Differentially expressed transcripts related to digestion in Seriola lalandi. Median transcript counts over three biological replicates per experimental treatment group are shown. Experimental groups include large or small fish at 2, 7, or 17 days post-hatch.

			Day		
	Gene	Size	2	7	17
Trypsin/ Chymotrypsin/ Antitrypsin	trypsin_transcript_42399	Large	42,211	189,335	409,879
		Small	62,746	180,324	232,070
	trypsinogen_transcript_11516	Large	21,282	65,207	142,996
		Small	27,989	93,753	112,708
	trypsinogens-1_transcript_423..	Large	13,867	67,168	155,659
		Small	22,720	67,632	76,700
	chymotrypsinogen-2_transcript..	Large	12,106	17,206	63,425
		Small	19,145	20,666	49,949
	chymotrypsinogen-1-like-protein	Large	1,079	18,725	44,745
		Small	1,672	13,008	17,654
	alpha-1-antitrypsin_transcript_	Large	3,626	16,109	28,126
		Small	5,524	13,014	15,296
Pepsin/ Proton Pump	pepsinogen_transcript_10433	Large	5	5	1,589
		Small	4	7	12
	proton-pump-beta-subunit_tran..	Large	22	17	125
		Small	12	26	26
Amylase	alpha-amylase_transcript_154..	Large	13,430	20,311	3,794
		Small	24,020	43,484	29,904
	amylase-2_transcript_15469	Large	1,404	1,914	486
		Small	2,052	4,221	3,011
Lipase	bile-salt-activated-lipase_tran	Large	7,256	4,196	2,308
		Small	9,775	7,636	8,411
	bile-salt-activated-lipasebal_t	Large	931	1,324	4,850
		Small	2,008	2,490	5,306
	bile-salt-activated-lipase-BAL_	Large	1,002	1,429	3,915
		Small	1,374	2,550	5,065
	bile-salt-activated-lipaseBAL_1	Large	1,002	1,429	3,915
		Small	1,374	2,550	5,065
	bile-salt-activated-lipase-like	Large	602	887	1,864
		Small	943	1,105	1,744
	hepatic-lipase_transcript_11663	Large	34	574	1,499
		Small	13	268	562

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In this study the authors generated and assessed the transcriptome and gene expression profile of five developmental stages of the Atlantic bonito. Aquaculture on this species is just starting and although there were not many genetic resources available for this non-model species, the authors wanted to use molecular tools to help produce high quality juveniles for aquaculture production. Pooled larval bonito were collected at 0 dph, 5 dph, 10 dph, 20 dph, and 30 dph. Samples were extracted and prepared for sequencing on the Illumina HiSeq 2000. A total of 169,326,711 paired-end reads were generated, resulting in a transcriptome with 68,220 contigs. This study describes the clusters formed by the differentially expressed transcripts and how gene expression patterns changed over the developmental periods. The approaches taken in this study and the examination of different larval stages are of interest for our work on larval yellowtail gene expression.

Valente, L.M.P., K.A. Moutou, L.E.C. Conceição, S. Engrola, J.M.O. Fernandes, and I.A. Johnston. 2013. What determines growth potential and juvenile quality of farmed fish species? Reviews in Aquaculture, 5 (Suppl. 1): S168-S193.

This review focuses on the research related to the biology, physiology, and genetics that are relevant to larval fish growth and quality. Specifically, this review goes into great detail on understanding fish skeletal muscle ontogeny, and the impact of that on larval and juvenile fitness. Genes and gene pathways involved in the development of these systems and in muscle growth processes are described in detail, along with the current state of knowledge from research on non-salmonid fish species in aquaculture. This research is of interest for our work on exploring growth variation in larval Seriola lalandi using RNA-Seq data.

Srichanun M, Tantikitti C, Utarabhand P, Kortner TM. 2013. Gene expression and activity of digestive enzymes during the larval development of Asian seabass (*Lates calcarifer*). Comparative biochemistry and physiology, Part B. 165:1-9.

The authors characterize gene expression and activity patterns during larval development from 2 to 30days post hatch (dph) in the Asian seabass. Increases and decreases in key enzymes involved in digestion of starches, lipids, and proteins were tracked and major developments of the digestive system were noted. This research is of interest for our work on exploring major patterns of development in larval *Seriola lalandi* using RNA-Seq data. Differences in development of the digestive system between fast and slow growing Seriola lalandi larvae may affect protocols for weaning off of live-food.

Exploring the Genetic Risks Posed to Natural Populations by Escaped Cultured Marine Fish: A Reintroduction to the OMEGA Model

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ABSTRACT

The rapid development worldwide of offshore marine finfish aquaculture has raised concerns, at least in part, because aquaculture programs have the potential to create various fitness effects if cultured fish escape and come in contact with wild conspecifics. Physical contact alone may result in fragmentation, competition, and disease transmission, while reproduction between cultured and wild fish may have negative genetic consequences for the mixed (wild plus cultured) population. Genetic impacts may include reduced genetic variability, introduction of non-native or maladapted alleles, swamping of the gene pool, inbreeding or outbreeding depression, and domestication, each of which can compromise the adaptive potential of the mixed population, making it less fit or able to respond to environmental change.

Fish escape for a variety of reasons, such as using improper mesh size or the presence of holes from normal wear and tear in net pen cages; carelessness during fish transfer among cages; washout from high wind and sea conditions during storms; and cage breaches by large predators. When evaluating the threat to genetic integrity these escaped fish pose, several factors merit consideration, including but not limited to the population genetic structure and phenotypic variability of the species in the wild; size of the local (affected) wild population relative to the magnitude, frequency, and survival rate of the escapees; age and growth characteristics of the wild and cultured populations; encounter rate between wild and cultured fish; management practices at the farm, including broodstock selection and breeding protocols; and the likelihood of genetic drift and domestication resulting from farm practices.

Unfortunately, little information is available to reliably assign the risk to population fitness due to escapes. Because scientific data is lacking, escape standards are largely theoretical or qualitative rather than quantitative. To address this technical and scientific barrier and advance sustainable US marine aquaculture, the NOAA Fisheries Office of Aquaculture (OAQ) fostered a research initiative to explore the interactions between wild fish and escapees and how to minimize the genetic impact. As part of this initiative, the Offshore Mariculture Escapes Genetics Assessment (OMEGA) model was developed by NOAA Fisheries and ICF International to simulate, identify, and quantify the fitness risk posed by escapees over time. Ultimately, OMEGA is intended to provide insight into factors affecting risk, help identify research priorities, explore options for design or modification of culture programs, and inform policy and management decisions related to mitigation of genetic impacts. To realize these objectives, the OAQ is focused on fostering external collaborations to develop model scenarios, evaluate model parameters, and validate the model with data from current and planned domestic and international marine aquaculture operations.

INTRODUCTION

The rapid development of offshore marine finfish aquaculture worldwide has raised concerns due, in significant part, to the potential negative fitness impact(s) escaped cultured fish may have on natural populations (Tufto 2010; Waples et al. 2012). Fitness is inversely related to the level of domestication in escapees (Baskett et al. 2012), and fitness declines due to domestication can occur rapidly in the wild (Araki

et al. 2008). Cultured fish escape from aquaculture holding systems for a variety of reasons. Operators may use a poor mesh size for net pen cages or holes in the nets may arise from normal wear and tear. Fish may be lost during transfer among cages (e.g. for grading, harvesting, etc.), due to high wind and sea conditions during storms, or when predators breach cages. Even spawning of reproductively mature individuals within cages may result in escaped gametes and fertilized eggs.

Non-genetic impacts of offshore aquaculture occur because of spatial proximity and physical contact between cultured and wild conspecifics and include the fragmentation of populations, competition, disease transmission, and pollution (IWG-A 2014). Genetic impacts occur due to gene flow when individuals from cultured and wild populations interbreed (Baskett et al. 2013). The introduction of hatchery genetic backgrounds, which may include non-native or maladapted alleles, can change the genetic composition of the wild population, potentially resulting in reduced genetic variability, inbreeding or outbreeding depression, and domestication (Tringali and Bert 1998; Tringali et al. 2007; Baskett and Waples 2012). Larger escape events could even swamp the wild population with hatchery-based alleles. These impacts threaten the adaptive potential of the wild population and may make it less able to respond to and survive fluctuations in the environment (Tringali and Bert 1998), including both stochastic events and long-term trends like climate change, ocean acidification, and warming ocean temperatures.

To address the issue of escapes from offshore aquaculture operations, the NOAA Fisheries Office of Aquaculture (OAQ) developed a research initiative designed to explore the genetic interactions between wild and cultured fish. As part of this initiative, the OAQ first sanctioned a comprehensive in-house review of the genetic risks of marine aquaculture (Waples et al. 2012). The OAQ then solicited the help of scientists at ICF International, Inc., (ICF) to develop the Offshore Mariculture Escapes Genetics Assessment (OMEGA) model. OMEGA is a Microsoft Excel-based program designed to simulate the risk posed to wild population fitness by escapes from marine aquaculture programs. ICF scientists Greg Blair and Jason Volk and collaborators originally introduced the OMEGA model at UJNR Aquaculture 2013 in Sapporo, Hokkaido, Japan, discussing the program and its guiding principles, as well as the related and precursory All-H Analyzer (AHA) model (Volk et al. 2015). Volk et al. (in press) then described how OMEGA was originally developed, parameterized, and verified using a hypothetical US domestic aquaculture program for sablefish (*Anoplopoma fimbria*). Here, we reintroduce OMEGA, touching again on the model's theoretical basis; describe user inputs and model outputs; discuss potential applications; and make a call for domestic and international collaboration toward validation of the model.

DISCUSSION

OMEGA was developed to provide insight into factors affecting risk, help identify research priorities, explore options for the design or modification of culture programs, and inform policy and management decisions related to the mitigation of genetic impacts. To evaluate risk, several factors – describing threats to and vulnerabilities of the system – are important, including 1) the genetic structure and phenotypic variability of the species in the wild; 2) local wild population size relative to the number and survival rate of escapees, as well as frequency of escape events; 3) age and growth characteristics of the wild and cultured populations, including maturation schedule and fecundity; 4) amount of contact between wild and cultured fish; 5) hatchery practices, including broodstock selection and breeding protocols, which affect the likelihood and magnitude of genetic drift and domestication. Once threats and vulnerabilities have been identified and quantified, risk (here, the probability of a negative fitness impact due to escapes from aquaculture) can be assessed. Subsequent risk reduction may then be fulfilled through applied research, design and modification of culture programs, and informed policy and management decisions, aspects of which may be fed back into OMEGA for further evaluation.

Structure and inputs

OMEGA is organized around three interacting components that incorporate the above factors: 1) the aquaculture operation itself, including the frequency and magnitude of escape events, and the cultured population characteristics, such as the number of fish, broodstock source, maturation, and growth; 2) wild population characteristics, such as abundance, distribution, survival, age and size at maturity, age composition, and age-specific harvest rates; and 3) the potential for interaction between escapes and wild conspecifics, which is affected by characteristics like the location of pens relative to the wild population and the survival rate, movement, and reproductive

success of escapees in nature. Under these components, OMEGA employs a modular format to describe assumptions used to model the potential interactions and impacts of escapees in the wild. There are nine modules requiring user input. The aquaculture operation and cultured population component contains modules describing the fish culture program itself and escape scenarios, while the wild population component has modules for natural production, growth and maturity, and harvest. Under the interactions component are modules for the relative survival of escapes, encounter rate, and fitness. Finally, there is an optional regulatory standards module, which helps the user determine whether the aquaculture program may be in compliance with or violation of certain regulations, if they exist.

Each module contains a variety of user inputs, ranging from basic settings and preferences for saving the workspace and running simulations to wild and cultured population data (e.g. Fig. 1). Currently, there are 103 possible user input variables (Index of User Inputs available upon request). Some modules and inputs are optional, while other inputs represent alternatives for which the user chooses one of two or more options. Ultimately, appropriate default values will be assigned for certain parameters, with the intent of making the model more user friendly, as well as time efficient.

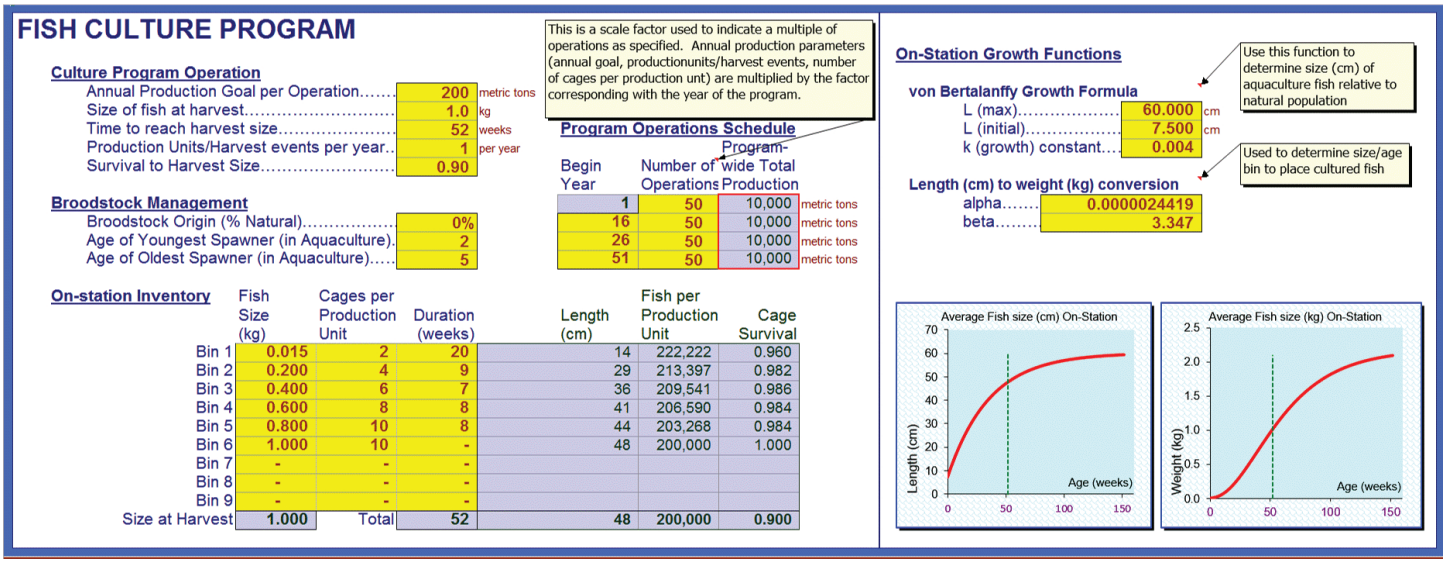

Figure 1. User input interface for cultured population characteristics. Inputs include information, such as annual production goals, size of fish at harvest, annual inventories, von Bertalanffy growth parameters, and the proportion of natural origin broodstock.

Theoretical basis

Once the model has been parameterized, OMEGA employs a single trait phenotypic fitness model developed by Ford (2002) to simulate risk. The assumptions of Ford (2002) are: 1) a single trait is under selection with different optimum values in the two environments; 2) the trait is normally distributed and subject to bell-shaped (Gaussian) selection; 3) mating is random, meaning the escapees and wild fish do not sort by origin; 4) population size is large, so drift, phenotypic plasticity, and other stochastic forces are ignored; 5) changes in mean trait value are deterministic, based on selection and gene flow; and 6) selection does not reduce population size, variance, or heritability of the trait over time. Ford (2002) originally developed it to assess the effects of supportive breeding on wild population fitness, and the model was first incorporated by an earlier Excel-based analytical tool called the All H Analyzer (AHA). The AHA was used to explore ways to balance the "4-Hs" – habitat, hatcheries, harvest, and hydroelectric effects – toward restoration and management of Pacific Northwest salmon populations.

In terms of OMEGA, Ford (2002) underlies a quantitative two population analysis of differential selection regimes and the effect of gene flow between the populations on the mean trait value. Initially, each of the two populations is exposed to a separate environment (hatchery or wild) and, therefore, selective regime (domestication or natural). The mean trait value of each population is equal to its environmental (and fitness) optimum. In one scenario, gene flow is unidirectional from the hatchery to the wild as escapees breed with the wild population. The resulting case is a change in the wild population mean trait value as it shifts toward the hatchery optimum. It should be noted that it is assumed any shift away from either environmental optimum leads to a reduction in fitness (Lande 2007; Tufto 2010). An alternative scenario encompasses the first but also includes gene

flow from the wild population to the cultured population as determined by the percent broodstock sourced from the wild population. The resulting case is a more nominal to nonexistent change in the wild population mean trait value.

Outputs

OMEGA outputs the simulation results in graphical format, and the figures include total biomass and total spawning biomass, plus percent escapee biomass and hatchery origin spawners; total harvest and percent wild yield; change in wild abundance due to escapes; and the change in wild fitness due to escapes. The original verification scenarios, for example, explored a low incidence of escapes versus a high incidence with a high encounter rate for a sablefish (*Anoplopoma fimbria*) hatchery program in the Pacific Northwest (Volk et al. 2015). Over the default 100-year timespan of the simulations, the low escapes scenario resulted in escapee percentage peaking at ≤5% of the total population and the spawning biomass soon after each escape event, with larger and/or successive events resulting in higher percentages, but gradually shrinking to <1% of the population (Fig. 2).

High chronic escapes coupled with several acute escape events, in contrast, had a longer lasting impact on population make-up. The high escape scenario resulted in escapees comprising a significantly higher proportion, averaging 5-7%. Correspondingly, fitness in the wild was reduced (shifted toward the hatchery optimum) more under the high escape scenario, whereas the low escape scenario remained near the wild optimum (Fig. 3). These results are in keeping with what is expected according to Ford (2002), as well as results from related research (e.g. Baskett et al. 2013).

Future research

Aquaculture must simultaneously support commercial interests; increase the availability of safe and nutritious fish, shellfish, and other products for consumers; and protect wild populations (IWG-A

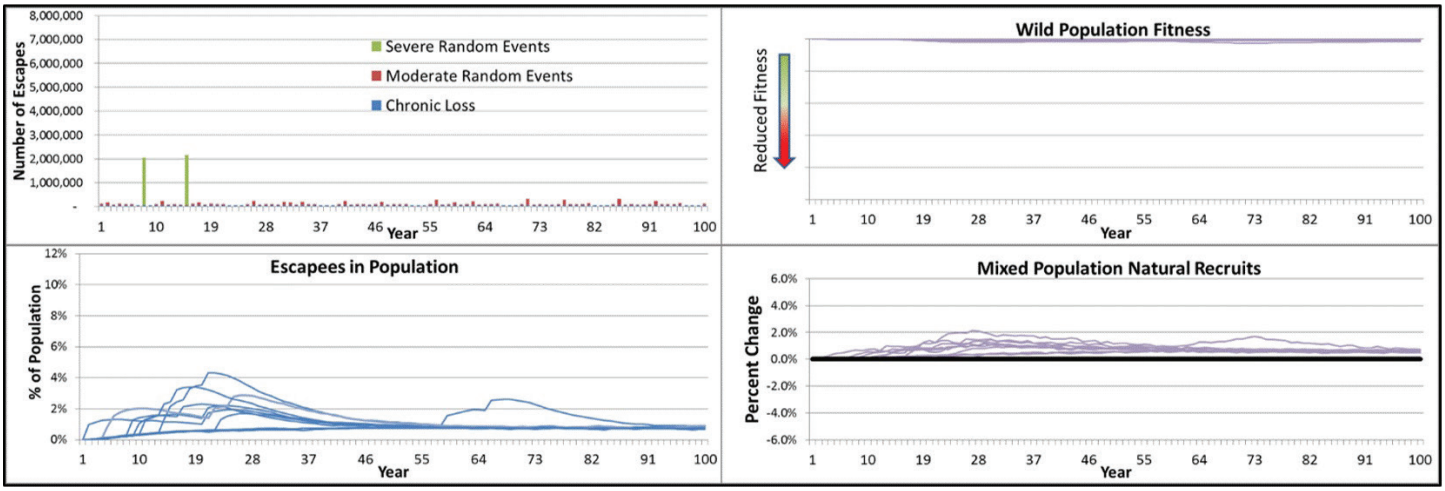

Figure 2. Simulation results for low escapes scenario. The number of escapees, based on escape event type, over time (here, 100 years) is in the upper left panel; escapees as a proportion of the population is at lower left; trends in wild population fitness at upper right; and the percent change in natural recruitment due to escapees is at lower right.

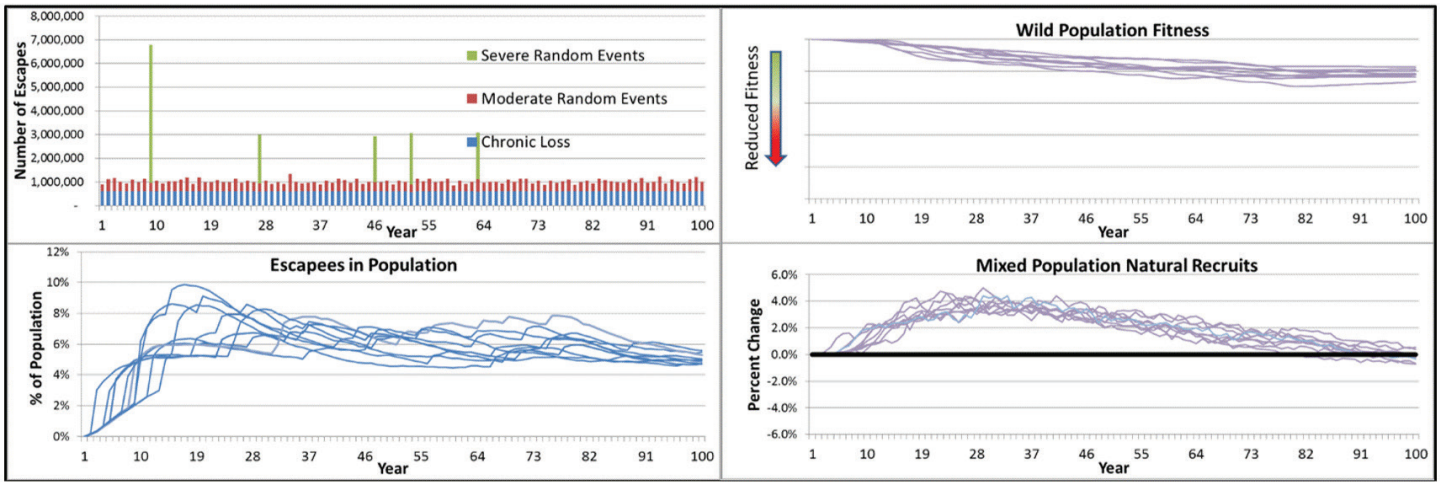

Figure 3. Simulation results for high escapes scenario. The number of escapees, based on escape event type, over time (here, 100 years) is in the upper left panel; escapees as a proportion of the population is at lower left; trends in wild population fitness at upper right; and the percent change in natural recruitment due to escapees is at lower right.

2014). OMEGA was designed to as a decision-making tool to assist in the assessment and management of marine aquaculture operations such that they may remain both commercially viable and environmentally responsible. Toward that end, there are four key questions that OMEGA may be used to address as marine aquaculture moves forward:

1. How significant are the impacts of escapees surviving to reproduce?
2. What are the effects of our decisions about aquaculture operations?
3. How do we define an acceptable risk to marine resources?
4. How do we balance the dual goals of economically viable aquaculture and conservation of marine resources?

First, in the shorter-term, we can use the model and the best available scientific information to explore the single-generation effects of reproductively-mature escapees. Longer-term, we would couple the use of OMEGA with monitoring and evaluation to improve our understanding of the realized effects of these escapees over time, both as more escape events occur, as later generations of offspring of mixed background also mature, and if and when hatchery practices change.

Next, in terms of economic viability, many aquaculture operators will eventually develop and implement various genetic improvement programs, including selective breeding, to increase the prevalence of desirable characteristics in their cultured populations. Moreover, selective breeding programs have the opportunity to enhance sustainability by improving feed and labor utilization efficiencies (Gjedrum 2012). In US marine aquaculture at present, genetic modification and other more manipulative technologies (e.g. cloning, transgenics) are either not commonly employed or not permitted for offshore stocking in federal waters (e.g. Gulf of Mexico Aquaculture Fishery Management Plan); conventional and marker assisted selective breeding and hybridization, although still rare are permitted (i.e., Atlantic salmon culture). Evaluation (with OMEGA) of genetic improvement programs is more likely to entail modeling various scenarios involving domesticated, or even inbred, broodstocks; non-chemical sterilization (e.g. triploidy, irradiation); or intraspecific hybridization. In terms of the final two questions, defining "acceptable" requires a qualitative systematic review of the best-available science that, when coupled with decision support tools like OMEGA, will enable informed discussion about escapes and their risks to sustainability of wild populations.

CONCLUSION

OMEGA v1.0 and its user guide are freely available for download on the OAQ website at www.nmfs.noaa.gov/aquaculture/science/omega_model_homepage.html. However, the model is still a work in progress. OMEGA requires further testing, including a sensitivity analysis of model inputs, using multiple real systems. The next steps toward finalizing the OMEGA model package for broader use are 1) to identify collaborators to develop case studies for current and future aquaculture programs for marine finfish species, 2) complete a sensitivity analysis of case studies, including determining acceptable default parameters and proxies; and 3) develop an economic analysis module.

The OMEGA team is extremely interested in pursuing potential collaborations with Japanese scientists. Japan supports several longer-term and prolific aquaculture programs for economically important taxa of marine finfish, from groundfish to pelagic, such as olive flounder (*Paralichthys olivaceus*), red sea bream (*Pagrus major*), and several yellowtail jacks (*Seriola* spp.). The OMEGA development and test team would like to collaborate with one or more Japanese researchers having an interest in modeling the potential fitness effects of escapes from current and planned marine aquaculture operations in the country. Japanese researchers involved with these programs represent excellent potential sources of real-world data (e.g. natural stock structure, life history, demographic, and ecological information; culture program history and scope; and regulatory requirements) for parameterizing OMEGA, developing model scenarios, and validating model assumptions. Moreover, these scientists possess the knowledge and experience to assess model output, which will help identify the model's strengths and weaknesses.

Since OMEGA is freely available, support levels from collaborators may range from supplying data or model results only, to active involvement in developing various real and/or simulated aquaculture program scenarios, to problem-solving and decision-making for future aquaculture planning, and up to mitigation planning within the Japanese regulatory framework. Preferentially, the teams would work closely with one another to develop the

most mutually beneficial and comprehensive evaluations of OMEGA, the species of interest, and the current or proposed aquaculture program.

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The author presents a single-trait phenotypic model that assumes different optimum trait values for hatchery and natural environments. Hence, due to the various effects of genetic drift and domestication selection in a hatchery environment, escaped cultured fish may cause a potential loss of genetic fitness if breeding occurs between them and wild conspecifics. The Ford model describes how mean phenotype values of the mixed population (captive plus wild fish) may shift relative to the optimum values for each environment, based on the presence/absence and amount of gene flow (interbreeding) between the cultured escaped and wild fish. The overall fitness effect depends on details, such as magnitude of the difference in optimum trait value, trait heritability, and selection pressure in the wild, as well as habitat capacity, number of escapes, and population demographics and dynamics. This model has been used in several studies, most notably in salmonids, to explore the potential fitness consequences of aquaculture, whether from supplementation of wild populations or the unintended straying of cultured fish to wild populations.

Tringali M.D., T.M. Bert, F. Cross, J.W. Dodrill, L.M. Gregg, W.G. Halstead, R.A. Krause, K.M. Leber, K. Mesner, W. Porak, D. Roberts, R. Stout, and D. Yeager. 2007. Genetic policy for the release of finfishes in Florida. Florida Fish and Wildlife Conservation Commission. Florida Fish and Wildlife Research Institute, Publication No. IHR-2007-1, St. Petersburg.

The authors of this document provide a comprehensive overview, including key references, of the potential genetic effects aquaculture may have on natural populations, such as a loss of fitness and viability, altered genetic diversity, and a reduction in long-term adaptive potential. The document is also intended to build a policy-based framework for

genetic management planning for species subject to supplementation in the state of Florida. Tringali et al. (2007) describe the various types of purposeful introductions that may occur, touching on escapes from commercial aquaculture, as well, and the authors also provide descriptions of options for evaluating, monitoring, and mitigating the negative genetic effects that may be seen in mixed populations of wild and cultured conspecifics. Finally, the document provides a step-by-step template useful to (non-geneticist) aquaculture managers, both within and outside of the state of Florida, to develop responsible and sustainable genetic management plans for supplemented species.

Waples R.S., K. Hindar, and J.J. Hard. 2012. Genetic risks associated with marine aquaculture. NOAA Technical Memorandum NMFS-NWFSC-119. US Department of Commerce NOAA NMFS, Washington, D.C.

The authors of this NOAA technical memorandum intended to "provide managers with a better understanding of the genetic effects of marine aquaculture on natural populations, so that these factors can more effectively be incorporated into informed decisions pertaining to federal marine aquaculture policy and regulatory decisions." Waples et al. (2012) is a comprehensive overview of the genetic issues and impacts to wild populations associated with aquaculture. The document attempts to synthesize relevant information and provide key references, identify areas where additional research is needed, provide a framework for assessing and balancing risks, and give examples of how monitoring and evaluation might be done toward developing responsible and sustainable aquaculture. While Waples et al. (2012) focuses on commercial aquaculture of marine finfish, it also discusses information gathered from salmon hatcheries and marine stock enhancement, which are related to marine aquaculture, and provide arguably more comprehensive scientific data due to their comparably longer history in the USA.

Suspended Culture of Asari Clam, *Ruditapes philippinarum*, and Their Roles in the Ecosystem

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Keywords: Asari clam, *Ruditapes philippinarum*, growth, suspended culture, oyster culture raft

ABSTRACT

Asari clam, *Ruditapes philippinarum*, is one of the most important fisheries species in Japan. Most of the clam's production depends on wild catch although the culture production in tidal flats is occupied only 3 to 4% of the total production. The suspended culture of Asari clam is not prevalent yet in Japan and any other countries. In order to develop the practical method of the suspended culture, the culture experiment was performed at an oyster culture raft in Ohnoura Bay, Toba City, Mie Prefecture. Prior to the culture experiment, for the collection of natural seed of Asari clam, 60 x 30 cm Nylon mesh bags with 2x3 mm mesh size were set on the tidal sand flat in Ohnoura Bay. Fine gravels and Care-Shells (oyster shell processed materials) were put in mesh bags. The mesh bag could collect more than 100 individuals/bag with the medium size of 20 mm in shell length after several month. For suspended culture, these seeds were put in plastic containers (41x31x14 cm). In each container 150 seeds (average shell length was 22 mm, wet weight was 2.0 g) were put with fine gravels and Care-Shells (oyster shell processed materials) in five different ratio of these substrates, namely 0, 20, 50, 80, or 100 % Care-Shell contents, setting to 6 cm in thickness. The containers were suspended in 2 m from the water surface. The culture started on April 18, 2011. After the five month culture, average SL and WW of the clams reached 33 to 35 mm and 8.3 to 9.7 g, and the survival rate was higher than 90%. Total wet weight of clams in the containers having started from 300 g reached 1,100 to 1,300 g. In terms of substrates, 50% of Care-Shell contents or over maintained the pore water pH of more than 8.0 during the culture period. The growth of the clams in 50 and 80% of Care-Shell contents seemed to be better result, but not significant. The growth of Asari clam in suspended culture showed much higher than on the tidal flat in Ise Bay. The results suggest that suspended culture of the clam has a potential for commercial clam production both on the high growth and survival rates.

According to the filtration experiment, the clam can filtrate 1.5 to 2.5 L/hr/g soft tissue dry weight. On the other hand, chlorophyll concentration adjacent the net pens of the red sea bream culture in Gokasho Bay, Mie showed in the range of 1.7-27.5 μg-Chl/L 2 - 8 m beneath the surface. By the estimation of nitrogen budget around the suspended culture of the clam, one kg of the clam has ability to absorb 4 – 200 mg-Nitrogen/day depending on the chlorophyll concentration. The harvesting the clam with increased one kg corresponds to 3.4 g nitrogen removal from the system. It is suggested that the introduction of the suspended clam culture has positive ecological impacts in terms of controlling water quality and biological production.

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Fujiwara M., S. Tsuji, M. Tanaka, Y. Imanishi and M. Nakanishi. 2008. Growth of Manila clam *Ruditapes philippinarum* on hanging culture using containers. Bull. Kyoto. Inst. Ocean. Fish. Sci. 30:49-53. (in Japanese)

In order to examine the growth of the manila clam *Ruditapes philippinarum*, a hanging cultivation experiment using containers was conducted in Kunda and Maizuru Bay. We covered the upper part of the culture containers with a fine mesh net, and put anthracite soil (particle size: 2 - 3 mm) into the containers for bed material. The wild juvenile clams used in this experiment were collected from hanging cockle culture containers in Kunda Bay. We could recognize from the time of discovering juvenile clams

whether they were autumn or summer broods. Autumn broods grew well from spring to summer, but hardly grew in autumn. Summer broods grew well from the first autumn to the summer of the following year, but hardly grew in autumn of the next year. The mean shell length of 1-year-olds was 32 - 42 mm in autumn broods, and 45 mm in summer broods. These results revealed markedly high growth rates not reported to date.

Mizuno T., T. Maruyama and J. Higano. 2009. Transition and Prospect of Asari clam (*Ruditapes philippinarum*) Fisheries in Ise Bay, Mie Prefecture. Bull. Mie Pref. Fish. Res. Inst. 17:1-21. (in Japanese)

The authors took an overview on the history of the clam fishery in the western-side region of Ise Bay, a large bay (2,342 km^2 with 660 km coastline) in the central part of Japan. Major commercial clams produced in the bay are Asari clam *Ruditapes philippinarum* living in shallow coastal areas, freshwater clam *Corbicula japonica* and hard clam *Meretrix lusoria* in brackish-water.

The annual yield of these 3 species decreased in response to fishing efforts and environmental changes. Until early 1960s, tidal flats at river mouths covered in excess of 6,000 ha, and eelgrass beds grew there as well as in other parts along the coastline. After the annual catch of Asari clam peaked at ca. 15,000 tons in the late 1960s, it decreased rapidly when coastal developments destroyed tidal flats and eelgrass beds during 1970s. In this period, the structure of coastal fisheries changed then laver culture and fisheries on clams and pelagic fish were continued. The second peak of Asari yield was seen in 1980s when clams in the subtidal zone were also exploited. Aged fishermen recruited into clam fisheries during 1990s avoiding intensive labors in the laver culture. In 2000s, the 70% of fishermen are working in clam fisheries, and the yield of Asari clam became stagnant in 1,000 to 3,000 tons due to the strong fishing pressure.

In the early 2000s, very few Asari clam larvae, less than 200 individuals/m^3 in the main reproductive period (spring and autumn in this region), were observed at the river mouth in the southern part of the bay. This abundance of larvae was contrastingly smaller than that in Mikawa Bay adjacent to Ise Bay where stable annual yields and high abundances of larvae, thousands of individuals in 1 m^3, were reported. In the survey point in Ise Bay, it was observed that juveniles immediately after settlement rarely survived after the flood in June through August, while juveniles occurred in autumn survived once in a few years. These survivors were exploited soon after their shell length reached at 25 mm, the minimum size allowed to catch, in 2 years.

Catch per unit effort (CPUE) of hard clam and freshwater clam recovered when the society of local fishermen became aware of the importance of regulating their catch and protecting fishing grounds in the light of field surveys. As contrasted with cases of these 2 species living in or around rivers, the spatial scale necessary for the fishing management of Asari clam should be much larger. In 2009, fishing cooperatives along the bay were organized to conduct resource surveys and activities to protect fishing grounds from undesirable predators. It has been also planned to enhance the quality of some fishing grounds in the bay by creating artificial tidal flats and adjusting the ground height to the known suitable level for the habitat of Asari clam.

Goulletquer, P., J.M. Deslous-Paoli, M. Héral. 1989. Ecophysiologie et bilan energétique de la palourde Japonaise d'élevage *Ruditapes philippinarum*. Jour. Exp. Mar. Biol. Ecol. 132:85-108.

Rates of filtration and respiration both follow a nonlinear model based on temperature of the form: with maximal values at 15 and 20 °C, respectively. Quantities of seston varying from 0 to 30 mg $\cdot$ 1^{-1} have no effect in reducing the filtration rate > 8 mg $\cdot$ 1^{-1}, ingestion is regulated by the production of pseudofaeces. Maximal assimilation efficiency is ≈ 78%, but this is considerably reduced when the mineral content of the water increases. Assimilation efficiency for the Manila clam is reduced at both high (> 10 $mm^3 \cdot h^{-1}$) or low (< 2 $mm^3 \cdot h^{-1}$) values of ingested ration. The estimated value of growth efficiency (75%) and values of growth efficiency derived from the model K1 = 33%, K2 = 51% are optimized when ingested volumes are between 1 and 2 mm^3. Standard metabolism is estimated as 0.11 ml $O_2 \cdot h^{-1}$. Zero growth efficiency occurs at a ration level of 2 J $\cdot$ h^{-1} for an adult. The individual energy budget shows that production is dependent more on temperature than on the energy value of the food. Comparison of calculated and measured production reveals differences resulting from the higher levels of seston found in the field. In particular, during the winter when the mineral content of the seston is high

(90 mg · 1^{-1}), there is a continuous loss of weight. This results from a lower assimilation efficiency together with production of pseudofaeces. Excretion of organic nitrogen varies throughout the year, ammonia representing no more than a mean of 29.8% of the total nitrogen excretion.

Magni P., S. Montani, C. Takada and Tsutsumi H. 2000. Temporal scaling and relevance of bivalve nutrient excretion on a tidal flat of the Seto Inland Sea, Japan. Mar. Ecol. Prog. Ser. 198:139-155

Using an indirect and integrated approach, we quantified the magnitude and temporal variability of the contribution of macrozoobenthos to the upward flux of inorganic nitrogen and phosphorus on a sandy tidal flat of the Seto Inland Sea, Japan. From April 1994 to April 1996, we conducted monthly field surveys on the abundance and faunal composition of the macrozoobenthic communities inhabiting the lower part of the intertidal zone. Subsequently, we carried out 24 h day/night laboratory experiments on the nutrient excretion rate by various size-classes of the dominant species. We then obtained the animal nutrient excretion over a 2 yr period, multiplying the species-specific excretion rates by the actual animal biomass found on the tidal flat. Additionally, for all different seasons, we calculated the upward diffusive flux from the sediments from the vertical profiles of nutrient concentrations in the porewater. On the flat, the bivalves *Ruditapes philippinarum* (Veneridae) and *Musculista senhousia* (Mytilidae) were dominant, making up 86 ± 5.6% when the total biomass exceeded 100 g DW (dry weight) m^{-2}. From our laboratory experiments, the mean (day and night) nutrient excretion rates at 20 to 22 °C by the 2 bivalve species were 18.9 μmol NH_{4+}-N g^{-1} DW h^{-1}, 4.8 μmol (NO_{3-} + NO_{2-})-N g^{-1} DW h^{-1} and 3.3 μmol PO_{43-}-P g^{-1} DW h^{-1}. In addition, NH_{4+}-N excretion by *R. philippinarum*, but not that by *M. senhousia*, was significantly higher during the day than during the night. This occurred while the food (*Thalassiosira* sp.) offered in spikes was rapidly taken up irrespective of the concentration (within a field relevant spring-summer range of 10 to 60 μg l^{-1} chl a) and in day/night treatments. The release of nitrogen (N) and the release of phosphorus (P) were highly correlated with each other, for both *R. philippinarum* and *M. senhousia*, with an N/P ratio of 7.8 ± 3.0 and 9.9 ± 3.5, respectively. In the field, the bivalve excretion rates of nutrients were calculated to be up to 35.2 mmol NH_{4+}-N m^{-2} d^{-1}, 8.8 mmol (NO_{3-} + NO_{2-})-N m^{-2} d^{-1} and 5.8 mmol PO_{43-}-P m^{-2} d^{-1}. These values rank in the upper range of nutrient excretion by intertidal macrozoobenthos and are comparable to those found on dense assemblages (800 to 2000 g ash free DW m-2) of the most investigated mussel, *Mytilus edulis*. In addition, nutrient fluxes through bivalve excretion varied strongly within a few months, up to 10-fold (*R. philippinarum*) and 100-fold (*M. senhousia*) between April 1994 and August 1994, as related to the temporal change of bivalve standing stock. The extent of nutrient regeneration through diffusive flux was comparable to that reported for other intertidal zones of Japan and in the eutrophic Seto Inland Sea, within a range of 0.2 to 1.5 mmol NH_{4+}-N m^{-2} d^{-1} and 0.01 to 0.05 mmol PO_{43-}-P m^{-2} d^{-1}, thus more than 1 order of magnitude lower than that due to the excretory activity by *R. philippinarum* and *M. senhousia*. Our results indicate that the dominant bivalves, *R. philippinarum* and *M. senhousia*, play a major role in the processes of benthic nutrient regeneration within the intertidal zone, rapidly and efficiently recycling the inorganic forms of N and P available to primary producers. This study highlighted the importance of considering the temporal scaling of intertidal macrozoobenthos distribution in the quantification of the processes of benthic nutrient regeneration in these highly variable sytems.

Interpreting the Microbial Ecology Found within Marine Fish

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ABSTRACT

The natural microbial communities found within vertebrate digestive tracts are composed of a multitude of different microorganisms, collectively known as the microbiome. Recent work in humans and model organisms has shown that the microbiome is a dynamic and vital element of host health—contributing to nutrition and digestion processes, as well as immune system development. Technological advances in sequencing now allow us to look at these communities as a whole, promoting the use of ecological theory to understand how these fish-microbiome systems are structured. Here we investigate the microbiome of wild fishes by looking at species local to the Southern California Bight: the sport fish and aquaculture target *Seriola lalandi* (California yellowtail) the forage fish *Scomber japonicus* (Pacific chub mackerel) and the omnivorous *Atherinops affinis* (Topsmelt silverside). Using high throughput sequencing, we analyze the microbial composition of the intestinal contents of individual fish. Initial results confirm that fish microbiomes have distinct taxonomic signatures unique from the environment, including the presence of key host-associated taxa such as segmented filamentous bacteria (SFB), the phyla Tenericutes, and the phyla Spirochaetes. Furthermore, differences between the communities associated with the epithelial and the lumenal regions indicate the importance of spatial organization within the gut environment. These results demonstrate early steps and methods to characterize marine fish gut microbiomes within an ecological framework. Ultimately, this knowledge can be applied to understanding the importance of the gut microbiome to fish health in aquaculture and ecosystems management.

Development of Integrated Multi-Trophic Aquaculture Using Sea Cucumber

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Keywords: integrated multi-trophic aquaculture, nitrogen budget, sandfish (*Holothuria scabra*), milkfish (*Chanos chanos*), Elkhorn sea moss (*Kappaphycus alvarezii*)

ABSTRACT

In Southeast Asian countries, aquaculture production continues to increase. Environmental deterioration associated with water and sediment eutrophication by aquaculture effluent has been problematic, sometimes resulting in disease outbreaks and fish kills due to hypoxia and hydrogen sulfide poisoning. Integrated multi-trophic aquaculture (IMTA) is one of the promising measures for sustainable aquaculture. In this study, a box model estimation of nitrogen (N) budget based on experimental data and values from literature was made for a system of sandfish (*Holothuria scabra*) in sea cage IMTA with milkfish (*Chanos chanos*) and Elkhorn sea moss (*Kappaphycus alvarezii*).

Information on stocking density, stocking size, mortality, growth, feed ration, feed assimilation, NH_4-N production and NH_4-N absorption of these species was obtained from a series of experiments and existing literature. In the production system 26 g milkfish were cultured in a 5 x 5 x 4 m cage at the stocking density of 36.7 ind/m^3 with the initial feeding ration of 10% of body weight which was gradually decreased to 4% over time; 10 g sandfish were cultured in a cage with the same bottom area as milkfish cage hanged under the milkfish cage to trap particulate N waste (i.e. feces and leftover feed) at the stocking density of 35 ind/m^2; the stocking weight of Elkhorn sea moss line culture was 10 kg; culturing period was 200 days.

It was estimated that milkfish culture cumulatively produced 145 kg of particulate N, and milkfish and sandfish together produced 60 kg of NH_4-N in 200 days of culture. Daily assimilation rate of the particulate N by sandfish ranged 3.4 - 12.4%, and 4.3% of the particulate N was estimated to be removed by sandfish in 200 days of culture. Daily absorption rate of NH_4-N by Elkhorn sea moss increased exponentially with time and reached 100% after 125 days of culture. Cumulative NH_4-N was estimated to be depleted after 162 days of culture. For complete utilization of particulate N by the end of culture, sandfish stocking density should be 805 ind/m^2, which is 200 times as high as that in existing sandfish aquaculture operations.

INTRODUCTION

In Southeast Asian countries, aquaculture production continues to increase. Environmental deterioration associated with water and sediment eutrophication by aquaculture effluent has been problematic, sometimes resulting in disease outbreaks and fish kills due to hypoxia and hydrogen sulfide poisoning. To minimize the effect of intensive aquaculture production on the surrounding environment and to potentially increase economic income, integrated multi-trophic aquaculture (IMTA) has been practiced in several temperate regions of the world and is increasingly getting attention in Southeast Asian countries including the Philippines.

IMTA is the polyculture of organisms in different trophic levels (i.e. primary producer, primary consumer, secondary consumer, etc.). Typically, combinations of fed species (e.g. finfish), inorganic extractive species (e.g. seaweed) and organic extractive species (e.g. benthos) are used (Troell et al. 2009; Abreu et al. 2009, 2011). IMTA mitigates the effects of cultivation on the environment through recycling of nutrients (excess feeds, feces and metabolites) from organisms in higher trophic levels by organisms in lower trophic levels.

Sea cucumbers have been intensively harvested for processing into *bêche-de-mer* (dried product) in many Southeast Asian countries for export to the Chinese market (Carpenter and Niem 1998; Conand 2004; Hamel et al. 2001; Uthicke et al. 2004), causing depletion of wild stocks in many species. There has been interest in developing hatchery and aquaculture techniques for sandfish, *Holothuria scabra*, one of the most valued tropical sea cucumber species (Battaglene et al. 1999; Purcell and Kirby 2006). There has also been interest in developing IMTA methods using sea cucumbers (Ahlgren 1998; Slater and Carton 2007; Pitt et al. 2004; Watanabe et al. 2012; Watanabe et al. 2013).

This paper introduces an example of a box model analysis to estimate the nitrogen budget based on experimental data and values from literature for a production system of sandfish in sea cage IMTA with milkfish (*Chanos chanos*) and Elkhorn sea moss (*Kappaphycus alvarezii*) which are both commonly cultured in the Philippines. The results help in deciding stocking density and weight of these species in IMTA.

MATERIALS AND METHODS

For the box model analysis of nitrogen (N) budget in polyculture of milkfish, sandfish and Elkhorn sea moss, data from our own experiments and literature information were used. In this closed-box model, milkfish feed was the sole N input source to the polyculture system. Sandfish consumed excess feed and feces (particulate N) from milkfish culture. Elkhorn sea moss absorbed ammonium (NH_4) excreted from milkfish and sandfish for growth. Unused N accumulated in the system; neither decomposition nor denitrification was taken into account for the model calculations.

Milkfish were reared in a floating net cage 5 × 5 × 4 m (length × width × depth) at Igang Marine Station (IMS) of Aquaculture Department, Southeast Asian Fisheries Development Center (SEAFEC/AQD). Milkfish fingerlings (26 g, 61 days of age) were stocked at 36.7 ind/m³ (3670 ind, i.e. standard stocking density in the Philippines) on November 21, 2011. A commercial milkfish diet (Oversea Feeds, Cebu, Philippines) was fed to the fish throughout the rearing period. Daily feeding ration was as follows: days of culture (DOC) 1 - 77: 4% of the mean body weight, DOC 78 - 161: 3.5%, and DOC 162 - 200: 3%. Body weight (BW) was measured monthly to the nearest 1 g under anesthesia with 2-phenoxyethanol by random sub-sampling of 300 individuals. Amount of feed was calculated and adjusted after each sampling. Mortality was recorded daily and monthly survival was recorded.

A logistic growth curve was fitted to the BW data of milkfish from DOC 1 to DOC 200 (i.e. 61 to 260 days of age): $BW\ (g) = 400.5 / (1 + e^{-0.0202 \times (t-192.0)})$ ($r^2 = 0.996$), where *t* is age in days. Daily BW growth rate (g/d) was calculated from the logistic growth curve (i.e. $BW_t - BW_{t-1}$). Daily survival rate was estimated by linear interpolation between the monthly sampling data.

The relationship between BW and ammonium-N (NH_4-N) excretion rate of milkfish was estimated by regression analysis of data reported by Sumagaysay-Chavoso (2003): $NH_4\text{-N}\ (mg/kg/d) = 1.49 \times 10^3 \times BW^{-0.47}$ ($n = 4$, $r^2 = 0.99$), where BW is fresh body weight of milkfish (g).

Daily BW growth pattern of sandfish followed the Gompertz model reported by Watanabe et al. (2014): BW (g) =1961 × e^(-22.5 × e^(-0.00661 × t)), where t is age in days. The relationship between BW and NH_4-N excretion of sandfish followed the equation reported by Kodama et al. (2015): $NH_4\text{-N}\ (g/d) = (0.0153 \times BW^{0.81})/1000$.

Sandfish was assumed to be cultured in a cage with a floor hung underneath the milkfish cage at the stocking size of 10.5 g and stocking density of 35 ind/m². The sandfish cage had the same bottom area as the milkfish cage (25 m²), and excess feed and milkfish feces (particulate N) accumulate 100% in the sandfish cage. Mortality of sandfish was assumed to be linear at the rate of 20%/year (Agudo 2006).

The whole body dry matter content of milkfish (n = 3) and sandfish (n = 10) obtained from the sea cucumber hatchery of SEAFDEC/AQD was measured after drying in a 50 °C oven for two weeks. N content of the milkfish whole body (n = 10), milkfish feed (n = 3) and sandfish whole body (n = 10) was analyzed using an EA-1108 elemental analyzer (Carlo Erba, Italy) at National Research Institute of Fisheries Science, Japan.

Daily particulate N production of milkfish (PNPM, excess feed and feces) was calculated as: $PNPM\ (g/d/cage) = (Feed\text{-}N - (growth\text{-}N + NH_4\text{-}N)) \times N_{milkfish}$, where growth-N is the amount of N gained in one day of body growth, and $N_{milkfish}$ is the number of milkfish. Daily N requirement of sandfish (NRS) was obtained as: $NRS\ (g/d/cage) = ((growth\text{-}N + NH_4\text{-}N)/0.8) \times N_{sandfish}$, where 0.8 is apparent digestibility coefficient of animal protein in sandfish (Orozco et al. 2014) and $N_{sandfish}$ is the number of sandfish.

Growth rate and NH_4-N requirement of Elkhorn sea moss were assumed to be 4.6%/d (specific growth rate) and 0.03 mg/g fresh plant, respectively (Rui et al. 1990). Fresh weight of Elkhorn sea moss on a specific day of culture (Wd) was calculated as: $Wd = e^{(0.046+\ln Wd-1)}$. Stocking weight of Elkhorn sea moss was 10,000 g (10 kg).

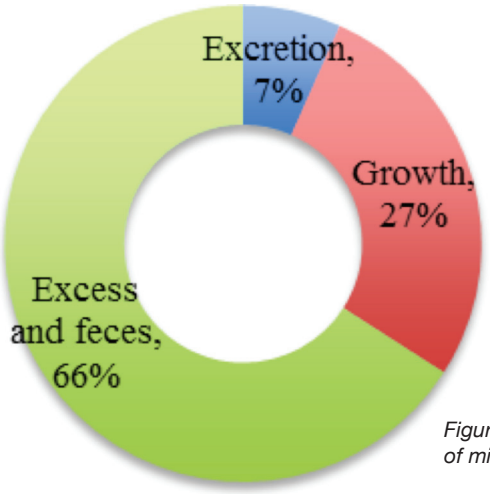

Figure 1. Nitrogen budget of milkfish aquaculture.

RESULTS AND DISCUSSION

The mean milkfish whole body dry matter content was 51.8% ± 4.1 (mean ± SD, n = 3), and the mean N content was 11.2% ± 1.5 (dry wt/wt, n = 10). The mean whole body dry matter content of sandfish was 18.4% ± 5.6 (n = 10), and the mean N content was 7.0% ± 2.9 (n = 10). The mean N content of milkfish feed was 7.0% ± 0.23 (n = 3).

Milkfish grew from 26 g to 320 g on average in the 200-day culture period. Feed conversion rate (FCR) of milkfish culture was high (2.9), and it was estimated that 66% of N in feed was wasted as excess (uneaten) and feces, and 7% was excreted as NH_4-N during 200 DOC (Fig. 1). In other words, 145 kg of particulate N and 60 kg of dissolved N were calculated to be wasted and released to the environment from a small (5 × 5 × 4 m) milkfish cage over 200 days. This is considered to have a high impact on environmental eutrophication. Not only is waste from milkfish culture harmful to the environment, but it is also uneconomical for the fish farmers.

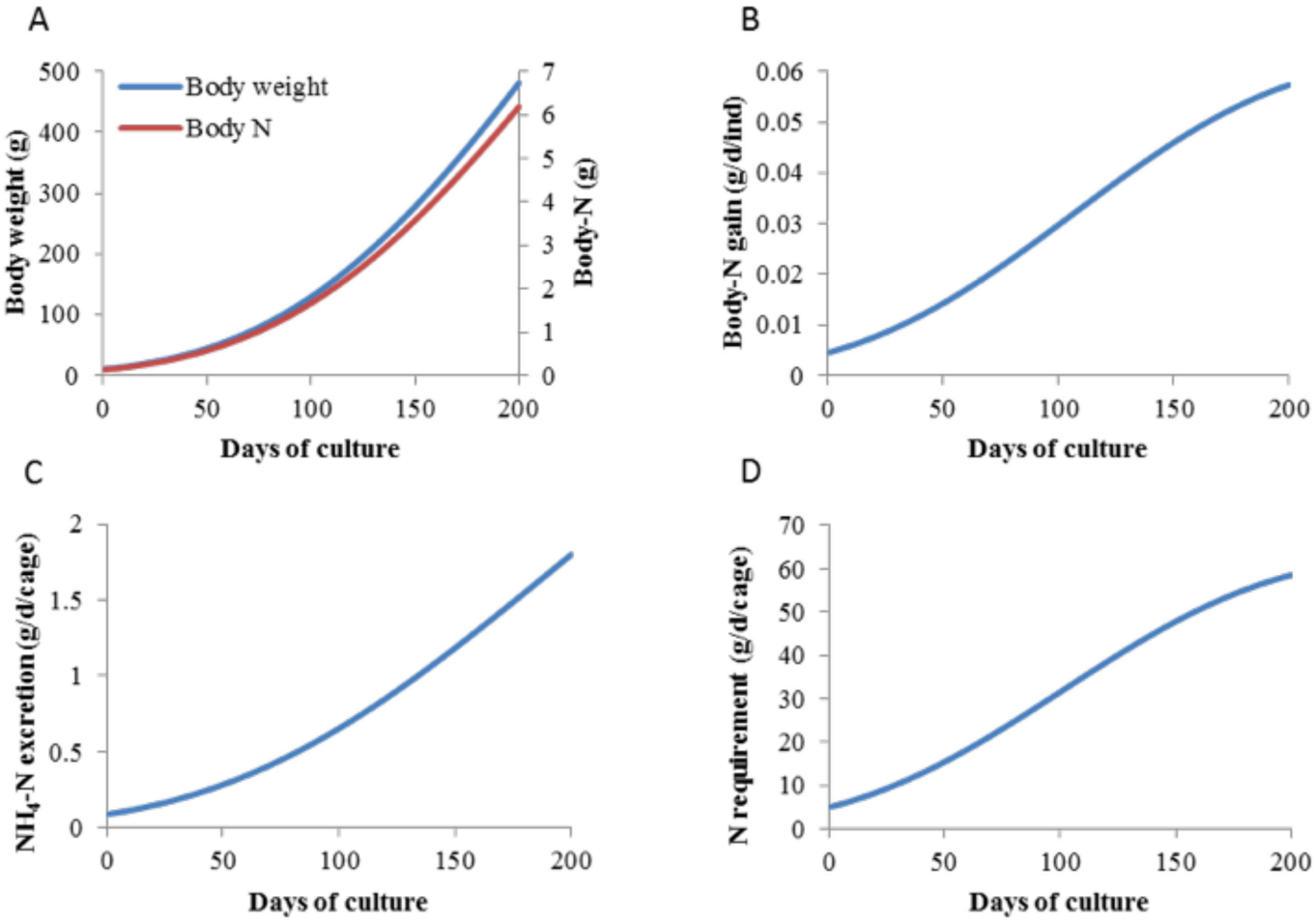

Figure 2. Growth (A), daily body-N gain (B), daily NH_4-N excretion (C) and daily N requirement (D) of sandfish during 200 days of polyculture with milkfish and Elkhorn sea moss.

Milkfish sea cage culture usually has a higher FCR (around 2) than pond culture (around 1.5 or less) where natural feeds are available (Sumagaysay-Chavoso and San Diego-McGlone 2003). The FCR of 2.9 obtained in this study was very high perhaps owing to the unusual experimental rearing season that started in November. Milkfish are usually stocked at the onset of the dry season around March or April in the Philippines. The growth rate of milkfish in this study was slower than those reported for marine pens and comparable to those reported for extensive pond culture (FAO web page). Although there is a possibility that the amount of wasted N may be lower during the regular culturing season, intensive milkfish culture may have detrimental effects on the surrounding environment, especially in semi-closed embayment with low water exchange.

Based on the Gompertz model (Watanabe et al. 2014), sandfish was calculated to grow from 10 g to 481 g in 200 DOC (Fig. 2A). Moisture content of sandfish was 84%, and the N content was 7.0% (dry wt/wt). Based on a linear mortality condition (Agudo 2006), the number of sandfish decreased from 870 to 784 in the 200-day period. Based on these, daily body N gain of sandfish is shown in Fig. 2B, and daily NH_4-N production is shown in Fig. 2C. Daily N requirement is obtained as the sum of growth and excretion shown in Fig. 2D.

Growth and N requirement of Elkhorn sea moss are shown in Fig. 3A and Fig. 3B, respectively.

Box model calculations were based on the above parameters. On DOC 200 for instance, one milkfish (320 g, 260 days of age) provided enough particulate N to support the N requirement of 5.8 sandfish (481 g, 420 d). NH_4-N excretion from one milkfish and one sandfish was estimated to sustain growth of 1068 g and 76 g of Elkhorn sea moss respectively.
Under the polyculture scheme, the recycle rate of NH_4-N excreted by milkfish and sandfish by Elkhorn sea moss increased exponentially with DOC and exceeded 100% by DOC 125 (Fig. 4A), and cumulative NH_4-N was expected to be depleted on DOC 162 (Fig. 4B). Thus, elkhorn sea moss seemed to have high potential to assimilate NH_4-N. However, in the wild sea cage conditions, these estimations may not hold true since NH_4–N excreted from the animals is quickly diluted and transported by the current. Therefore, the amount of Elkhorn sea moss sustainable by the NH_4-N excretion from milkfish and sandfish might have been overestimated. On the other hand, availability of naturally occurring dissolved inorganic N in the seawater should also be incorporated in the model analysis. The specific growth rate of Elkhorn sea moss reported by Rui and Chaoyuan (1990) was based on a 36-day rearing experiment, and the growth rate may not stay constant for 200 days. The effect of stocking density on growth rate (Thirumaran and Anantharaman 2009) should also be included in the model.

Cumulative particulate N did not greatly differ between monoculture and polyculture (Fig. 4D) due to the low particulate N recycle rate by sandfish, which fluctuated between 3.4% and 12.4% due to the stepwise milkfish feeding regime (Fig. 4C). The total amount of particulate N removed by sandfish during the 200 DOC was 4.3% of the total particulate N accumulation from milkfish culture. Thus, although sea cucumbers are known to play an important role in recycling of nutrients of sediments (Uthicke 1999), particulate N recycling rate by sandfish did not seem to be able to keep up with particulate N accumulation from milkfish culture.

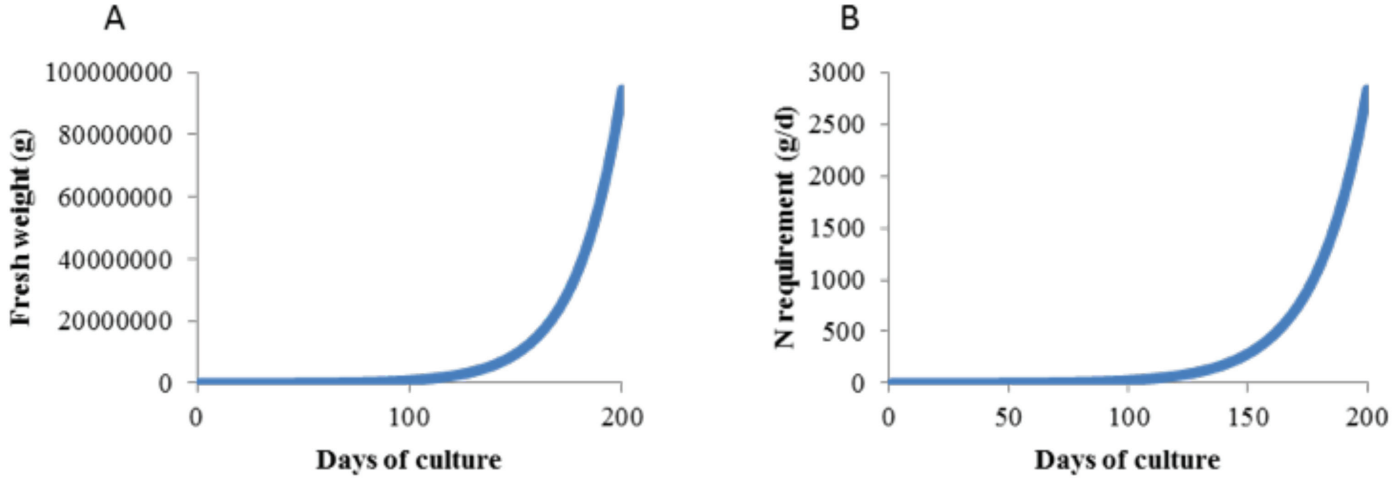

Figure 3. Growth (A) and N requirement (B) of Elkhorn sea moss during 200 days of polyculture with milkfish and sandfish.

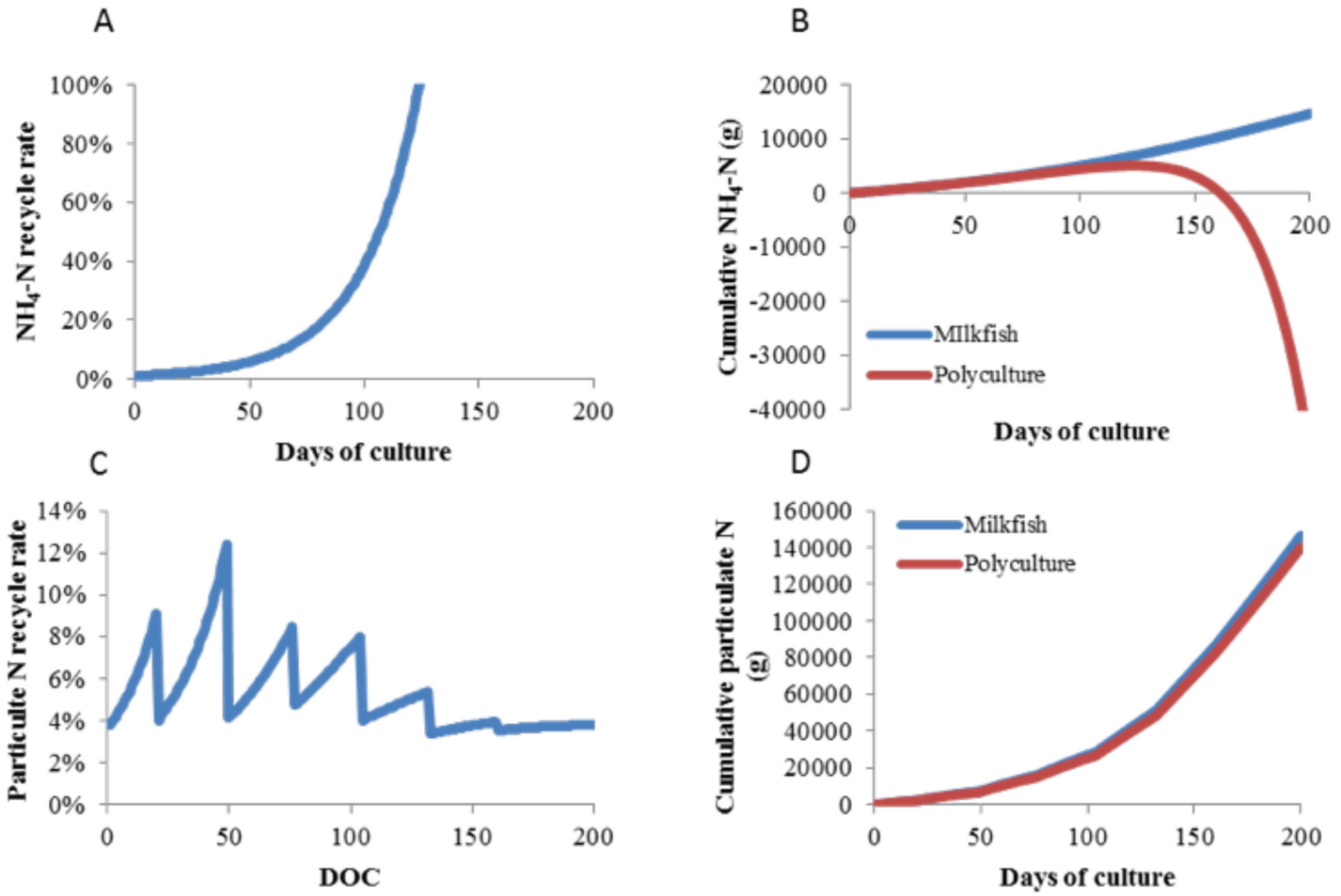

Figure 4. NH_4-N recycle rate (A), cumulative NH_4-N (B), particulate N recycle rate (C) and cumulative particulate N (D) in monoculture of milkfish and polyculture of milkfish, sandfish and Elkhorn sea moss.

Based on the box model, stocking weight of Elkhorn sea moss at which NH_4-N from milkfish and sandfish culture on DOC 200 is completely absorbed would be 2351 g, and the stocking density of sandfish (10 g, weight at stocking) should be 805 ind/m2 to completely consume particulate N from milkfish culture on DOC 200. In existing sandfish aquaculture operations, however, stocking densities are much lower: e.g. 4 ind/m² (20 g) in New Caledonia and 0.73 ind/m² (50 g) in Vietnam (Agudo 2006). The model estimated stocking density of 805 ind/m² seems too high to be practical.

IMTA is a method to recycle wasted nutrients into harvestable commodities. Although sandfish may not be an effective bioremediator for removing nutrients from milkfish culture effluents, IMTA using sandfish may still be economically feasible due to its high commercial value. Sandfish culture cages should be designed in such a way that water exchange rate is high enough for particulate N level to remain low. The highest organic matter level in the sediment tolerable by sandfish is 1.8% when aquaculture feed is the only source of organic matter (Novilla, unpublished data).

This study focused only on estimation of the N budget in the IMTA system. Since sandfish culture methods in sea cages have not been well established, more rearing experiments should be done to build on the data resulting from this study.

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Watanabe S., J.G. Sumbing, and M.J.H. Lebata-Ramos. 2014. Growth pattern of tropical sea cucumber, *Holothuria scabra*, under captivity. JARQ 48: 457-464.

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S. Watanabe, J. G. Sumbing and Ma. J. H. Lebata-Ramos (2014) Growth pattern of tropical sea cucumber, *Holothuria scabra*, under captivity. Japan Agricultural Research Quarterly 48:457-464.

The growth of the juvenile sea cucumber, *Holothuria scabra*, was studied under captivity to elucidate the growth variation pattern and determine the best-fit growth model to estimate age- and size-specific growth rates. Individual growth was extremely variable, with some individuals below the mean initial weight and some expanding their original body length (L) and weight (W) by up to 6.4 and 156 times, respectively; during 84 days of culture starting at 127 days of age. Some of the smallest individuals showed a higher condition factor than larger individuals in the presence of ample food, indicating that lack of food may not be the only impediment to growth. Among the three growth models compared (von Bertalanffy, Gompertz and logistic), the Gompertz model was considered optimal to express *H. scabra* growth; both in L and W. The age- and size-specific daily growth rate for L and W up to 365 days of age, as estimated by the Gompertz model, had a range of two and nine orders of magnitude in L (0.035 – 0.96 mm/day) and W (3.4 × 10-7 – 3.5 g/day), respectively. Use of the Gompertz model over the linear model, which tends to overestimate growth rates, is encouraged to estimate the growth of *H. scabra* more accurately.

Z. G. A. Orozco, J. G. Sumbing, Ma. J. H. Lebata-Ramos and S. Watanabe (2014) Apparent digestibility coefficient of nutrients from shrimp, mussel, diatom and seaweed by juvenile *Holothuria scabra* Jaeger. Aquaculture Research 45:1153–1163.

The ability of *Holothuria scabra* to digest nutrients, such as organic matter (OM), protein and carbohydrate from animal and plant feed ingredients was investigated. Four test feeds prepared by mixing sand with single ingredients from animal sources (shrimp and mussel) and plant sources (diatom and seaweed) were fed to *H. scabra* to estimate apparent digestibility coefficient (ADC). The total assimilated nutrient (TAN) increased with ADC, whereas ingestion rate (IR) varied slightly among the feeds suggesting that ADC might be a good indicator of nutrient availability to *H. scabra*. The ADCOM of shrimp and mussel was significantly higher than that diatom and seaweed: 86.2%, 77.1%, 55.1% and 32.3% respectively. ADCprotein was similar for shrimp (88.7%), mussel (84.8%) and diatom (75.2%), but significantly lower in sea- weed (34.4%). ADCcarbohydrate was similar in mussel (58.5%) and diatom (58.3%) as well as in seaweed (31.6) and shrimp (28.0%). ADCprotein was relatively higher than ADCcarbohydrate suggesting that *H. scabra* generally digests more protein than carbohydrate. Furthermore, results indicated that nutrients from animal-based feeds are more efficiently digested by *H. scabra*; thus, animal ingredients rich in easily digestible protein could potentially provide an efficiently balanced diet for *H. scabra* fed with diatom containing high easily digestible carbohydrate.

S. Watanabe, M. Kodama, J. G. Sumbing and Ma. J. H. Lebata-Ramos (2013) Diet-tissue stable isotopic fractionation of tropical sea cucumber, *Holothuria scabra*. Japan Agricultural Research Quarterly 47:127-134.

To provide a basis for a stable carbon and nitrogen isotope ratio (δ13C / δ15N) analysis to determine the assimilated organic matter in sea cucumber, *Holothuria scabra*, diet-tissue fractionations were experimentally determined by mono-feeding rearing with diatom. While δ15N fractionation of the whole body wall (2.4‰) was similar to the commonly accepted value (2.6 – 4‰), δ13C fractionation of the body wall (4.2‰) showed considerable discrepancy with the commonly accepted value (0 – 1‰) due to the high content (35% dry wt/wt) of calcareous spicules (CaCO3) in the body wall, which had significantly higher δ13C (-8.6‰) than the organic fractions. Computational elimination of spicules based upon spicule content and spicule δ13C reduced the δ13C fractionation of

the body wall to 1.5‰, close to the common value. δ13C fractionation after spicule removal by acid decarbonation and subsequent rinsing (3.2‰) did not agree with the common value, and δ15N fractionation was significantly elevated by decarbonation. δ15N and δ13C fractionations of the intestine (1.5 and 2.2‰, respectively) did not agree with the common values. Since δ13C and δ15N of the feces did not differ significantly from those of the diet, feces may be used to determine ingested organic matter in the wild.

S. Watanabe, J. M. Zarate, J. G. Sumbing, Ma. J. H. Lebata-Ramos and M. F. Nievales (2012) Size measurement and nutritional condition evaluation methods in sandfish (*Holothuria scabra* Jaeger). Aquaculture Research 43:940-948.

The aims of this study were to establish an accurate size measurement method and a nutritional condition evaluation method of *Holothuria scabra* (Jaeger). Although 0.5% KCl and 0.05% MgSO4 did not induce anaesthesia, 2% menthol-ethanol for 20 min was found to be effective and harmless. The anaesthetization significantly reduced the coefficient of variation of the mean body length and weight by 68% and 43% respectively. During starvation, body size and weight decreased concomitantly, resulting in an unchanged condition factor (body weight/volume), suggesting that the condition factor cannot be used as an index of nutritional condition. Protein, cholesterol and carbohydrate concentrations in the body fluid were analysed to study the relationship with starvation. As the protein and cholesterol concentrations initially increased and then decreased during the starvation period, it is difficult to use them as an index of nutritional condition. The carbohydrate concentration showed a gradual one-fold increase during 10 days of starvation, and it may be used as a proxy for nutritional condition; however, further physiological studies are needed. Body fluid density and volume relative to body size gradually increased and decreased, respectively, during starvation. These methods may be used to correctly monitor the conditions of *H. scabra* in studies for aquaculture and stock enhancement techniques.

S. Watanabe, M. Kodama, J. M. Zarate, Ma. J. H. Lebata-Ramos and M. F. J. Nievales (2012) Ability of sandfish (*Holothuria scabra*) to utilise organic matter in black tiger shrimp ponds. ACIAR Proceedings 136:113-120.

Due to frequent viral disease outbreaks, a large proportion of shrimp aquaculture in South-East Asian countries has switched from black tiger shrimp (*Penaeus monodon*) to ***P. vannamei***, an exotic species originally imported from Latin America. One of the causes of disease outbreaks is thought to be poor water and sediment conditions in the shrimp ponds, which may aggravate disease symptoms. To obtain basic information for co-culture methods of black tiger shrimp and sandfish (*Holothuria scabra*) for possible mitigation of shrimp-pond eutrophication and prevention of disease outbreaks, basic laboratory experiments were conducted at the Southeast Asian Fisheries Development Center - Aquaculture Department in Iloilo, the Philippines. A feeding trial of juvenile sandfish showed that they do not grow well with fresh shrimp feed on hard substrate. Another trial indicated that sand substrate enhances the growth of juvenile sandfish fed with shrimp feed. A feeding trial using shrimp tank detritus, shrimp faeces and *Navicula ramosissima* (a benthic diatom) as food sources showed that sandfish grew fastest with the faeces, followed by detritus and *N. ramosissima*. Dissolved oxygen consumption and acid-volatile sulfur levels in the shrimp tank detritus were reduced by sandfish feeding. This suggests that sandfish are capable of growing with organic matter in shrimp ponds, and can bioremediate shrimp-pond sediment.