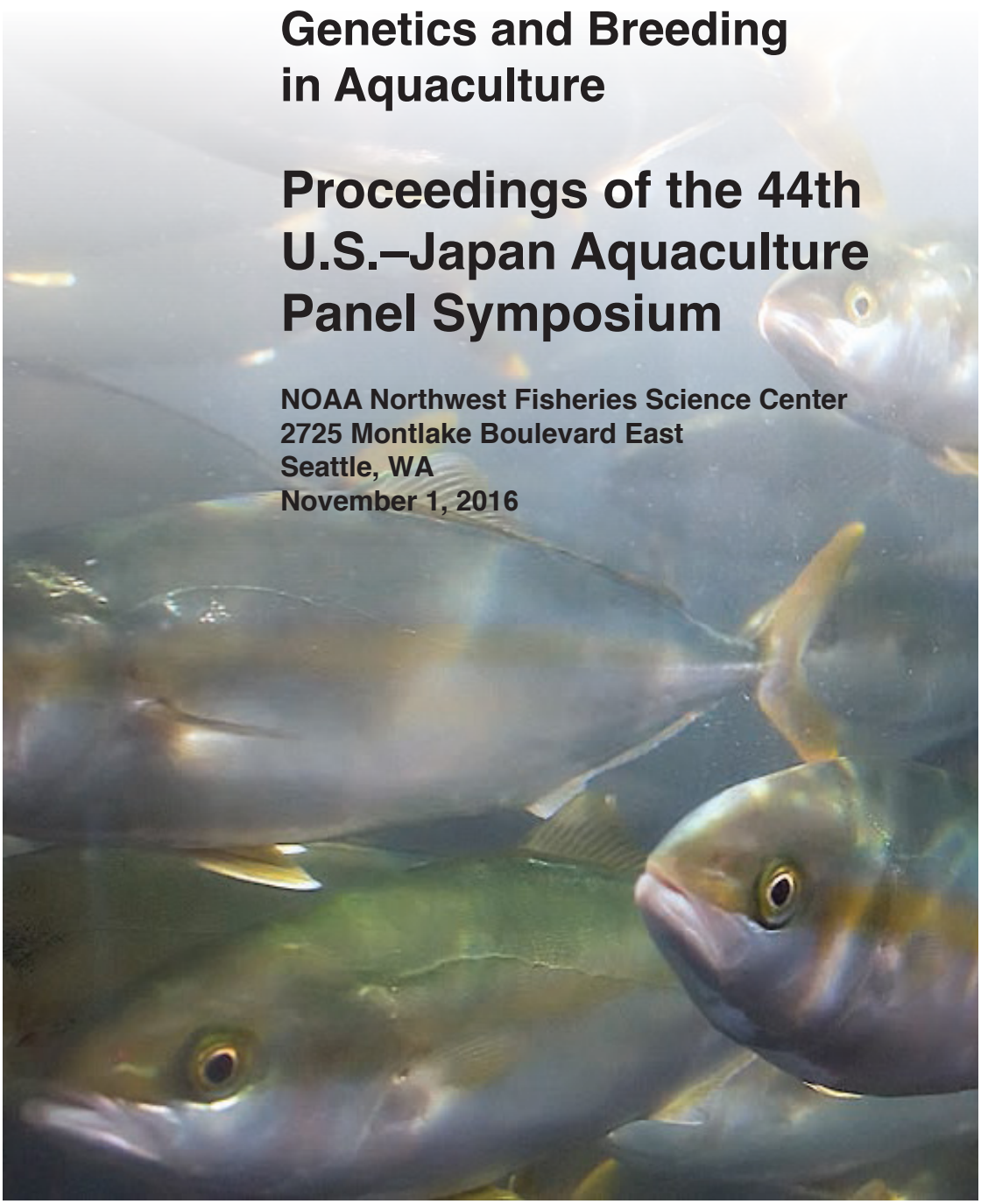

Genetics and Breeding in Aquaculture

Proceedings of the 44th U.S.–Japan Aquaculture Panel Symposium

NOAA Northwest Fisheries Science Center
2725 Montlake Boulevard East
Seattle, WA
November 1, 2016

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

NOAA Technical Memorandum NMFS-F/SPO-175

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U.S. Department of Commerce
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Cover photo of juvenile California yellowtail, *Seriola dorsalis*, swimming in a research tank at Hubbs–SeaWorld Research Institute in San Diego, California. Photo credit: Mark Drawbridge, Hubbs–SeaWorld Research Institute.

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Introduction

Genetic improvement has contributed significantly to advances in aquaculture production. Traditional selective breeding has a long history of improving production traits in livestock and is having progressively greater impacts in aquaculture. However, a new alphabet soup of technologies—QTLs, MAS, CRISPR, TALENs, gBLUP, and Big Data!—that can be used to improve production efficiency, product quality, and fish health will significantly accelerate this process. In health management, for example, genetic tools used to improve disease resistance and immune response, better understand pathogens, and develop vaccines have strengthened the aquaculture industry. In terms of sustainability, understanding genetic interactions between wild and cultured stocks and genetic and epigenetic resilience to environmental change are also research priorities shared by the Fisheries Research and Education Agency (FREA) of Japan, the National Oceanic and Atmospheric Administration (NOAA), and the U.S. Department of Agriculture. Opportunities created by this science will be of even greater importance in the future. This Proceedings concludes three UJNR Symposia focused on these interrelated topics. Proceedings from the previous two Symposia were published by NOAA (42nd UJNR Proceedings) and FREA (43rd UJNR Proceedings). Some common themes include genetic, genomic, and transcriptomic resource development; improvement through selective breeding using genetic, genomic, pedigree, and phenotypic data; chromosome set manipulation such as triploid induction; emerging technologies like genome editing and epigenetics; and assessing competitive, genetic, and fitness impacts on wild populations using empirical data and modeling. Finally, these meetings have facilitated the exchange of scientists toward developing competitive new and enhancing existing aquaculture industries on both sides of the Pacific. We hope this Proceedings and the previous two will serve as a good launching point for those wanting more information on these exciting topics.

Michael B. Rust
NOAA Fisheries Office of Aquaculture
United States Panel Chair

Integrated Multi-Trophic Aquaculture (IMTA) as a Countermeasure for Coastal Oligotrophication in Japan

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Keywords: IMTA, eutrophication, oligotrophication, feed conversion ratio

ABSTRACT

Intensification of aquaculture production has caused environmental deterioration associated with water and sediment eutrophication, resulting in sporadic mass fish kills in some parts of the world. In contrast, eutrophication has been reduced in coastal waters of Japan because of diminished production and improved feeding efficiency of finfish aquaculture and/or mitigation efforts that regulate the allowable amount of terrestrial nitrogen/phosphorus discharge to the sea. Red seabream, *Pagrus major*, aquaculture production, for instance, peaked in the early 1990s and has been in a decreasing trend since 2000 in Japan. The levels of surface dissolved inorganic nitrogen (DIN) have gradually decreased, while phosphorus levels have been rather constant in Ise Bay since the 1990s, possibly causing a nutrient imbalance for primary production. Surface chlorophyll *a* levels have been in a decreasing trend since the 1990s, and the occurrence frequency of harmful algal bloom has decreased by 90% since 1979 in Ise Bay. The oligotrophication (relative to the past) of coastal waters is considered to have brought about a decline in carrying capacity of the coastal environment, causing in part a continuous decrease in some coastal fishery resources and reduced productivity in molluscan and algal aquaculture. The national production of the Manila clam, *Ruditapes philippinarum*, for example, has continuously declined for thirty years, falling below 10% of the peak value marked in the mid-1980s. In Ariake Bay, DIN deficiency causes bleaching of cultured nori, *Pyropia yezoensis*, severely reducing the market value especially towards the end of the culture season in early spring. Integrated multi-trophic aquaculture (IMTA) is an aquaculture approach that combines culture of economically important species from different trophic levels, typically finfish, organic extractive species (e.g. bivalve) and inorganic extractive species (e.g. seaweed). Along with reinforcement of economic stability, one of the important goals of IMTA is to mitigate effluent load from finfish culture for environmental integrity. In contrast, we are trying to develop techniques to use IMTA to compensate for the reduced aquaculture productivity of bivalves and seaweeds associated with the coastal oligotrophication. The amount of NH_4^+-N excretion from the red seabream aquaculture in Hasamaura Cove, Mie Prefecture was estimated to be 9.2 t/year. The amount of nitrogen extracted by green alga, *Monostroma nitidum* (Japanese common name is Hitoegusa) aquaculture in the cove was estimated to be 2.9% of the red seabream NH_4^+-N excretion. *M. nitidum* was found to have a nitrogen content twice as high in Hasamaura Cove than those cultured in nearby bays without finfish aquaculture. The red seabream NH_4^+-N excretion is equivalent to the amount of nitrogen in 2071 t/year (wet weight) of the Manila clam suspended culture production. Thus, IMTA has a big potential to enhance aquaculture production by using what would otherwise be fish waste.

INTRODUCTION

Intensification of aquaculture production has caused environmental deterioration associated with water and sediment eutrophication and consequent harmful algal bloom, hypoxia and sulfide production, resulting in sporadic mass fish kills in some parts of the world. Eutrophication is harmful for not only the cultured fish but also surrounding environments. In contrast, eutrophication has been reduced in coastal waters of Japan because of diminished production and improved feeding management of finfish aquaculture and/or enforcement of the Total Pollutant Load Control System, issued by the Ministry of the Environment in 1979, which regulates the allowable amount of terrestrial nitrogen/phosphorus discharge to the sea. The oligotrophication (relative to the past) of coastal

waters is considered to have brought about a decline in carrying capacity of the coastal environment, causing in part a continuous decrease in some coastal fishery resources and reduced productivity in molluscan and algal aquaculture. Benthic fish fisheries production in Seto Inland Sea, for instance, has decreased markedly, and the fishery production of the benthic fish was found to be negatively correlated with the dissolved inorganic nitrogen (DIN) level in the coastal water (Handa and Harada, 2012).

In this paper, the trends in environmental factors in Ise Bay and southern coast of Mie Prefecture, Japan, will be introduced based on the statistical data provided by the prefectural and national government. The relationship between the environmental factors and fisheries production will be discussed. Finally, our attempt to use integrated multi-trophic aquaculture (IMTA) to enhance unfed aquaculture production in recent oligotrophic environments will be introduced.

MATERIALS AND METHODS

Statistical data for environmental parameters in Ise Bay, central Japan, were obtained from the report of *Senkai Teisen Chosa* (shallow sea fixed line survey), a monthly monitoring survey conducted at 16 stations by Mie Prefecture Fisheries Research Institute (Mie Prefecture Fisheries Research Institute, 1972-2016). The monthly data for dissolved inorganic nitrogen (DIN, i.e. the sum of NH_4^+, NO_2^-, NO_3^-), PO_4^-P, chlorophyll *a* and sea surface temperature were smoothed by 13-month moving average operation. Where the data collections were not complete in some stations, missing data were ignored, and the grand mean of all stations were obtained to analyze the change in trend.

Aquaculture production data were obtained from the Ministry of Agriculture, Forestry and Fisheries web site, *Kaimen Gyogyou Seisan Toukei Chosa* (marine fisheries production statistics survey): http://www.maff.go.jp/j/tokei/kouhyou/kaimen_gyosei/index.html.

Ammonium (NH_4^+) excretion rate (TAN) of red seabream (*Pagrus major*) is reported by Takashi et al. (2017) as follows: TAN (mg-N/day) = $0.013\exp^{0.07T}W^{1.01}$, where T is water temperature (°C) and W is fish weight (g). This formula was used to estimate annual NH_4^+ excretion by red seabream aquaculture in Hasamaura Cove, Mie Prefecture. Information on the red seabream production in the area was obtained from Mie Prefectural Government statistics, details of which will not be disclosed in this paper. For feed conversion ratio (FCR) of red seabream aquaculture, the common value of 2.5 was used for calculations.

Green alga, *Monostroma nitidum* (Japanese common name is Hitoegusa) specimens were collected from aquaculture sites in Hasamaura Cove and adjacent bays, Mie Prefecture. Nitrogen content of *M. nitidum* was analyzed by an elemental analyzer, Flash EA112 (ThermoFisher Scientific).

RESULTS AND DISCUSSION

The Total Pollutant Load Control System (TPLCS) has been implemented to mitigate the eutrophication of the closed sea areas in Japan (i.e. Tokyo Bay, Ise Bay and Seto Inland Sea) since 1979. Allowable amounts of terrestrial nutrient discharge to the sea areas are set every five years, and for instance the target values for N and P discharge to Ise Bay during the seventh term (i.e. 2011 to 2016) were 115 t/day and 8.7 t/day, respectively. Eutrophication caused by aquaculture effluent has been regulated by Sustainable Aquaculture Production Assurance Act (APAA) issued by the Fisheries Agency since 1999. With the assistance of the prefectural extension workers, fisheries cooperative associations as a group or individual farmers having a demarcated fishery right submit an Aquaculture Area Improvement Plan, which prescribes (i) the water area concerned and the species of the farmed aquatic animals and plants, (ii) improvement goal of the aquaculture area, (iii) measures to be taken to improve the aquaculture area and their implementation time, and (iv) establishment of the facilities and organizational system necessary to improve the aquaculture area, to the respective government office every five years. Typically, appropriate maximum culturing density is set to be less than 95% of the trimmed mean excluding the largest and the smallest values of the density from the previous five-year term. The farmers may receive subsidy for pension plans to compensate for the income loss incurred by the reduced aquaculture production. The reduced production also intends to prevent oversupply and subsequent price drop of the products. This may be one of the causes of Japan's decreasing aquaculture production, along with competition with cheaper labour in developing countries despite the continuous and rapid global expansion of aquaculture production.

Because of the mitigation efforts, water quality has been gradually improving along the coast of Japan. In Ise Bay, the DIN level has a long term decreasing trend with big fluctuations over the past thirty years (Fig. 1). For example, the annual mean DIN level was 11.2 μM and 4.6 μM in 1985 and 2015, respectively (i.e. 58.9% reduction in 30 years). Phosphate level, on the other hand, did not decrease between 1985 (0.59 μM) and 2003 (0.63 μM) and had a decreasing trend with large fluctuations between 2004 (0.79 μM) and 2015 (0.27 μM) (Fig. 2). The difference observed in nitrogen and phosphate levels between 1985 to 2004 might be attributable to the introduction of phosphate-free detergents and establishment of coagulation sedimentation method in the late 1970s, which had already reduced the phosphate level before

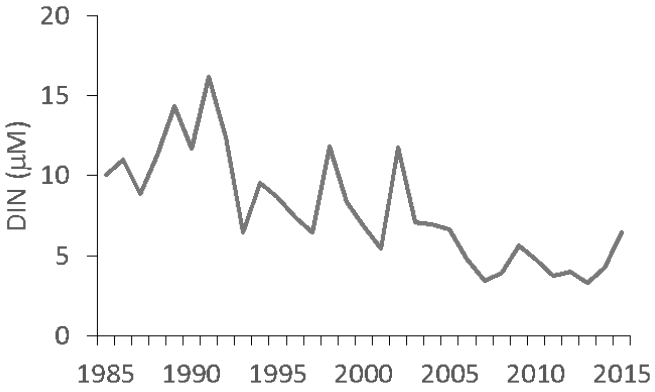

Figure 1. Surface water dissolved inorganic nitrogen (DIN) level in Ise Bay. DIN is the sum of ammonium, nitrate and nitrite.

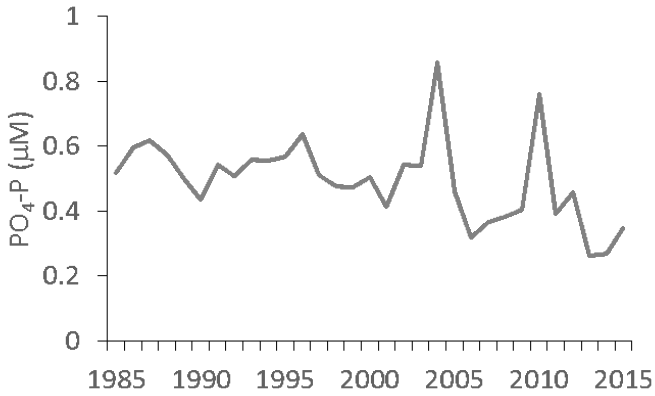

Figure 2. Surface water phosphate level in Ise Bay.

1985 (Washio 2015). The denitrification method for nitrogen removal, on the other hand, did not become widely used until 1990s, resulting in nitrogen over supply relative to phosphate (i.e. N-P imbalance) during this period. The ratio of nitrogen and phosphate in seawater is known to alter phytoplankton species compositions (e.g. Rhee 1978), which may subsequently affect aquaculture production of bivalves that feed on the phytoplankton. Reasons for reduction in phosphate with large fluctuation over the past decade are unknown.

Occurrence of harmful algal bloom (HAB) is tightly linked with seawater nutrient levels (Honjo 1994). Mie Prefecture Fisheries Research Institute regularly monitors occurrence of HAB; the HAB species are as follows: *Heterocapsa circularisquama*, *Chattonella marina*, *C. antiqua*, *Dictyocha fibula* (syn *C. globosa*), *Heterosigma akashiwo*, *Karenia mikimotoi* (syn *Gymnodinium mikimotoi*), *Ceratium furuca*, *Prorocentrum dentatum*, *Noctiluca scintillans*, and *Akashiwo sanguinea*. The frequency of HAB has been markedly reduced in Ise Bay (Fig. 3). HAB was observed 31 times (i.e. gross number and not necessarily equals the actual occurrence) in 1985, whereas it was observed only once in 2015. Reduced nutrient level is probably one of the key causes of the long-term subsiding HAB. Similarly, the surface chlorophyll *a* level has been in a decreasing trend in Ise Bay (Fig. 4), indicating the reduced primary production of not only HAB species but also other phytoplankters which may play important roles in supporting the food web in the area. Occurrence of HAB is more frequent along the southern coast of Mie Prefecture facing the Pacific Ocean.

While the reduced occurrence of HAB is favorable to fisheries and aquaculture, the reduced primary production started to be conceived as a problem in many parts of Japan. It is considered that reduced benthic fish and bivalve production, as well as bleaching of cultured nori (*Pyropia yezoensis*) thalli (i.e. darker color commands a higher price) are in part attributable to insufficient supply of nutrients to the coastal waters (Handa and Harada 2012; Yamamoto 2003). The Manila clam (*Ruditapes philippinarum*) production has drastically dwindled in Mie Prefecture from 1224 t/year in 1985 to 106 t/year in 2015 (Fig. 5) concomitantly with the drop in the DIN level (r^2 = 0.54, $p < 0.0001$) and the chlorophyll *a* level (r^2 = 0.31, $p < 0.01$). Although there are complex, multiple factors involved in the diminution of the clam production (e.g.

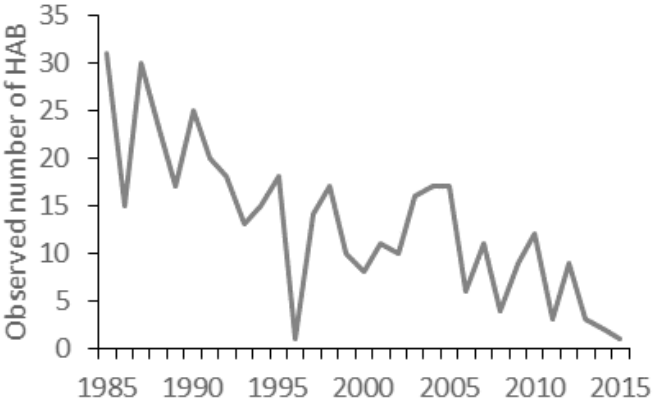

Figure 3. Observed number of harmful algal bloom (HAB) in Ise Bay.

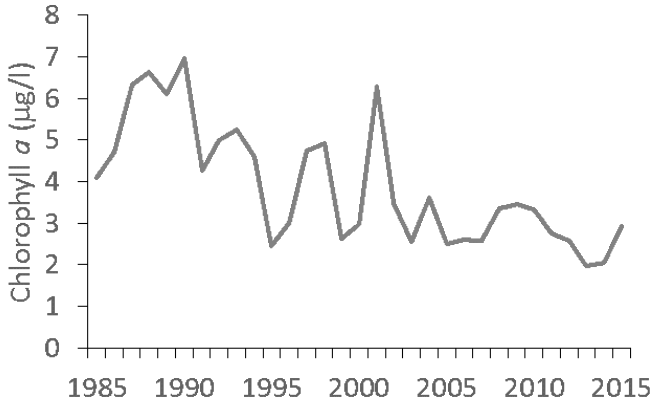

Figure 4. Surface water chlorophyll a level in Ise Bay.

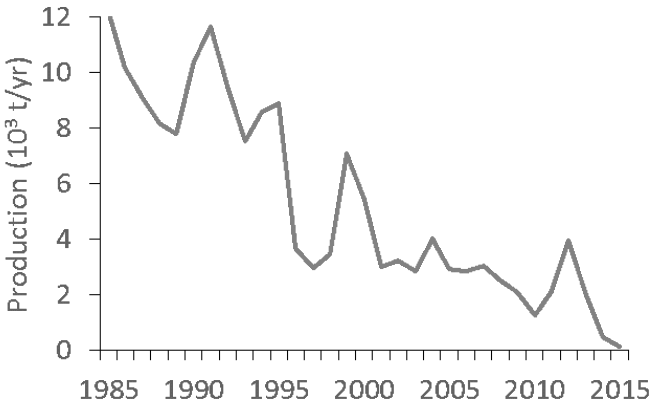

*Figure 5. Fisheries production of the Manila clam (*Ruditapes philippinarum*) in Mie Prefecture.*

Okamoto 2009) and causative relationship between the nutrient environment and fishery production is not fully elucidated, reduction in food supply seems to be at least partially responsible for the diminution. Similar oligotrophication problems are also reported from freshwater systems in North America (Stockner et al. 2000).

Water quality has also been improving in some semi-closed bays along the ria coastline of southern Mie Prefecture, where the TPLSC is not applied, largely because of diminished finfish aquaculture production and improved feeding managements. These areas used to have large amounts of red seabream and yellow tail (*Seriola quinqueradiata*) aquaculture production, and eutrophication derived from the aquaculture effluent was problematic (Abo 2000). However, red seabream production in Mie Prefecture is in a decreasing trend in both volume and value (Fig. 6) because of varied reasons, such as the enforcement of the APAA since 2004, reduced profitability due to international competition, aging of the operators, as well as damage caused by the 2011 Great East Japan Earthquake. The number of management bodies (mostly self-owned businesses) of red seabream aquaculture was reduced from 287 in 2003 to 115 in 2014 in Mie Prefecture (Fig. 6). Yellowtail aquaculture sites have been relocated from inner bay to offshore for better water exchange. Switch of aquaculture feed from raw bait to pellets significantly contributed in the fish productivity and reduction in effluents.

Thus, oligotrophication rather than eutrophication is an issue in Japan, as is the reduced aquaculture profitability of some finfish. Establishing a method to make use of fed aquaculture effluent to enhance unfed aquaculture productivity is desirable. Integrated multi-trophic aquaculture (IMTA) is an aquaculture approach that combines culture of economically important species from different trophic levels, typically finfish, organic extractive species (e.g. bivalve) and inorganic extractive species (e.g. seaweed)

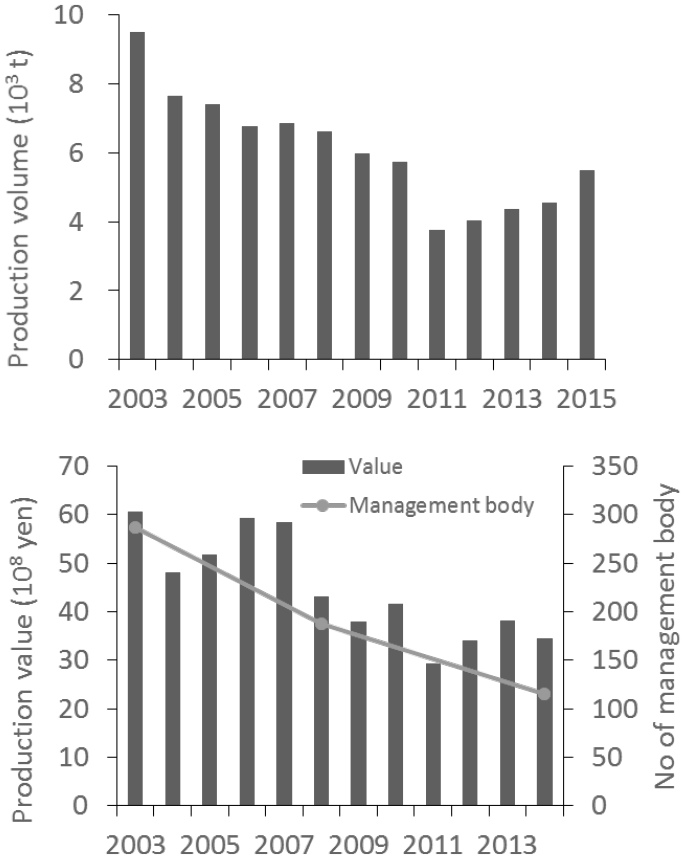

*Figure 6. Production volume (upper) and value (lower) of the red seabream (*Pagrus major*) aquaculture in Mie Prefecture.*

(Robinson and Chopin 2004; Chopin 2006). Along with reinforcement of economic stability, one of the important goals of IMTA is to mitigate effluent load from finfish aquaculture for environmental integrity. In contrast, we are trying to develop techniques to use IMTA to compensate for the reduced aquaculture productivity of bivalves and seaweeds associated with the coastal oligotrophication.

The nitrogenous waste production rate from red seabream aquaculture was estimated in Hasamaura Cove, a semi-closed bay in southern part of Mie Prefecture. As of 2012, there were 13 management bodies, and approximately 157 t of red seabream was produced annually in Hasamaura Cove. The red seabream seeds are stocked at around 60 g in body weight and harvested at around 1.4 kg after three years of culture with 85% survival rate. Assuming an FCR of 2.5, the amount of nitrogen given to the fish in the feed was estimated to be 24.2 t/year, 40% of which was calculated to be used for the fish growth, 17% wasted as leftover feed, 38% excreted as NH_4^+-N, and 5% excreted as feces (Fig. 7). Therefore, the total nitrogen load to the environment from the red seabream culture was estimated to be 14.5 t/year, in which 9.2 t/year was NH_4^+-N that could be readily used for primary

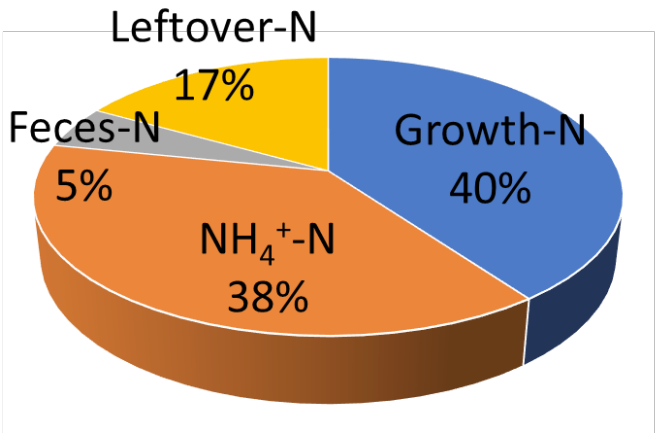

Figure 7. Nitrogen budget of red seabream aquaculture in Hasamaura Cove, Mie Prefecture estimated by a mass balance model.

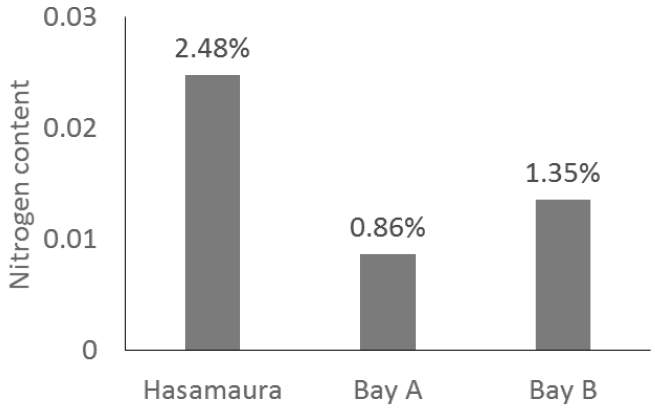

*Figure 8. Nitrogen content of green alga Hitoegusa (*Monostroma nitidum*) in Hasamaura Cove and nearby bays in Mie Prefecture. The names of Bay A and B are not disclosed.*

production. The chlorophyll *a* level was observed to be higher near the red seabream cages, with the maximum layer expanding horizontally towards the inner part of the cove.

Despite the relatively small amount compared to the TPLCS target value of 115 t-N/day in Ise Bay, the nitrogenous wastes from red seabream aquaculture seemed to have an influence on the product quality of *M. nitidum* cultured in Hasamaura Cove. Darker *M. nitidum* with higher nitrogen content is considered a better quality product and fetches higher unit price (e.g. farm gate price ranging from approx. JPY 3,500 to 13,000 per kg in dry weight). The mean nitrogen content of *M. nitidum* cultured in Hasamaura Cove was more than twice as high as those cultured in nearby bays along the same coastline without finfish aquaculture (Fig. 8). Furthermore, there was a negative correlation between the nitrogen content of *M. nitidum* and the distance between the red seabream cages and *M. nitidum* culture sites in Hasamaura Cove. These imply that the effluents from the red seabream aquaculture have a potential to fertilize *M. nitidum*. The amount of nitrogen extracted by *M. nitidum* culture in Hasamaura cove (i.e. 264 kg/year of harvest in 2014) was equivalent to 2.9% of the red seabream NH_4^+-N excretion.

Because of severely dwindled Manila clam fisheries production in many parts of Japan including Mie Prefecture, suspended culture of the Manila clam has recently been drawing much attention (Higano and Asao 2017). A simple box model analysis estimated that the amount of NH_4^+-N excretion from the red seabream aquaculture in Hasamaura Cove was equivalent to the amount of nitrogen in 2071 t/year (wet weight) of Manila clam production, based on the ongoing experimental data on growth, survival (Hasegawa unpublished data), food consumption and metabolism (Watanabe unpublished data) of the Manila clam under suspended culture conditions.

Considering that the fisheries production of the Manila clam in Mie Prefecture has fallen below 1000 t/year (Fig. 5), the nutrient provision from red seabream aquaculture via primary production might have huge potential to enhance bivalve production.

Thus, IMTA has great potential to enhance aquaculture production by using what would otherwise be fish waste. Further studies are underway on aquaculture techniques to provide useful information for establishment of effective IMTA systems to utilize aquaculture effluent in oligotrophic waters.

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This is one of the first papers on IMTA (referred to as integrated culture) combining salmon cage culture and *Porphyra* (currently *Pyropia*) species culture to alleviate the seasonal nutrient depletion by using the significant loading of salmon farms, which is then valued (wastes become fertilizers) and managed (competition for nutrients between desirable algal crops and problem species associated with severe disturbances). The development of integrated aquaculture systems is a positive initiative for optimizing the efficiency of aquaculture operations, while maintaining the health of coastal waters.

Watanabe, S., M. Kodama, and M. Fukuda. 2009. Nitrogen stable isotope ratio in the manila clam, *Ruditapes philippinarum*, reflects eutrophication levels in tidal flats. Marine Pollution Bulletin 58: 1447–1453.

The authors revealed that the nitrogen stable isotope ratio ($\delta^{15}N$) in the soft tissues of the manila clam, *Ruditapes philippinarum*, could be used as an indicator of anthropogenic eutrophication levels in tidal flat environments. In addition, they found that the acid insoluble fraction of the shell organic matrix, conchiolin, could be used as a proxy for the soft tissues in $\delta^{15}N$ analyses, which would result in easier storage handling and the expansion of chances for sample acquisition. Understanding the effects of anthropogenic eutrophication on coastal fisheries may help in the enhancement of fishery production by effective utilization of sewage effluents, as well as in the consequent reduction of eutrophication.

Interactions Between Shellfish Aquaculture and the Environment in the Northeastern U.S.

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ABSTRACT

Opposition to new shellfish aquaculture operations in the Northeastern U.S. frequently is based upon the premise that these commercial operations have negative environmental consequences. This perception is not consistent with the myriad of ecosystem services that are attributed to shellfish, including improving water clarity, reducing excess nutrients, providing habitat, stabilizing sediments, and serving as a food for wildlife and humans. Researchers at the Milford Laboratory have worked to quantify the complex interactions between shellfish aquaculture and the natural environment across the Northeastern United States region and beyond.

From 2008-2010 experiments were conducted in East Creek, Long Island, New York to quantify the environmental footprint of a floating upweller system (FLUPSY) within the surrounding embayment. Metrics such as dissolved nutrients, phytoplankton abundance and community composition, chlorophyll *a*, temperature, salinity, and dissolved oxygen were measured. Results of the multi-year study indicate that the FLUPSY had a very small role in ecosystem function compared with the range of natural environmental variation within the East Creek System. From 2009 to 2013, studies were conducted to measure the consequences of hydraulic dredging for clam harvest upon sediment chemistry and the benthic community in coastal Milford Connecticut. Milford Laboratory data indicated that hydraulic dredging caused initial, short-lived changes in sediment chemistry that resolved within a few weeks. Disturbance of the benthic community was minimal and the community that inhabited this nearshore zone was naturally resilient to a dynamic physical environment.

Milford research also has quantified the environmental benefits of shellfish aquaculture. Starting in 2011, the biodeposition method was employed to measure nitrogen removal and water clarity improvements by shellfish throughout New England and on the west coast of the Korean peninsula. U.S. study locations have included NY, CT, RI and MA and species included ribbed mussels, blue mussels, clams and oysters. Research indicated that shellfish are able to adapt to a wide range of environmental conditions, but careful site selection is needed to maximize environmental benefits of aquaculture operations. Ultimately, this work demonstrates that shellfish aquaculture can have a positive role in eutrophic ecosystems. We plan to continue our work on interactions between aquaculture and the environment by expanding into multi-trophic and off-shore aquaculture systems in New England.

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Land-based Aquaculture of Red-Spotted Grouper (*Epinephelus akaara*) Using the Closed Recirculation System

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ABSTRACT

The red-spotted grouper, *Epinephelus akaara,* is a commercially valuable fish in Japan. Because of its high value, this species is expected to be a new target fish for aquaculture. However, its culture is thought to be difficult because of its slow growth (four or five years until the fish are of commercial size). To achieve faster growth and higher feed efficiency for the red-spotted grouper, we investigated different water temperatures using a closed recirculation system. In higher water temperatures between 16 °C and 31 °C, growth was faster. The best feed efficiency was obtained at 25 °C. We also carried out similar experiments with salinity. Together, we were able to determine the optimum water temperature and salinity conditions for the effective growth of red-spotted grouper.

INTRODUCTION

The red-spotted grouper belongs to the family Serranidae, which is great interest in this species as new target fish for aquaculture due to its high commercial price in Japan (Nambu 2014). However, because it takes four to five years for this species to reach commercial size, we needed to develop methods for shortening the rearing period to decrease the cost of culturing the fish (Morita 2014). In Japan, research on closed recirculation systems was started in the 1950s. Dr. Saeki and Dr. Hirayama were pioneers in research on closed recirculation systems, including development of the bio-filter (Yamamoto et al. 2008). We started technical development of the closed recirculation system for seed production of red sea bream in 2000 at the Yashima Laboratory (Yamamoto et al. 2013). In this study, to shorten the culture period of the red-spotted grouper, we investigated optimum water temperature and salinity by using the closed recirculation system, which allowed us to maintain optimal water conditions.

MATERIALS AND METHODS

Growth Due to the Difference in the Rearing Water Temperature

We compared the growth, daily feeding rate and feed conversion efficiency of the red-spotted grouper using the closed recirculation system at the Yashima Laboratory of the National Research Institute of Fisheries and Inland Sea in August (to September) 2013. Twenty specimens were held for 30 days in two 80 L tanks (containing 75 L of seawater) in each experiment. The mean body weight was 88 g at the beginning. These experiments were performed at 16, 22, 25, 28 and 31 °C.

Growth Due to the Difference in the Rearing Salinity

We compared the growth and feed conversion efficiency in different salinity conditions. Thirty specimens were held for 32 days in two 80 L tanks (containing 75 L of seawater) in each experiment. The mean body weight was 23 g at the beginning. These experiments were performed in 6, 13, 19, 26, 32 and 38 psu.

RESULTS

Growth Due to the Difference in the Rearing Water Temperature

The rates of the increased weight are shown in Fig. 1. The rates of the increased weight (final body weight / initial body weight x 100) were 101 % (16 °C), 104 % (19 °C), 108 % (22 °C), 116 % (25 °C), 116 % (28 °C) and 129 % (31 °C). Those fish in the controls (16 °C) didn't increase in weight (101 %).

The daily feeding rate (average feed weight / body weight over the 30 days) was 4 % (16 °C), 7 % (19 °C), 16 % (22 °C), 18 % (25 °C), 24 % (28 °C) and 35 % (31

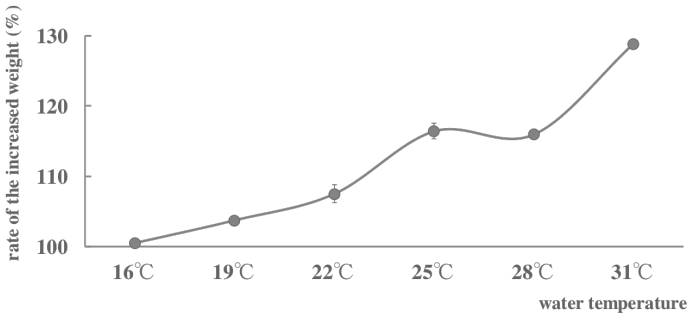

Figure 1. Growth due to the difference in the rearing water temperature. All statistics were done by independent t-test. No statistically significant difference was found among temperatures.

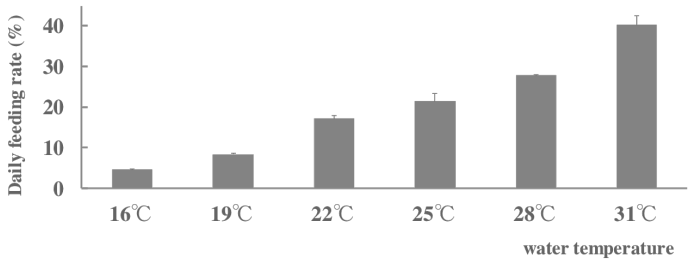

Figure 2. Daily feeding rate due to the difference in the rearing water temperature. All statistics were done by independent t-test. No statistically significant difference was found among temperatures.

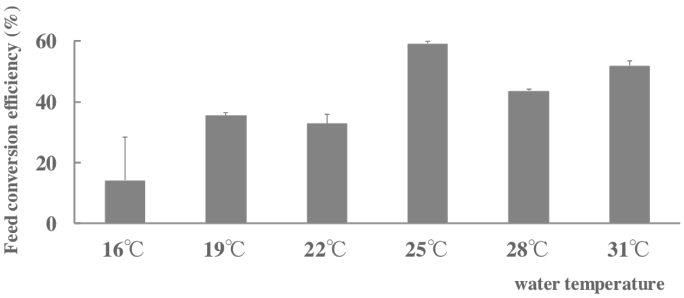

Figure 3. Feed conversion efficiency due to the difference in the rearing water temperature. All statistics were done by independent t-test. No statistically significant difference was found among temperatures.

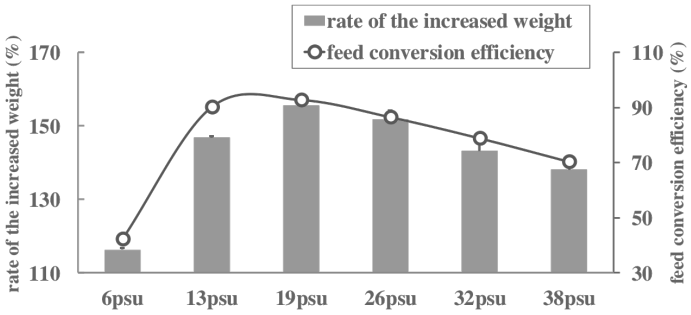

Figure 4. Growth due to the difference in the rearing salinity. The line graph (open circle) is feed conversion efficiency, the bar graph is rate of the increased weight. All statistics were done by independent t-test. No statistically significant difference was found among salinities in each experiment.

°C). The daily feeding rate is shown in Fig. 2. From 16 °C to 31 °C, feeding rate rose as the rearing water temperature is higher.

The feed efficiency was calculated using following formula: feed conversion efficiency (%) = ((final weight - initial weight) / total feed weight) x 100. The feed conversion efficiencies were 13 % (16 °C), 53 % (19 °C), 49 % (22 °C), 89 % (25 °C), 65 % (28 °C) and 78 % (31 °C). The feed conversion efficiency is shown in Fig. 3. From 16 °C to 31 °C, the highest of the feed conversion was obtained at 25 °C.

Growth Due to the Difference in the Rearing Salinity

The rates of the increased weight and the feed conversion efficiency are shown in Fig. 4. The trends in weight increase and feed conversion across different salt concentrations were similar. The rate of the increased weight was 116 % (6 psu), 147 % (13 psu), 156 % (19 psu), 152 % (26 psu), 143 % (32 psu) and 138 % (38 psu). The feed conversion efficiency was 42 % (6 psu), 90 % (13 psu), 93% (19 psu), 87% (26 psu), 79 % (32 psu) and 70 % (38 psu). The growth rate was faster in water of 19 psu and 26 psu compared with 32 psu and 38 psu. Some specimens died in the water below 6 psu.

Example in Larger-scale Culture Tanks

From these results, we conducted the breeding experiment in the larger 5 kL culture-scale tank (total water quantity was 8 kL, including in the closed recirculation system) in September 2013. The body weight at the beginning was 8.6 g ± 1.5 g (5.3 g - 11.7 g). Two thousand specimens were used in each treatment. The change of the body weight from 4 days to 66 days is shown in Fig. 5.

Three treatment groups (25 °C, 30 °C and 25 °C (low salinity)) were reared in closed recirculation systems. In all three treatments, a difference in weight gain was apparent early when compared to the control, where the water temperature and the salinity was not adjusted. Among the three treatments, the weight gain was the largest in the test group with water temperatures of 25 °C plus low salinity (20-29 ‰). These conditions (25 °C plus low salinity) would allow growth of red-spotted grouper up to the commercial size (500 g) within two years.

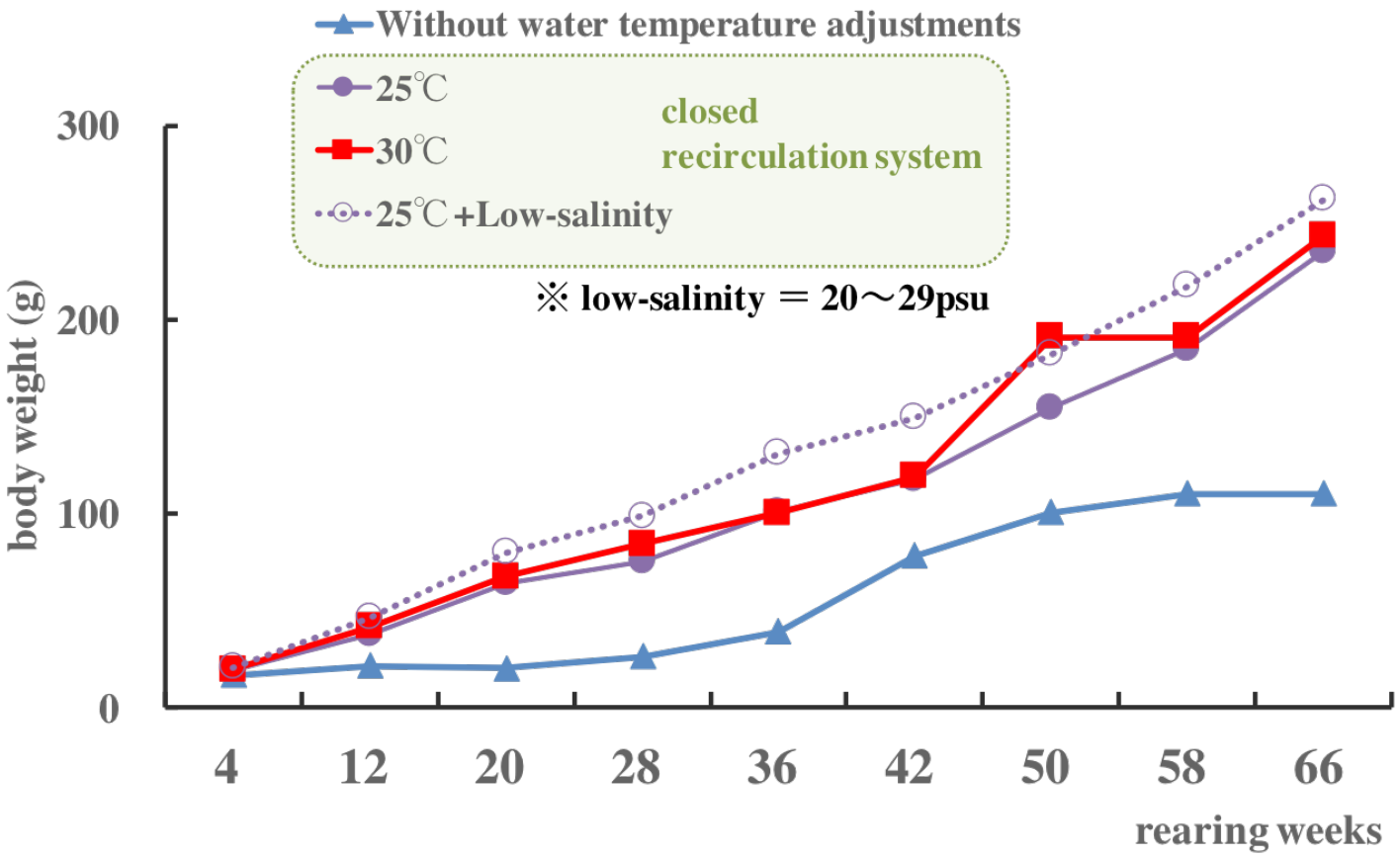

Figure 5. Change in body weight in larger scale culture tanks (5 kL). Closed circle and Purple lines show results at 25 °C. Open circle and Broken line show results at 25 °C + low-salinity. Square with Red line shows results at 30 °C. Three treatments (purple, broken, and red) were reared using closed recirculation systems. Triangle and Blue lines show results where water temperature and salinity were not adjusted. Significant differences, using Tukey's test (p = 5 %), were found between the 25 °C + low salinity treatment and the other treatments for all weeks after the 4th week.

DISCUSSION

The red-spotted grouper is a new potential target for aquaculture because of its high commercial value in Japan. However, its culture is thought to be difficult due to its slow growth where four to five years are needed to reach commercial harvest size (Nambu 2014). Our results suggest that the closed recirculation system can assist in the expansion of land-based aquaculture. The traditional flow-through system is expensive to maintain in optimum condition, but the closed recirculation system solves this problem by reusing drainage. (Yamamoto et al. 2008). In addition, we were able to improve rearing techniques for a slow growth fish with great potential for aquaculture. Our investigation revealed the optimal water temperature and salinity for growth of red-spotted grouper to commercial size is only two years in a closed recirculating aquaculture system.

Furthermore, mass mortalities associated with viral nervous necrosis (VNN) have occurred in red-spotted grouper reared in a traditional flow-through system not only in Japan but also in other countries (Lin et al. 2001). It has been suggested that closed recirculation systems would be suitable for breeding research to compare environmental conditions and study control of fish diseases.

However, since the breeding conditions of the red-spotted grouper using traditional flow-through systems had only been studied in larger tanks (Nambu, 2014) and recirculation systems in smaller tanks, economic analysis of production in closed recirculation systems versus conventional flow-through systems has not yet been investigated. To address this question, we also needed to investigate breeding conditions in a larger tank at a scale suitable for practical application. Finally, it will be necessary to perform a cost analysis in the future in order to develop practical aquaculture technology using the closed recirculation system.

CONCLUSION

The results of this study demonstrated the potential for large-scale production of red-spotted grouper using a closed recirculating aquaculture system. However, in order to enable commercial adoption of

this technology, we need to develop more practical technologies and cost analyses. Thus, it is necessary to further develop appropriate rearing techniques for fish grown in the closed recirculation system.

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This is one of the first reports on effectiveness using low-salinity treatment of red-spotted grouper's seed production. The author suggested that examining the optimum salinity setting and the effective period at rearing of the red-spotted grouper for the improvement of the survival rate, also reported the possibility of the cost saving effect by using the closed recirculation system in low-salinity rearing.

Using Physiological Tools to Assess and Optimize Aquaculture of California Yellowtail, *Seriola dorsalis*

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ABSTRACT

The California yellowtail (*Seriola dorsalis*) is a promising candidate for offshore commercial aquaculture in southern California. However, best rearing practices for this and other *Seriola* species are still under development, and our work has shown that yellowtail spawned and reared in captivity are typically less healthy than their wild-caught counterparts. Our research thus seeks to use various physiological tools and metrics to monitor fish health, fitness, and growth in an attempt to enhance rearing techniques. Over the past few years, our work has focused on 1) examining fitness differences between wild-caught and aquaculture-reared individuals through measures of critical swimming speed and metabolic rate, 2) determining the adverse effects of an extremely common deformity (an uninflated gas bladder) in hatchery-reared yellowtail on their critical swimming speed, metabolic rate, growth rate, and feed conversion ratio, and 3) investigating the potential growth and health benefits of forced exercise on early juvenile-stage yellowtail. Our results suggest that although wild-caught fish tend to show better health and metabolic efficiency, great strides in yellowtail health and fitness in aquaculture have occurred during the duration of our research, and eliminating deformities such as uninflated gas bladders greatly increases growout efficiency. While our swimming exercise studies are still underway, preliminary data suggest that a short duration of exercise (3-4 weeks) followed by growout in standard rearing tanks produces a growth advantage. Such exercise regimes may thus represent not only a mechanism for improving fish health and fitness, but also a non-genetic and non-hormonal treatment to accelerate growth of fish for food or wild stock enhancement.

INTRODUCTION

The carangid genus *Seriola*, or amberjack species, has been identified as a group with ideal characteristics for warm water aquaculture. *Seriola* species have fast growth rates, demonstrate adaptability to hatchery production and pen culture, and provide high value sashimi products as well as premium fish fillets. However, unlike salmonids, which have a much longer history in aquaculture, basic research is required to inform best practices for rearing yellowtail in a healthy, sustainable way. Historically, the culture of *Seriola* has involved collecting early life stage juveniles from the wild that are transferred to pens and reared to market size. However, the regional and, in many cases, the declining availability of wild seed (Nakada 2002, 2008) has led to more recent efforts to produce eggs and larvae in a hatchery setting from wild-captured broodstock (Moran et al. 2007; Stuart and Drawbridge 2013).

Recent research in larval rearing techniques has opened the opportunity for expanding *Seriola* aquaculture development; however, further research is needed to understand and optimize the rearing process. Over the past five years, research at the Southwest Fisheries Science Center has used a variety of physiological tools to assess the health and fitness of the California yellowtail (*Seriola dorsalis*) to enhance rearing protocols. Specifically, this has included three studies: 1) examination of differences in metabolic and swimming fitness between aquaculture-reared and wild-caught fish to provide baseline metrics and achievable health standards for hatchery-reared yellowtail, 2) determination of the adverse effects of an uninflated gas bladder (a common hatchery deformity) on the fitness of aquaculture-reared yellowtail, and 3) examination of the potential beneficial effects of forced exercise on the growth and health of yellowtail reared in captivity.

This work builds upon preliminary research presented at the 2014 UJNR workshop which compared aquaculture-reared and wild-caught *S. dorsalis* in terms of swimming performance (e.g., critical swimming speed) and metabolic fitness (e.g., standard metabolic rate) as indicators of fish "physiological well-being." Having established baseline metrics for yellowtail, we apply these to examine the physiological costs of *S. dorsalis* lacking a properly inflated swim bladder. While the exact cause of this deformity is not well understood, it is important to understand potential health impacts (and associated production costs) associated with rearing fish with this deformity.

We test the hypothesis that, because the gas bladder regulates buoyancy and thus fish with this deficiency are negatively buoyant, this deformity results in substantial metabolic costs that likely influence energy allocation and growth.

Finally, exercise has been shown to be a promising non-invasive, non-hormonal method to stimulate growth (Brown et al. 2011; Palstra et al. 2015; Yogata and Oku 2000), lower feed conversion ratios (Palstra et al. 2015; Yogata and Oku 2000), and potentially affect metabolic rate (Brown et al. 2011), resulting in improved fitness. However, the results of exercise conditioning for *S. dorsalis,* and for the closely related *S. lalandi*, have varied in terms of swimming speed, exercise duration, and feeding parameters, so it is unclear how much exercise is needed to elicit a favorable response. This study thus aimed to make preliminary evaluations on the effect of exercise duration on growth and fitness in *S. dorsalis*.

METHODS

Fish Collection and Husbandry

Aquaculture-reared California yellowtail used in this work were provided by Hubbs-SeaWorld Research Institute (HSWRI) in San Diego, CA, and were transferred to the experimental aquarium facility at the Southwest Fisheries Science Center (SWFCS) for physiological experimentation. Wild juvenile yellowtail associated with drifting kelp in local offshore waters were captured by hook and line. All fish were fed commercial pellet fish feed to satiation six days a week and were kept for a minimum of five weeks in the aquarium facility before any experimentation began.

Study 1: Swimming and Metabolic Fitness of Aquaculture-reared and Wild-caught Yellowtail

In order to examine potential differences in the swimming and metabolic fitness of aquaculture-reared and wild-caught yellowtail, measures of critical swimming speed and metabolic rate were made using a small 5.4 l Brett-style swim tunnel respirometer. Following a 1 h acclimation period, fish were forced to swim at incremental velocities (5-10 cm s^{-1}) for 30 minutes until the speed was too fast for the fish to maintain its position within the tunnel (i.e., to exhaustion). The critical swimming speed (U_{crit}) was estimated by the equation:

$$U_{\mathrm{crit}} = V_{\mathrm{p}} + \left(\frac{t_{\mathrm{f}}}{t_{\mathrm{i}}} \times V_{\mathrm{i}} \right) \qquad (1)$$

where U_{crit} is the critical swimming speed (cm s^{-1}), V_p is the highest speed sustained for a full 30-minute increment (cm s^{-1}), t_f is the time the fish swam (minutes) at the fatigue velocity, t_i is the time interval for each velocity increment (30 minutes), and V_i is the last incremental speed increase (either 5 cm s^{-1} or 10 cm s^{-1}). Following experimentation, swimming speed was corrected for the solid blocking effect of the flow meter during calibration and for the fish in the chamber (Bell and Terhune 1970).

Oxygen consumption rates ($\dot{M}_{O_2}$) were determined for each fish at each swimming speed using a fiber optic oxygen sensor. Shortly after the step increase in swimming speed, the respirometer was temporarily sealed from 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 and then $\dot{M}_{O_2}$ measurements were repeated at that speed, if possible. To compensate for differences in fish mass and water temperature between trials, $\dot{M}_{O_2}$ measurements were adjusted to a water / body temperature of 18°C (using $Q_{10} = 2$) (Pirozzi and Booth 2009) and to a body mass of 80 g (using $\mathrm{mass}^{0.80}$). Metabolic data were then used to create an exponential regression relationship for $\dot{M}_{O_2}$ vs. swimming speed for each individual. Each regression line was extrapolated to a swimming speed of 0 cm s^{-1} to estimate the standard metabolic rate for that fish.

Study 2: Fitness and Growth of Yellowtail with a Swim Bladder Deformity

Swimming and metabolic fitness as well as the somatic growth and efficiency of food use were examined in aquaculture-reared yellowtail with both properly inflated (functional) and non-inflated (non-functional) swim bladders in comparison to wild-captured individuals. Each group (inflated n=40, uninflated n=40, wild n=39) was kept in a separate oval tank (3200 l) with consistent water and feed conditions for an 8-month growout period to track fitness and growth over time.

Methods for determining critical swimming speed and standard metabolic rate were repeated as in Study 1 at two time points during growout: once at the beginning of the growout period using a 5.4 l swim tunnel respirometer and once approximately four months later using a 30 l swim tunnel respirometer. These time points were selected based on optimal fish size for proper utilization of the two available swim tunnels. Swim tunnel trials were conducted on 7-9 individuals randomly selected from each group at each time point.

Somatic measurements were taken for all individuals in each group at the beginning and end of the 8-month growout period, and growth rate was calculated as the average initial mass subtracted from the average final mass (g), divided by the number of days of growth. All fish were hand fed to prevent over feeding. The

feed conversion ratio (FCR) was calculated using the equation:

$$\text{FCR} = \frac{\text{dry feed consumed (g)}}{\text{mass gained (g)}} \qquad (2)$$

Study 3: Examination of the Effect of Exercise on Metabolism and Growth

For the final study, aquaculture-reared yellowtail were exercised in custom-designed fish raceways (Fig. 1) in order to examine the potential beneficial effects of exercise on metabolic fitness and growth. Fish were forced to swim against a current in the raceways at predetermined optimal swimming speeds (6-8 BL s^{-1}) for different durations of time (two, three, or four weeks). Following exercise, fish were removed from the raceways to separate growout tanks with the same parameters as a non-exercised control group (3200 l oval tank). Metabolic rate, growth, and feed conversion ratio were then tracked over a six-month growout period. Metabolic rates were determined for a subset of fish (n = 8) randomly selected from each group measured in the week following exercise and at two other time points (1.5 and three months post-exercise) using incremental velocity trials in swim tunnel respirometers as previously described. After swim tunnel experimentation, fish were measured as described above and sacrificed. The rest of the fish were grown out and somatic measurements were taken on a subset of 30 fish from each group every two weeks.

Statistical Analyses

Swimming and metabolic metrics in Study 1 were compared between aquaculture-reared and wild-caught yellowtail using a two-tail t-test. A single factor ANOVA with a post-hoc Tukey test was performed on critical swimming speed data, standard metabolic data, and somatic measurement data for Studies 2 and 3. Statistical significance was established at $P \leq 0.05$.

RESULTS AND DISCUSSION

Study 1: Swimming and Metabolic Fitness of Aquaculture-reared and Wild-caught yellowtail

The mean maximum sustainable swimming speed for 10 aquaculture-reared yellowtail (77.08 ± 9.36 cm s^{-1}, 4.16 ± 0.62 BL s^{-1}) was significantly slower than that determined for seven wild caught individuals of similar size (92.93 ± 13.32 cm s^{-1}, 4.80 ± 0.52 BL s^{-1}). Aggregate data showing the relationship between $\dot{M}_{O_2}$ and swimming velocity for all metabolic measurements for 10 aquaculture-reared and seven wild-caught yellowtail are shown in Fig. 2. Extrapolation of regression equations for individual fish to a swimming

Figure 1. Custom-designed fish raceway used for exercise training of California yellowtail in Study 3.

speed of 0 cm s^{-1} shows that the standard metabolic rate of aquaculture-reared yellowtail (7.31 ± 2.32 mgO_2 kg^{-1} min^{-1}) was significantly greater than that of wild-caught individuals (3.94 ± 1.60 mgO_2 kg^{-1} min^{-1}).

These data show reduced fitness of aquaculture-reared yellowtail in comparison to wild-caught individuals as manifested through differences in critical swimming speed and standard metabolic rate. Such reductions in fitness are likely associated with rearing in a suboptimal aquaculture setting (e.g., lower water or diet quality, decreased physical activity) or may reflect suboptimal phenotypes having a higher chance for survival in captivity. Both scenarios are likely at work and highlight the need for both genetic studies for the selection of broodstock that produce the highest quality offspring as well as experimentation to determine best rearing practices (i.e., determination of feed, water, and other environmental impacts on fish fitness and growth). This study also suggests that aspects of both the swimming and metabolic fitness of *S. dorsalis* can be improved in culture growout operations. For example, a 50% reduction in the standard metabolic rate of aquaculture-reared yellowtail (to that observed in wild-caught fish) should result in lower feed requirements and substantial cost savings to aquaculture producers.

Study 2: Fitness and Growth of Yellowtail with a Swim Bladder Deformity

While the results of this study are still being assessed, preliminary data reveal that the standard metabolic rate and critical swimming speed for yellowtail reared in aquaculture with inflated and uninflated swim bladders did not differ significantly (Table 1). However, yellowtail with uninflated swim bladders were found to have higher feed conversion ratios and slower growth rates than those of yellowtail with properly inflated swim bladders. This less efficient resource allocation likely results from the need of yellowtail that lack a functional swim bladder to swim at faster speeds to generate

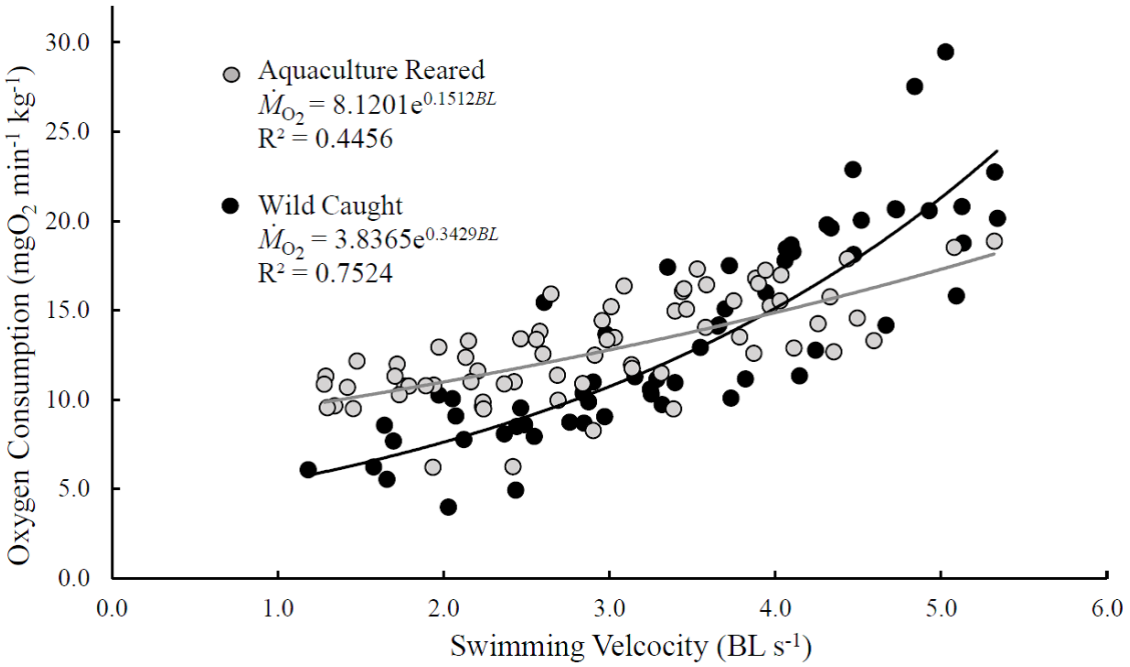

Figure 2. Oxygen uptake ($\dot{M}_{O_2}$) of aquaculture-reared (grey circles) and wild-caught (black circles) California yellowtail, S. dorsalis, *swimming at variable speeds in a swim tunnel respirometer in Study 1. For direct comparison all data were adjusted to a mass of 80 g using $M^{0.80}$ and water temperature of 18°C using Q_{10} = 2.0. Regression lines are for observational purposes only; standard metabolic rate was determined from individual fish data.*

Table 1. Metrics of physical fitness and growth for aquaculture-reared S. dorsalis *with properly inflated and uninflated swim bladders in comparison to wild captured yellowtail (Study 2).*

Group	U_{crit} (Start) (BL s^{-1})	U_{crit} (4 months) (BL s^{-1})	SMR (Start) (mgO_2 kg^{-1} s^{-1})	SMR (4 months) (mgO_2 kg^{-1} s^{-1})	FCR	Growth Rate (g day^{-1})
Wild	5.34 ± 0.59	4.31 ± 0.35	**2.97 ± 0.93**	2.96 ± 0.83	1.41	2.79
Inflated	5.12 ± 0.99	4.50 ± 0.37	5.17 ± 1.93	3.55 ± 1.95	1.50	3.20
Uninflated	5.20 ± 0.53	4.25 ± 0.62	5.43 ± 1.69	3.41 ± 1.59	2.08	2.64

Critical swimming speed (U_{crit}) and standard metabolic rate (SMR) are shown at the start of the experiment (mean mass = 63.79 ± 17.81 g) and again approximately four months later (412.15 ± 61.94 g). Statistically significant differences indicated in **bold**. Values given as means ± standard deviation.

Table 2. Mean size [fork length (FL), total length (TL) and mass] ± standard deviation of fish in each exercise group (2, 3, 4 weeks of sustained swimming at 6-8 body lengths per second), compared to a non-exercised control group after a 14-week grow-out period (Study 3).

Group	FL (cm)	TL (cm)	Mass (g)	% mass difference (above control)
Four Weeks	29.73 ± 1.39	32.80 ± 1.54	471.47 ± 73.79	14.66
Three Weeks	29.69 ± 1.14	32.86 ± 1.32	472.20 ± 61.81	14.83
Two Weeks	29.44 ± 1.54	32.52 ±1.76	452.13 ± 87.22	9.95
Control	29.30 ± 1.43	32.18 ± 1.52	411.20 ± 70.05	0.00

Note: Data are preliminary.

Table 3. Feed conversion ratio for three groups of exercised yellowtail in comparison to a non-exercised control (Study 3).

Group	Total Food Consumed (kg)	Total Mass Gained (kg)	FCR	% difference from control
Four Weeks	48.95	43.56	1.12	1.65
Three Weeks	56.30	51.03	1.10	3.44
Two Weeks	55.29	50.51	1.09	4.20
Control	48.66	42.59	1.14	0.00

Note: Data are preliminary.

hydrostatic lift in order to compensate for their negative buoyancy. The resulting increase in required food for yellowtail with this deformity likely signifies that it is not economical to rear them to market size.

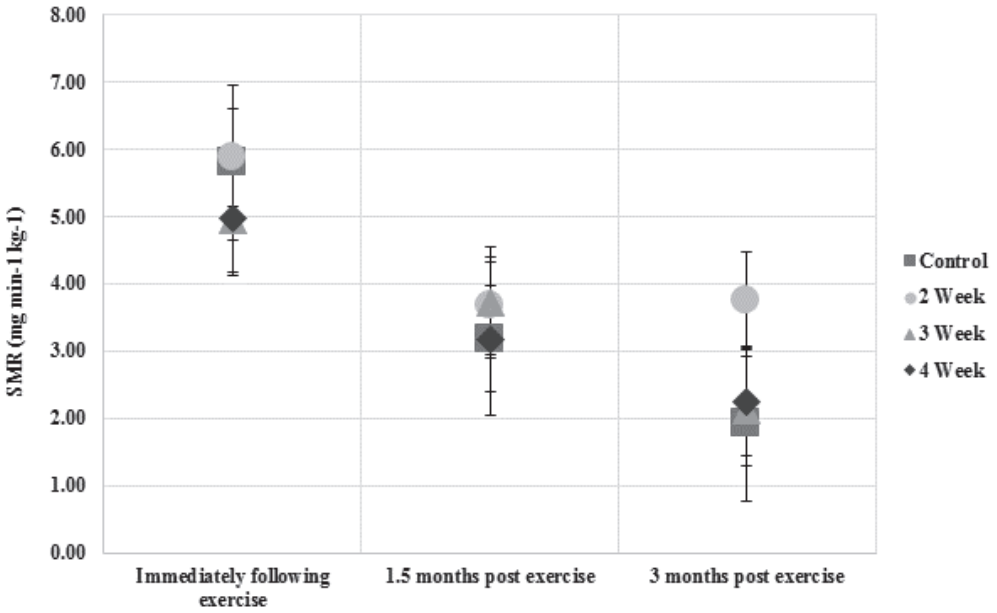

Figure 3. Mean standard metabolic rate (SMR) for each group (2, 3, 4 weeks of sustained exercise) in comparison to a control at three time points post exercise (Study 3). Means for each group are shown with 95% confidence intervals and were standardized to 22 °C (using Q_{10} = 2) and scaled to the mean mass at each time point (using $M^{0.80}$). Data are preliminary.

Like Study 1, this study also showed that aquaculture yellowtail (both with and without properly inflated gas-bladders) had a higher standard metabolic rate than that of wild-caught fish at the start of the experiment (Table 1). Interestingly, there was not a significant difference in the critical swimming speed of aquaculture-reared and wild caught fish in this study, and the difference in standard metabolic rate was less than that previously observed (i.e., in Study 1). This suggests that enhancements in rearing protocols between Study 1 (a 2012 cohort) and this study (using a 2015 cohort) have likely increased the fitness of yellowtail reared in aquaculture. While it appears that improvements have been made, the potential negative effects of captive rearing were seen in that after four months, wild-caught yellowtail no longer showed a metabolic fitness advantage in comparison to aquaculture-reared fish (Table 1).

This study thus demonstrates that there is continued room for improvement in aquaculture-reared fish in lowering their standard metabolic rates, which could improve efficiency and lower feed conversion ratios. Ideally, farmed fish would have metabolic rates and feed conversion ratios similar to those observed in wild fish while maintaining the high growth rates of the inflated group (Table 1). This combination would produce physiologically fit and healthy fish that could be produced more efficiently and sustainably.

Study 3: Examination of the Effect of Exercise on Metabolism and Growth

Preliminary somatic growth measurements taken 14 weeks following completion of the exercise trials show that the exercised fish are 10-15% larger than the controls (Table 2). Preliminary metabolic results (Fig. 3) suggest that exercise may also have positive effects on metabolism by lowering the standard metabolic rate of yellowtail exercised for three and four weeks by approximately 15%. This decrease in standard metabolic rate may allow more energy to be allocated toward growth resulting in the increased body size observed in Table 2. However, preliminary results also indicate this metabolic advantage is not retained over time and that within 1.5 months there is no longer a metabolic difference between groups. Preliminary feed conversion data (Table 3) suggests only slight differences in feed efficiency between groups, but a full analysis still needs to be completed.

While the results of this and previous studies on other *Seriola* species indicate exercise has a positive effect on growth, the magnitude of this effect seems to be highly variable, ranging from 10 to 46% (Yogata and Oku 2000; Brown et al. 2011; Palstra et al. 2015). The large variation in observed growth rates likely reflects variable conditions between studies including temperature, swimming speed, exercise duration, and the initial size of the fish in each experiment. Analysis and interpretation of our results is ongoing, and due to variability in the response to exercise, we plan to replicate these trials on future cohorts. Our results suggest exercise has a strong, immediate physiological effect, but timing (yellowtail life stage and duration of exercise) and environmental variables (temperature, flow speed) play important roles in optimizing the response.

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This study investigated the effect of swimming exercise on the growth of cultured New Zealand yellowtail kingfish, *Seriola lalandi*, at different water temperatures and swimming speeds. Two growth trials exposed fish to different exercise levels (determined by current speed) at 14.9° and 21.1°C. Lengths and weights were measured for each fish before and post exercise, along with metabolic rates using a respirometer. Results of these trials showed that exercise yielded a 10% increase in growth but only at low swimming speeds (0.75 BL s-1) and at a temperature of 21.1°C. Experiments using a swim tunnel respirometer indicated that exercise training had no effect on metabolic scope or critical swimming speeds but it did improve swimming efficiency.

Palstra, A.P., D. Mes, K. Kusters, J.A.C. Roques, G. Flik, K. Kloet, and R.J.W. Blonk. 2015. Forced sustained swimming exercise at optimal speed enhances growth of juvenile yellowtail kingfish (*Seriola lalandi*). Frontiers in Physiology 5: 506.

The swimming exercise trials in this study for *Seriola lalandi* resulted in 46% greater increases in mass and 92% larger increases in length compared to non-exercised controls. This study thus showed greater exercise benefits than those observed in the Brown et al. (2012) study (above), and these differences were likely related to using more optimal exercise swimming speeds. The exercise and control groups in this study were fed equivalent rations, indicating the exercised fish had more efficient resource allocation, resulting in a lower feed conversion ratio (1.21 vs. 1.74 for non-exercised yellowtail). These researchers thus suggest that growth rate can be greatly enhanced without increased feeding.

Palstra, A.P., and J.V. Planas (eds). 2013. Swimming Physiology of Fish: Towards Using Exercise to Farm a Fit Fish in Sustainable Aquaculture. Springer-Verlag, Berlin.

This multi-chaptered volume reviews the relevant advances in understanding the physiology of fish swimming and examines topics ranging from fish biomechanics and migrations to the potential benefits of swimming exercise in aquaculture resulting in changes in fish growth and flesh quality. It brings together both field and laboratory studies to better understand the physiology of fish swimming with the intent to apply such findings to enhance aquaculture practices. A chapter by Davison and Herbert summarizes research activities and advances on swimming-enhanced growth.

Paradigm Shift in Fish Breeding: Marker-Assisted Selection to Genomic Selection

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Keywords: marker-assisted selection (MAS), genomic selection (GS), polygenic QTL, yellowtail (*Seriola quinqueradiata*), EST-based SNPs

ABSTRACT

High density SNP arrays have become the tool of choice for quantitative trait locus (QTL) mapping, genome-wide association studies (GWAS), and marker-assisted selection (MAS). The essential conditions for DNA marker-assisted selection are 1) development of useful resource families to evaluate phenotypes and information about genetic linkages and 2) a large number of polymorphic genetic markers. Some of the cases have already reached a practical stage and have been used in genetic improvement of certain species of aquaculture fish. Earlier mapping studies have identified QTL for important commercial traits, such as disease resistance, and combining the resources of a high density genetic map with genome sequence data facilitates the fine mapping of these loci. Otherwise, traditional MAS has not resulted in widespread use of DNA information in animal breeding. The main reason was that the traits of interest in livestock production were much more complex than expected; they were determined by thousands of genes with small effects on phenotype. These effects were usually too small to be statistically significant and they were ignored. In aquaculture species, these very stringent tests result in only the largest QTL being found. Those large QTL results were often limited to disease resistance, including Japanese flounder resistant to lymphocystis disease, Atlantic salmon resistant to infectious pancreatic necrosis (IPN), and yellowtail resistant to Benedenia disease.

While it might be difficult to find the largest QTL for other traits like growth and tolerance of low fish meal feed and apply MAS, genomic selection (GS) has a possibility to improve such traits, which are dominated by polygenic QTL. GS assumes that all markers might be linked to a gene affecting the trait and concentrates on estimating their total effect rather than testing its significance. Therefore, these technological breakthroughs are resulting in the current wide-spread use of DNA information in animal breeding: the development of the GS technology, the discovery of massive numbers of genetic markers (usually SNPs), and high-throughput technology to genotype animals for thousands of SNPs in a cost-effective manner. It is anticipated that future application of genetics and breeding will shift to GS from MAS in aquaculture.

INTRODUCTION

Construction of genetic linkage maps and identification of QTL are beginning to make an impact on animal breeding by providing DNA markers linked to QTL affecting phenotypic traits of interest. Such molecular markers can be used in marker-assisted selection (MAS). The advantages of MAS, i.e., selection based partly or fully on DNA marker genotypes, are obvious as compared with the conventional selective breeding. MAS is 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 found through QTL mapping or association studies (Lande and Thompson 1990). Ideally, the DNA markers should be the linkage disequilibrium (LD) blocks underlying the phenotypic variation. QTL studies in aquaculture species have 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. However, of the QTL detected from experimental crosses, only a small number of them have been followed up by confirmation and fine-mapping, and the responsible genes have not been identified. Regardless, 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, for example, 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 are its relatives 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 have been cultured in southern areas of Japan using juveniles caught from natural stocks. Although basic 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.

To move genetic improvement for yellowtail amberjack forward, we are researching the practical application of selection for economically important traits based on genetic resources available in natural populations of yellowtail amberjack. More recently, high-density linkage maps generated by SNP array data have proven to be crucial for the accurate assembly of scaffolds and contigs in whole-genome sequencing efforts. High density SNP arrays have become the tool of choice for QTL mapping, genome-wide association studies (GWAS), MAS and genomic selection (GS). In this study, Affymetrix SNP array was used to genotype 460 samples collected across five families from a wild population of yellowtail in coastal waters of Goto Fukue-island. We then use those SNP data for QTL interval mapping and a GWAS for GS.

MATERIALS AND METHODS

To establish an EST (expressed sequence tag) -based SNP array, a cDNA library was generated from pooled RNA samples extracted from 11 tissues from a single individual. Sequencing on a Roche/454 GS FLX platform generated 1,353,405 reads. Sequencing for SNP identification produced 570,846 raw reads derived from the full-length library and 456,482 raw reads derived from the 3'-anchored library generated from 500 juveniles. Quality-based variant calling using CLC Genomics Workbench detected 9,356 putative biallelic SNPs in 6,025 contigs, with a minor allele frequency (MAF) >25 %.

A linkage analysis was performed using application package of LINKMFEX version 2.3. This application can separate alleles from male or female origin. In order to minimize genotyping error, the accuracy of genotypes in the progeny was checked against the male and female parental alleles. Genotype data were converted to a backcross format even though the grandparent genotype was unknown. Pairwise analysis was performed, and markers were sorted into linkage groups at a minimum LOD threshold of 5.0. Linkage phases were determined retrospectively by examining the assortment of alleles among linked markers.

ssGBLUP and ssGWAS were performed on the same EST-based SNP data set. Software used for evaluation included the BLUPf90 family of programs (Misztal et al. 2014).

RESULTS AND DISCUSSION

A total of 6,275 EST-based SNPs were mapped to 24 linkage groups (Fig. 1, Table 1). This map is currently being used to map QTL for a number of commercially important traits, giving us confidence that it will be possible to rapidly develop domesticated yellowtail strains, and will be used to improve the assembly of the yellowtail genome.

In this study, we performed QTL analyses using five F1 strains of yellowtail to find genetic evidence of resistance to Benedenia disease. By using a high-density linkage map based on SNP markers, we identified some chromosomal regions containing QTL that were reported in previous study (Ozaki et al. 2013). These previous QTL and new QTL discovered here, with medium to large effects on Benedenia disease resistance, were localized to specific linkage groups

Table 1. Numbers of EST-based SNPs in five analysis families. A total of 6,275 EST-based SNPs were mapped to 24 linkage groups using five families. LOD threshold was 5.0. Linkge phases were determined retrospectively by examining the assortment of alleles among linked markers. Male and female linkage maps were created separately.

Male	length(kosambi)	no. of markers	avg. of markers
LGR-MSNPGr1	1138.5cM	1449loci	0.79cM
LGR-MSNPGr3	1355.7cM	1237loci	1.10cM
LGR-MSNPRr1	1106.3cM	1418loci	0.78cM
LGR-MSNPWw2	1380.9cM	1362loci	1.01cM
LGR-MSNPWw7	1171.2cM	1301loci	0.90cM
	except overlap markers	Total 4587loci	
avg. of five male	1230.5cM		0.27cM
SSR Buri linkage map ver. 4.0.1	1224.0cM	1044loci	1.17cM
Female	**length(kosambi)**	**no. of markers**	**avg. of markers**
LGR-FSNPGr1	752.8cM	1304loci	0.58cM
LGR-FSNPGr3	1036.1cM	1299loci	0.80cM
LGR-FSNPRr1	1065.4cM	1352loci	0.79cM
LGR-FSNPWw2	986.1cM	1293loci	0.76cM
LGR-FSNPWw7	910.4cM	1445loci	0.63cM
	except overlap markers	Total 4444loci	
avg. of five female	950.2cM		0.21cM
SSR Buri linkage map ver. 4.0.1	1031.1cM	1042loci	0.99cM

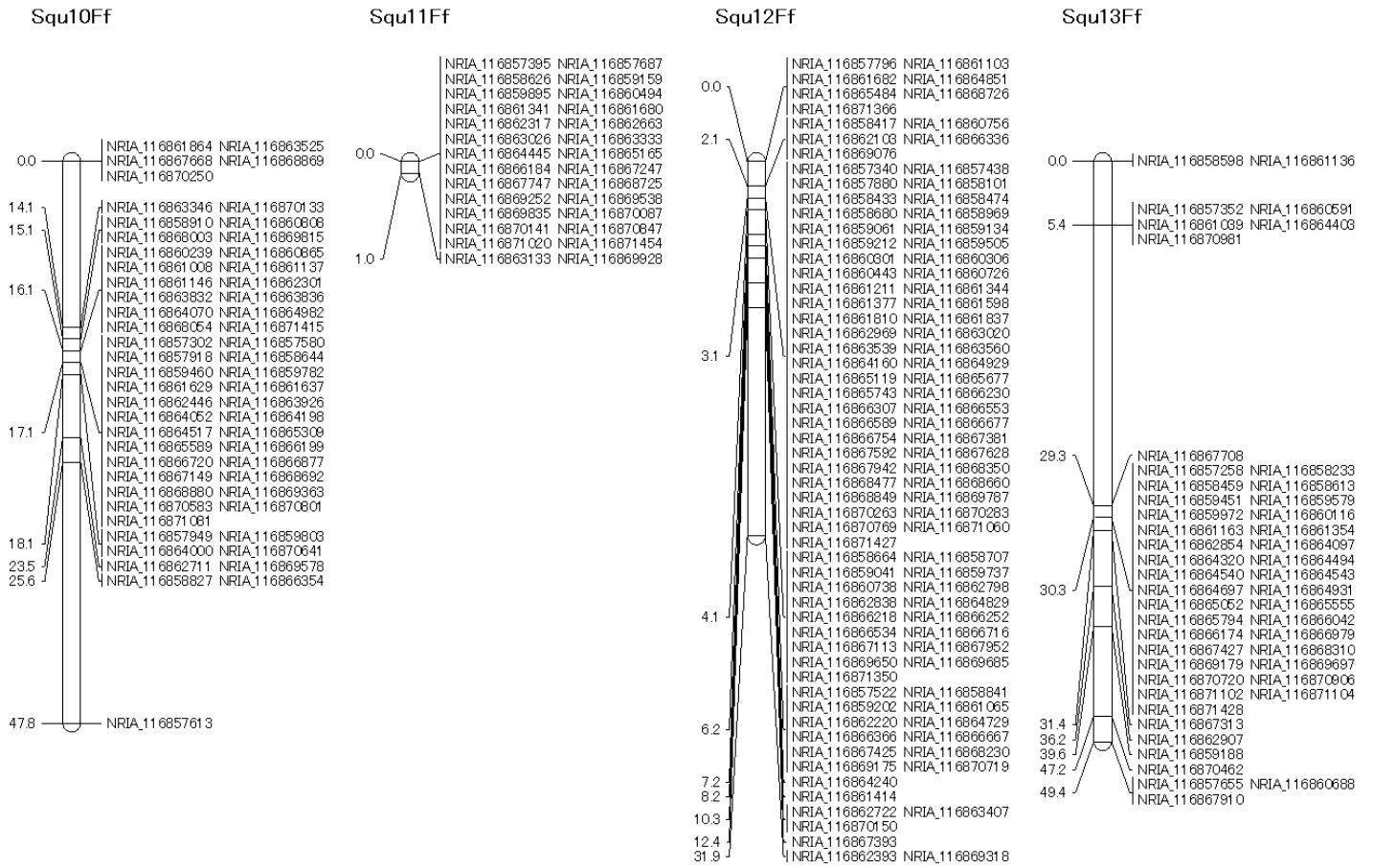

Figure 1. Example of a SNP linkage map in yellowtail. A total of 6.275 EST-based SNPs were mapped to 24 linkage groups. LOD threshold was 5.0. Linkage phases were determined retrospectively by examining the assortment of alleles among linked markers.

in the analysis families (Fig. 2). These loci were responsible for up to 81.2 % of the total phenotypic variation. Finding this QTL region strongly supports the potential for success of MAS for this disease.

GS appears to be a difficult method when compared with a QTL study, but it is not so different from traditional BLUP, wherein the A (allele) matrix is replaced with a G (genotype) matrix or H (haplotype) matrix composite of both.
The results showed SNPs with significant phenotypic associations exist in certain chromosomal regions (Fig. 3). Both interval QTL mapping and GWAS methods have advantages and disadvantages, but it is possible to use the same SNP data with each method. Reliability will depend on the quality of the reference sequence data and the SNPs themselves, which come from re-sequencing

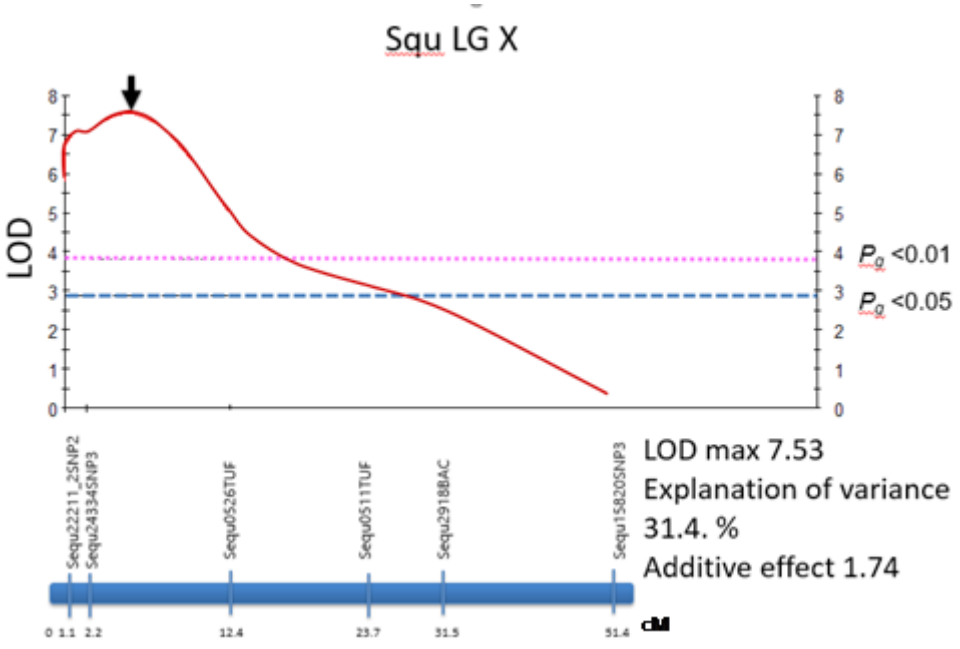

*Figure 2. Example of OTL interval mapping result within a linkage group. Quantitative trait loci (QTL) associated with resistance to parasitic disease (*Benedenia seriolae*) in yellowtail. Distance along x-axis in cM.*

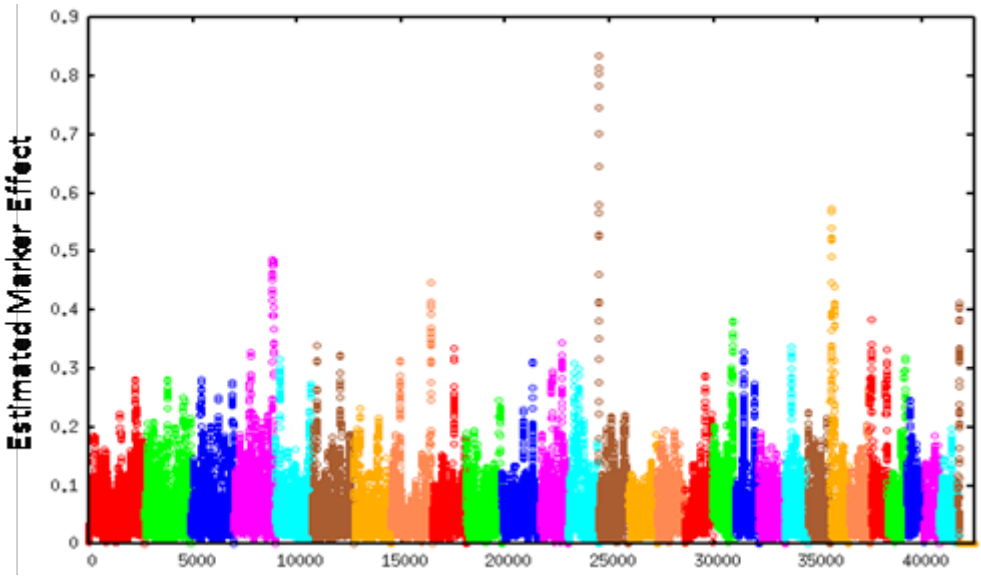

*Figure 3. Example gnuplot of weighted ssGWAS depicting individual SNP (open circles) effects using the same data set associated with resistance to parasitic disease (*Benedenia seriolae*) in yellowtail. SNPs are color-coded by linkage group. Distance along x-axis in cM.*

data. If sequence data continue to improve, the method used will shift from QTL to MAS, and genetic breeding methods will be able to shift from MAS to GS.

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Wedemeyer, G. 1996. Physiology of fish in intensive culture systems. London: Chapman & Hall.

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An allele of a microsatellite, Poli9-8TUF, has a dominant effect at a single major locus and is responsible for resistance to lymphocystis disease (LD-R) in Japanese flounder. We developed a new population of Japanese flounder produced by marker assisted breeding using this allele. A female that originated from the KP-B inbred line with LD-R that was homozygous for the favorable allele (B-favorable) and a male from a commercial stock bred for higher growth rate and good body shape were selected as parents. A female was selected as the LD-R-bearing parent because the recombination rate of females is lower in the region where the LD-R locus is located. As expected, the B-favorable allele was transmitted as a heterozygote to the progeny (LD-R+ population). The

LD-R+ population, when tested at two commercial fish farms that had LD outbreaks, showed no incidence of LD at either farm, while a control population without B-favorable alleles (LD-R–) had incidences of 4.5% and 6.3% at the two farms. These results show that marker-assisted breeding using molecular markers linked to an economically important trait is an efficient strategy for breeding.

Moen, T., Baranski, M., Sonesson, A.K. and Kjoglum, S. (2009) Confirmation and fine-mapping of a major QTL for resistance to infectious pancreatic necrosis in Atlantic salmon (*Salmo salar*): population-level associations between markers and trait. BMC Genomics 10, 368.

Infectious pancreatic necrosis (IPN) is one of the most prevalent and economically devastating diseases in Atlantic salmon (*Salmo salar*) farming worldwide. The disease causes large mortalities at both the fry- and post-smolt stages. Family selection for increased IPN resistance is performed through the use of controlled challenge tests, where survival rates of sib-groups are recorded. However, since challenge-tested animals cannot be used as breeding candidates, within family selection is not performed and only half of the genetic variation for IPN resistance is being exploited. DNA markers linked to quantitative trait loci (QTL) affecting IPN resistance would therefore be a powerful selection tool. The aim of this study was to identify and fine-map QTL for IPN-resistance in Atlantic salmon for use in marker-assisted selection to increase the rate of genetic improvement for this trait. The QTL confirmed in this study represents a case of a major gene explaining the bulk of genetic variation for a presumed complex trait. QTL genotypes were deduced within most parents of the 2005 generation of a major breeding company, providing a solid framework for linkage-based MAS within the whole population in subsequent generations. Since haplotype-trait associations valid at the population level were found, there is also a potential for MAS based on linkage disequilibrium (LD). However, in order to use MAS across many generations without reassessment of linkage phases between markers and the underlying polymorphism, the QTL needs to be positioned with even greater accuracy. This will require higher marker densities than are currently available.

Ozaki, A., K. Yoshida, K. Fuji, S. Kubota, W. Kai, J.Y. Aoki, Y. Kawabata, J. Suzuki, K. Akita, T. Koyama, M. Nakagawa, T. Hotta, T. Tsuzaki, N. Okamoto, K. Araki, and T. Sakamoto. 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 the 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 F1 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.

Meuwissen, T.H., B.J. Hayes, and M.E. Goddard. 2001. Prediction of total genetic value using genome-wide dense marker maps. Genetics 157(4): 1819-1829.

Recent advances in molecular genetic techniques will make dense marker maps available and genotyping many individuals for these markers feasible. Here, we attempted to estimate the effects of approximately 50,000 marker haplotypes simultaneously from a limited number of phenotypic records. A genome of 1000 cM was simulated with a marker spacing of 1 cM. The markers surrounding every 1-cM region were combined into marker haplotypes. Due to finite population size N(e) = 100, the marker haplotypes were in linkage disequilibrium with the QTL located between the markers. Using least squares, all haplotype effects could not be estimated simultaneously. When only the biggest effects were included, they were overestimated and the accuracy of predicting genetic values of the offspring of the recorded animals was only 0.32. Best linear unbiased prediction of haplotype effects assumed equal variances associated to each 1-cM chromosomal segment, which yielded an accuracy of 0.73, although this assumption was far from true. Bayesian methods that assumed a prior distribution of the variance associated with each chromosome segment increased this accuracy to 0.85, even when the prior was not correct. It was concluded that selection on genetic values predicted from markers could substantially increase the rate of genetic gain in animals and plants, especially if combined with reproductive techniques to shorten the generation interval.

Genomic Analyses to Inform Sablefish Aquaculture Research

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Keywords: Not available.

ABSTRACT

The marine fish species, sablefish (*Anoplopoma fimbria*, black cod), are long-lived, migratory species native to the west coast of the U.S. Sablefish is an ideal species for aquaculture because of their high market value and fast growth in culture. The development of the species for aquaculture will benefit from genomic technologies for a number of objectives, including: 1) an understanding of the genetic stock structure in the wild, from which progenitor broodstock is developed, 2) an understanding of the genetic basis of important production traits, and 3) the development of comprehensive genotyping assays for screening sablefish in culture. We discuss in this presentation our findings of genetic stock structure in wild populations, and future directions in genome sequencing and genomics of quantitative traits. Using Restriction Site Associated DNA sequencing (RADseq), we identified more than 100,000 SNPs in >400 individuals collected from a U.S. West Coast surveys from the Bering Sea in Alaska to southern California. After filtering for genetic analyses, 2661 SNPs were used to assess population structure and test for signatures of natural selection and association with environmental variables. Our results show a lack of population structure and adaptive variation in Sablefish, and are suggestive of a single panmictic population that is likely the result of a complex juvenile life history and long range movements as adults. Our current studies now focus on understanding the genetic basis of sexually dimorphic growth in Sablefish, and the development of a refined genome assembly to provide a reference for genomic studies in this species.

ANNOTATED BIBLIOGRAPHY OF KEY WORKS

Jasonowicz, A.J., F.W. Goetz, G.W. Goetz, and K.M. Nichols. 2017. Love the one you're with: genomic evidence of panmixia in the sablefish (*Anoplopoma fimbria*). Canadian Journal of Fisheries and Aquatic Sciences 74(3): 377-387.

This study examines genetic stock structure in Sablefish along the U.S. West Coast and Alaska using RADseq, and finds no evidence for population differentiation.

Luckenbach, J.A., and W.T. Fairgreve. 2016. Gonadal sex differentiation and effects of dietary methyltestosterone treatment in Sablefish (*Anoplopoma fimbria*). Fish Physiology and Biochemistry 42: 233-248.

An important characterization of sex differentiation in Sablefish, and identification of the developmental time period sensitive to sex reversal by dietary testosterone.

Rondeau, E.B., A.M. Messmer, D.S. Sanderson, S.G. Jantzen, K.R. von Schalburg, , D.R. Minkley, J.S. Leong, G.M. Macdonald, A.E. Davidsen, W.A. Parker, R.S. Mazzola, B. Campbell, and B.F. Koop. 2013. Genomics of sablefish (*Anoplopoma fimbria*): expressed genes, mitochondrial phylogeny, linkage map, and identification of a putative sex gene. BMC Genomics 14: 452.

An important paper providing evidence for the sablefish sex determining gene, which will be important in understanding genetic sex control in sablefish. This paper also provides the first draft genome sequence that we are currently improving with additional sequence data, and provides the first comprehensive genomic resource for this fish species.

Mutagenesis and Genome Editing for Aquaculture Fish Species: Modification of SCPP Genes in Tiger Pufferfish (*Takifugu rubripes*)

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Keywords: mutagenesis, TILLING, genome editing, CRISPR/Cas, tiger pufferfish (*Takifugu rubripes*)

ABSTRACT

There is increasing interest in manipulating the function of target genes to achieve production gains not only in agriculture but also in aquaculture. Of these, the two primary methods are chemical mutagenesis, including TILLING and genome editing. In order to reduce the need for performing a tooth cutting procedure to prevent mutual biting in tiger puffer farming, we developed the basic technology for creating individuals whose function of nine SCPP genes involved in tooth formation was suppressed. We comprehensively searched for mutants in the genes of about 850 progeny of a mutagenesis (TILLING) treatment male, but mutants with the suppressed function were not obtained. On the other hand, by genomic editing technology, we succeeded in producing pufferfish with mutations in one of the SCPP genes, and analysis using the variant homozygote individuals is expected in the future.

INTRODUCTION

Mutation breeding has been used in the agricultural field since the first half of the 20th century, and varieties that have been produced of rice, barley, cotton and so on have been put to practical use worldwide. For a long time in the field of fish farming, this method was limited to technical research testing, and practical application was hardly done. Although transgenic techniques are not often used, there are several alternatives.

In recent years, a technique called TILLING (Targeting Induced Local Lesions In Genomes) was developed to introduce a genetic saturation mutation (single nucleotide mutation) into fish sperm with a chemical mutagen; specific gene mutant individuals from a mutant population are then found by nucleotide sequence analysis (Wienholds et al. 2002; Taniguchi et al. 2006). Chemical mutagenesis has been widely used in agriculture, but there have been few reported successes in aquaculture. TILLING has been applied to experimental fish such as zebrafish and medaka. In these instances, researchers have subjected adult fish to a bath of strong alkylating agents (e.g., N-ethyl-N-nitrosourea (ENU)). Exposure to the chemical mutagen induces single nucleotide substitutions in the genomes of all cells but particularly in the gametes. Because the body size of most intensively cultured food fish is much larger than these experimental species, it is not practical to use whole-body baths of ENU solution. To address this, methods have been developed to induce mutagenesis by soaking eggs or sperm in ENU solution or injecting ENU solution into the abdominal cavity of ripe males. The single nucleotide substitution mutation will induce: 1) inhibition of gene function by generation of stop codons in protein coding regions, 2) reduction/modification of protein function by amino acid substitution, 3) increase/decrease of the gene expression amount due to mutation of the expression regulatory region. As such, it is one technique used for functional analysis of genes. It has been reported that mutant individuals doubled the muscle content of grass carp in China in 2011 (Jiang et al. 2011), and the utilization of mutation breeding technology in the world had begun.

Whereas chemical mutagens induce genome-wide random mutations, artificial nuclease for genome editing technology can target mutations to a specific locus with random-length deletion or specific nucleotide sequence replacement. The mutation induction ratio has been greatly improved using genome editing technologies like ZFN, TALEN or CRISPR, which is a mutation inducing technique for a specific gene, and fruitful results are being reported even in experimental fish (Ansai et al. 2012). These methods are attracting worldwide attention as a new breeding technology (NBT).

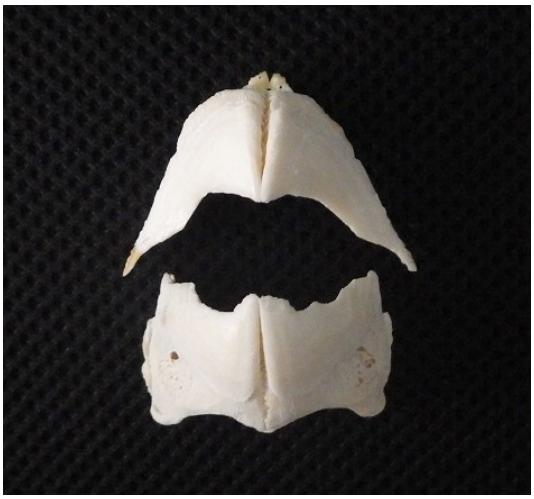

Figure 1. Teeth of tiger pufferfish.

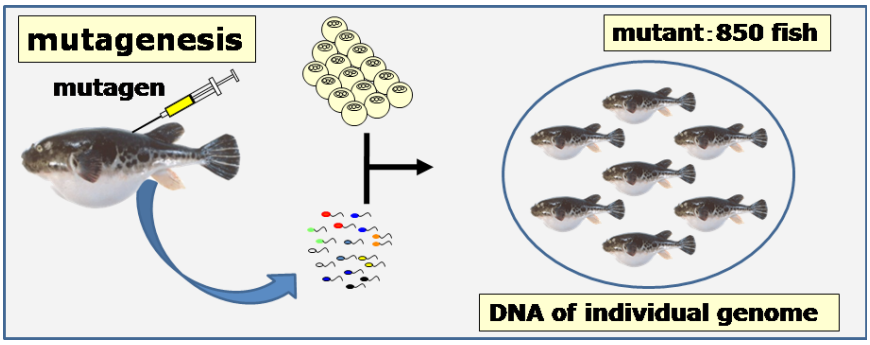

Figure 2. Production of a tiger pufferfish mutant population.

In tiger puffer farming, it is necessary to perform a tooth cutting operation to prevent a decrease in commercial value due to a loss of fin or mortality by mutual biting (Fig. 1). However, since tooth cutting is very hard work for fish farmers and stressful for fish, measures to reduce labor are desired. Teeth of teleost fish and mammalian teeth have the hardest substances in their outermost layers, such as enameloids (teleost fishes) and enamel (mammals). They are functionally and morphologically similar structures. However, the crystal structures of both were thought to be different from each other because the connective tissues from which they are derived are different (Kogaya and Iwaku 2004). Thus, the teeth of teleost fishes and mammals might be morphologically and functionally similar but evolved separately (parallel evolution). However, in 2005, the secretory calcium-binding phosphoprotein (SCPP) was clarified as a gene group involved in the formation of teeth in tiger pufferfish (*Takifugu rubripes*) (Kawasaki et al. 2005), and molecular evolutionary analysis revealed that the SCPP gene is a major gene group related to universal teeth mineralization in vertebrate teeth evolution (Kawasaki and Weiss 2008).

MATERIALS AND METHODS

Production of the Tiger Pufferfish Mutant Population

The following tests were conducted on the creation and selection of functionally deficient individuals for the SCPP gene using the mutation method, TILLING. The genomic DNA was extracted from each individual in a mutant population of about 850 fish produced using frozen sperm derived from male tiger pufferfish treated with the chemical mutagen, ENU (Kuroyanagi et al. 2013) (Fig. 2).

Detection of Mutated Bases on SCPP genes

For each of the nine secreted calcium-binding phosphoprotein SCPP genes, 48 regions (about 200 bp per region) from the 5 ‘ upstream were chosen, including SPARC (Exons 2, 3, 4, 5, 6: 445 bases), SPARCL1 (Exons 2, 3, 4: 1145 bases), SCPP1 (Exons 2, 3, 4, 5, 6, 7, 8, 9: 480 bases), SCPP2 (Exon 2, 3, 4, 5, 6, 7, 8: 661 bases), SCPP3A (Exons 2, 3, 4: 259 bases), SCPP3B (Exons 2, 3, 4: 289 bases), SCPP3C Exons 2, 3, 4: 277 bases), SCPP4 (Exons 2, 3, 4, 5, 6: 741 bases), SCPP5 (Exons 2, 3, 4, 5, 6, 7 + 8: 409 bases), and 48 pairs of sequence-specific amplification primers were designed (Fig. 3).

Four genomic DNAs extracted from each individual were pooled, and the 48 amplicons (4,706 bases total) were attached to a sample identification adapter using a multi-amplicon production system, Access Array™ (Fluidigm). For each amplicon, nucleotide sequence data was acquired using the next generation sequencer, Ion PGM ™ (Thermo Fisher Scientific). The data was analyzed (mutation screening) using genome analysis software, CLC genomics workbench (Filgen), to identify mutated amino acids and to select mutants.

Obtaining Sperm and Eggs of Tiger Pufferfish for Genome Editing

In order to obtain fertilized eggs necessary for genome editing for tiger pufferfish, we transferred unfertilized eggs and semen from the Mie Prefectural Fish Farming Center in Owase City to Tamaki Station FRA in Tamaki Town. We got fertilized eggs by artificial insemination. In addition, in Kamiura Station, early artificial maturation tests were conducted on one male and three females to obtain eggs in between January and March. The obtained eggs were used for the genome editing test.

	Exon	Int	Exon	Int	Exon	Int	Exon	Int	Exon	Int	Exon	Int	Exon	Int	Exon	Int	Exon	Int	Exon	bp Total
gene	1		2		3		4		5		6		7		8		9		10	11	
SPARC	64		66	218	48	864	88	639	122	86	121	190	134	338	149	75	149	81	360		3792
	RC.1		RC.2		RC.3		RC.4		RC.5		RC.6		RC.7		RC.8		RC.9				
SPARCL1	115	837	56	134	57	81	1032	348	109	301	122	75	121	69	137	109	149	522	149	108	328	4959
	CL1.1		CL1.2		CL1.3		CL1.4,1-6		CL1.5		CL1.6		CL1.7		CL1.8		CL1.9		CL1.10			
SCPP1	92	191	55	158	51	217	54	78	27	102	57	88	36	82	60	95	760					2203
	1.1		1.2		1.3		1.4		1.5		1.6		1.7		1.8		1.9					
SCPP2			160	86	36	98	48	65	207	93	57	75	120	513	33	71	63	92	169			1986
	2.1		2.2		2.3		2.4		2.5		2.6		2.7		2.8		2.9					
SCPP3A	54	75	62	85	57	99	405															837
	3A.1		3A.2		3A.3		3A.4															
SCPP3C	55	76	74	79	75	88	428															875
	3C.1		3C.2		3C.3		3C.4															
SCPP3B	46	97	74	99	63	64	334															777
	3B.1		3B.2		3B.3		3B.4															
SCPP4			126	79	219	96	57	405	39	166	337											1524
	4.1		4.2		4.3		4.4		4.5		4.6											
SCPP5	56	73	76	94	66	97	72	85	48	96	69	84	30	79	48	82	36	75	81	78	343	1768
	5.1		5.2		5.3		5.4		5.5		5.6		5.7		5.8		5.9		5.10			

Figure 3. Nucleotide sequence region (blue) of nine SCPP genes mutated by next generation sequencing.

Production of Base Deficient Individuals of SCPP Genes

The following tests were conducted on the creation and selection of individuals with deficient SCPP genes using the genome editing systems TALEN or CRISPR. TALEN or CRISPR/Cas, which can specifically inhibit the function of any target genes, were used. For TALEN, RNAs were synthesized by mMESSAGE mMACHINE (SP6) (Thermo Fisher Scientific) using TALEN A and B pair plasmids against the SPARCL1 gene donated from Hiroshima University. For designing guide RNA targeting the nucleotide sequences of SCPPs 1, 2, 3A, 3B, 3C, 4, and 5, SPARC and SPARCL1 genes used in CRISPR, a free design tool (https: / /wwws.blueheronbio.com/external/tools/gRNASrc.jsp) was used. The designed crRNA was synthesized with tracrRNA by Fasmac. For DNA cleavage enzyme Cas9, pCS2 + hspCas9 (Addgene) plasmid DNA was digested with NotI, and the RNA was synthesized using mMESSAGE mMACHINE (SP6). The crRNA, tracrRNA, and Cas9 RNA were adjusted to concentrations of 33, 67, and 200 ng/μl, respectively, and microinjected into each fertilized egg. Mutant individuals were confirmed by heteroduplex mobility assay (HMA) analysis using MultiNa (Shimadzu). In some eggs, RNA synthesized from pCS2mt-GFP (Addgene) was co-injected with CRISPR/Cas9 RNA for SCPP to monitor gene expression into embryoid bodies.

RESULTS AND DISCUSSION

Detection of Mutated Bases in SCPP Genes by Next Generation Sequencing

The genomic DNA sequence of the parent fish was used as a reference sequence to detect mutated bases in the mutant population. Mutations in the genomic DNA of the mutant population having an occurrence frequency of 5 % or less when compared to the parent sequence were regarded as sequencing error, and those exhibiting frequencies of 10 % or more must be errors other than mutagenesis. As a result of removing the in/del sites from the analysis data, nine mutations without amino acid substitutions (synonymous) were found in four genes, SCPP2, SCPP3A, SCPP3C and SPARC (Fig. 4), and five mutations with amino acid substitutions (non-synonymous) were found in two genes (SCPP3A: one site, SCPP3C: four sites). In the latter cases, cysteine was mutated to tryptophan in SCPP3A and threonine was mutated to alanine (three sites) or isoleucine (one site) in SCPP3C. No stop mutation was found in any of the genes. As a result of this analysis, the mutation introduction rate detected in the genomic region of the SCPP gene group was 0.0004%, which was lower than the initial expectation about 0.4% obtained with other genes so far. For this reason, we speculate that the SCPP gene may be in a genomic region where mutation induction is difficult.

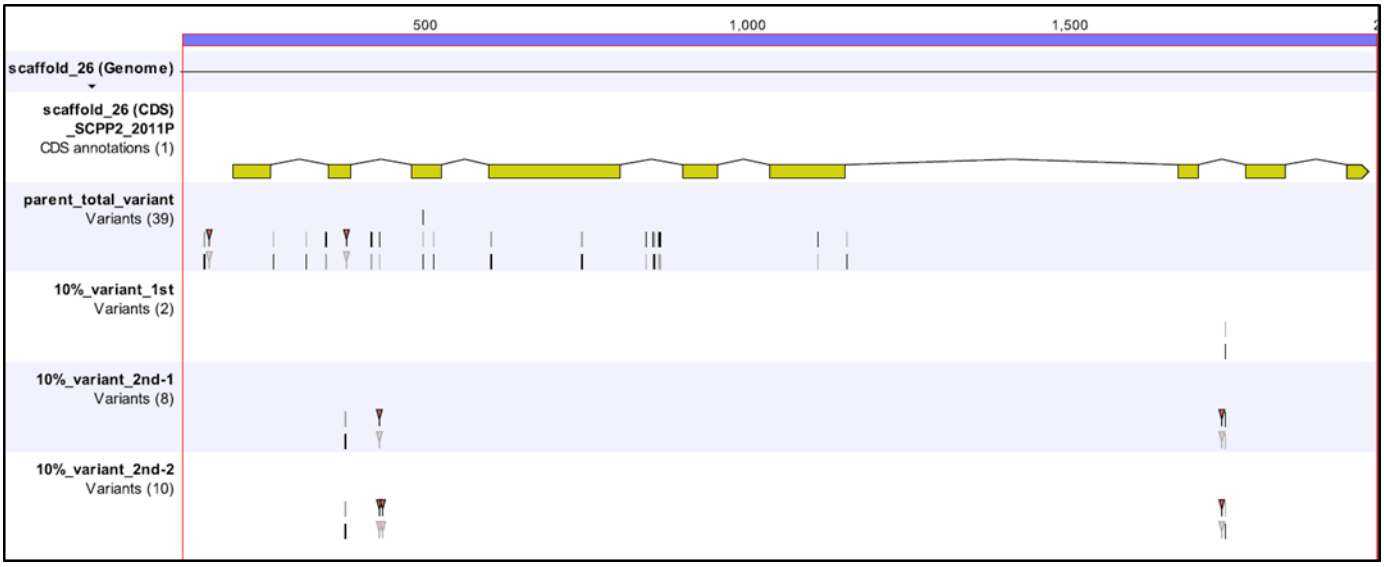

Figure 4. Mutation points detected in SCPP gene.

It was also clear that the above amino acid mutant types were supposed to be heterozygous and did not have a large influence on the teeth formation, since no single individual with incomplete tooth formation was observed in this test group. Possible reasons are that these mutations of the SCPP gene might be recessive or the mutations do not affect gene expression or protein function. It was also considered that a wild type SCPP gene might be responsible for compensating for any reduced function in the mutant SCPP.

Transportation and Microinjection Testing of Tiger Pufferfish Eggs

To ensure that microinjection opportunities occurred more than once in a year, we were donated tiger pufferfish unfertilized eggs from Mie Prefectural Fish Farming Center in Owase City. However, prior to conducting the genome editing test, we first needed to investigate whether transporting the eggs to the Tamaki Station near our institute from Owase Station in the Mie Prefectural Center for one hour by car, followed by microinjection after artificial insemination, were possible. It was found that hatching larvae can be obtained after artificial insemination of unfertilized eggs maintained at a water temperature of 17 °C for as long as three to seven hours after spawning. Hatching larvae were obtained without problem even after microinjection was carried out after one additional hour. At 48 hours after fertilization, the survival rate was 80-90 % as judged by methylene blue staining pattern. Even when eggs two or three days after fertilization were transported by car at 17.3-17.8 °C, the survival rate was 90 %, as also judged by methylene blue staining, with 51.6 % GFP signal positive individuals (n = 281). From these results, it was clear that the genome editing test would be possible to carry out even when receiving and transporting tiger pufferfish unfertilized eggs from a remote place.

Artificial Early Maturation Induction of Tiger Pufferfish

In order to increase the chances of microinjection, we tried early maturation of tiger pufferfish. In mid-November, a tank of pufferfish was cooled from 20 °C to 15 °C over 10 days at -0.5 °C/day, then maintained at 15 °C for 16 days. Later, the temperature was increased 0.5 °C every two weeks starting at the beginning of December, and then maintained at 17 °C from early February. The day length was natural day length. In early March, egg diameter was confirmed by cannula in three females at about 800-1,000 μm. When fish were injected one to two times with 2,500 IU of human chorionic gonadotropin for each 4-5 kg body weight, ovulation was observed four days after the first injection, with an initial egg diameter of 1,034 μm; 12 days after injection at 936 μm; and 23 days after injection at 857 μm. For each individual, the egg collection period was not stable, but it indicated the possibility to take eggs about 1-2 months early from all three fish, which will provide us more opportunities for microinjection.

Production of Tiger Pufferfish Individuals with Mutant SCPP Genes by Genome Editing

Since it was not possible to create mutants by TILLING mutagenesis, we attempted to create mutants by TALEN, one of the genome editing techniques. TALEN-RNAs, SPARCL1-TALEN-A and B pairs for the SPARLCL1 gene, a member of SCPP gene family, were microinjected in fertilized eggs artificially inseminated at the Tamaki Station using the transported eggs from the Owase Station. Screening for mutant individuals was performed by HMA analysis. Mutagenized individuals were searched for in approximately 80 hatching fish, but no characteristic mutational band shift was observed, and mutants could not be obtained.

However, the TALENs were confirmed to have DNA cleavage activity in cultured cells, so it was unclear why mutants could not be obtained.

Since the molecular weight of RNA used in CRISPR/Cas is smaller than that used in TALEN, RNA for many genes can be edited and microinjected into one egg at the same time. Therefore, CRISPR/Cas can perform efficiently with less laboratory materials and breeding spaces. In order to carry out genome editing by CRISPR/Cas, necessary guide RNA was designed and synthesized for nine kinds of SCPP genes. Microinjection was performed on fertilized eggs for each of 12 guide RNAs, and mutagenic activity was investigated. DNA was extracted from the egg two days after fertilization and HMA analysis was performed on the amplicon DNA of each gene. The cleavage activity of each guide RNA was strong for SCPP3A - 2, 3B - 1, 3B - 2, 3C - 1, and 4 and SPARC but weak for SCPP1, 2, 3A - 1, 3C - 2, and 5 and SPARCL1. A guide to SCPP5 – chosen because it is one of the genes expressed in various tissues during teeth development – and artificial nuclease RNA were microinjected into approximately 500 fertilized eggs. The developing ratio immediately before hatching was 35.5 %, and 50 mutant candidate juveniles were obtained at two months of age after hatching. Pit tags were installed at a body length of 5-7 cm in seven mutant individuals obtained by the HMA method (Fig. 5). We visually checked the external form of the tooth, but clear morphological defect was not observed.

In the obtained genome edited F0 individuals, we suspect that the heterozygous mutation was introduced in the somatic tissues as a mosaic, so any functional deficiency was considered to be limited. It is necessary to create subsequent generations of F1 and F2 individuals to evaluate the formation of teeth in whole cell (non-mosaic) homozygous mutant organisms. By creating whole cell homozygous mutants, we will advance our knowledge of the molecular mechanism of tooth formation and obtain a functional understanding of the genes involved. Eventually, we expect a reduction in the amount of tooth cutting work needed in future puffer farming as a result of this research.

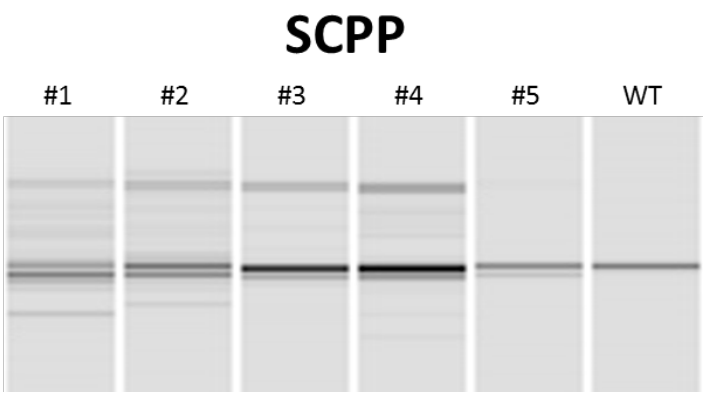

Figure 5. Confirmation of mutant individuals (#1-4: mutation, #5: no mutation, WT: wild type).

Genome editing is preferred when genes or candidate genes that are associated with desirable traits have been identified. In the absence of this information, or if there is a need to survey for alternative phenotypes, chemical mutagenesis is the preferred method. To date, few candidate genes have been identified for commercially important traits such as growth, disease resistance, and food conversion efficiency. This is because such traits are controlled by complex gene networks, with each gene having a very small effect individually. It is expected that improvements in our understanding of gene functions related to commercial traits could be made by further investment in mutagenesis and gene editing research.

ACKNOWLEDGMENTS

We thank Dr. Takashi Yamamoto and Dr. Tetsushi Sakuma at Hiroshima University for giving our team the TALEN plasmid and Kazuhiro Okada, Tohru Kasuya, Shouhei Sugiyama and their colleagues in the Mie Prefectural Fish Farming Center for their kind donation of tiger pufferfish sperm and eggs in part. We also thank Dr. Koichi Okuzawa and Dr. Hironori Usuki for valuable comments, and Naomi Yamasaki, Hideyuki Fujita, Takeshi Taguchi, Tomoyo Nozaki, Akemi Nakanishi Yukinori Shimada and Toshiya Yamaguchi for their assistance with our research. This work was supported in part by Grants-in-Aid for Scientific Research from Fisheries Research and Education Agency of Japan and JSPS KAKENHI Grant Number JP26450273.

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Ansai, S., and M. Kinoshita. 2014. Targeted mutagenesis using CRISPR/Cas system in medaka. Biology Open 3: 362-371.

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system-based RNA-guided endonuclease (RGEN) has recently emerged as a simple and efficient tool for targeted genome editing. In this study, we showed successful targeted mutagenesis using RGENs in medaka, *Oryzias latipes*. Somatic and heritable mutations were induced with high efficiency at the targeted genomic sequence on the *DJ-1* gene in embryos that had been injected with the single guide RNA (sgRNA) transcribed by a T7 promoter and capped RNA encoding a Cas9 nuclease. The sgRNAs that were designed for the target genomic sequences without the 5′ end of GG required by the T7 promoter induced the targeted mutations. This suggests that the RGEN can target any sequence adjacent to an NGG protospacer adjacent motif (PAM) sequence, which occurs once every 8 bp. The off-target alterations at 2 genomic loci harboring double mismatches in the 18-bp targeting sequences were induced in the RGEN-injected embryos. However, we also found that the off-target effects could be reduced by lower dosages of sgRNA. Taken together, our results suggest that CRISPR/Cas-mediated RGENs may be an efficient and flexible tool for genome editing in medaka.

Kuroyanagi, M., T. Katayama, T. Imai, Y. Yamamoto, S. Chisada, Y. Yoshiura, T. Ushijima, T. Matsushita, M. Fujita, A. Nozawa, Y. Suzuki, K. Kikuchi, and H. Okamoto. 2013. New approach for fish breeding by chemical mutagenesis: establishment of TILLING method in fugu (*Takifugu rubripes*) with ENU mutagenesis. BMC Genomics 14: 786.

Background: In fish breeding, it is essential to discover and generate fish exhibiting an effective phenotype for the aquaculture industry, but screening for natural mutants by only depending on natural spontaneous mutations is limited. Presently, reverse genetics has become an important tool to generate mutants, which exhibit the phenotype caused by inactivation of a gene. TILLING (Targeting Induced Local Lesions IN Genomes) is a reverse genetics strategy that combines random chemical mutagenesis with high-throughput discovery technologies for screening the induced mutations in target genes. Although the chemical mutagenesis has been used widely in a variety of model species and also genetic breeding of microorganisms and crops, the application of the mutagenesis in fish breeding has been only rarely reported.

Results: In this study, we developed the TILLING method in fugu with ENU mutagenesis and high-resolution melting (HRM) analysis to detect base pair changes in target sequences. Fugu males were treated 3 times at weekly intervals with various ENU concentrations, and then the collected sperm after the treatment was used to fertilize normal female for generating the mutagenized population (F1). The fertilization and the hatching ratios were similar to those of the control and did not reveal a dose dependency of ENU. Genomic DNA from the harvested F1 offspring was used for the HRM analysis. To obtain a fish exhibiting a useful phenotype (e.g. high meat production and rapid growth), fugu myostatin (Mstn) gene was examined as a target gene, because it has been clarified that the mstn deficient medaka exhibited double-muscle phenotype in common with MSTN knockout mice and bovine MSTN mutant. As a result, ten types of ENU-induced mutations were identified including a nonsense mutation in the investigated region with HRM analysis. In addition, the average mutation frequency in fugu Mstn gene was 1 mutant per 297 kb, which is similar to values calculated for zebrafish and medaka TILLING libraries.

Conclusions: These results demonstrate that the TILLING method in fugu was established. We anticipate that this TILLING approach can be used to generate a wide range of mutant alleles, and be applicable to many farmed fish that can be chemically mutagenized.

Jiang, X.-Y., C.-F. Sun, Q.-G. Zhang, S.-M. Zou. 2011. ENU-Induced mutagenesis in grass carp (*Ctenopharyngodon idellus*) by treating mature sperm. PLoS ONE 6(10): e26475.

N-ethyl-N-nitrosourea (ENU) mutagenesis is a useful approach for genetic improvement of plants, as well as for inducing functional mutants in animal models including mice and zebrafish. In the present study, mature sperm of grass carp (*Ctenopharyngodon idellus*) were treated with a range of ENU concentrations for 45 min, and then wild-type eggs were fertilized. The results indicated that the proportion of embryos with morphological abnormalities at segmentation stage or dead fry at hatching stage increased with increasing ENU dose up to 10 mM. Choosing a dose that was mutagenic, but provided adequate numbers of viable fry, an F1 population was generated from 1 mM ENU-treated sperm for screening purposes. The ENU-treated F1 population showed large variations in

growth during the first year. A few bigger mutants with morphologically normal were generated, as compared to the controls. Analysis of DNA from 15 F1 ENU-treated individuals for mutations in partial coding regions of igf-2a, igf-2b, mstn-1, mstn-2, fst-1and fst-2 loci revealed that most ENU-treated point mutations were GC to AT or AT to GC substitution, which led to nonsense, nonsynonymous and synonymous mutations. The average mutation rate at the examined loci was 0.41%. These results indicate that ENU treatment of mature sperm can efficiently induce point mutations in grass carp, which is a potentially useful approach for genetic improvement of these fish.

Russell, W. L., P. R. Hunsicker, D. A. Carpenter, C. V. Cornett, and G. M. Guinn. 1989. Effect of dose fractionation on the ethylnitrosourea induction of specific-locus mutations in mouse spermatogonia. Proceedings of the National Academy of Sciences of the USA 79: 3592-3593.

As measured by specific-locus mutations in mouse spermatogonia, fractionating a dose of 100 mg of ethylnitrosourea per kg of body weight into doses of 10 mg/kg injected intraperitoneally at weekly intervals greatly reduces the mutation frequency compared with that from a single dose of 100 mg/kg. Because there is independent evidence that the doses of 10 and 100 mg/kg reach the germ cells in amounts directly proportional to the injected dose, the lower mutational response with the fractionated dose is attributed to repair. The induced mutation rate expected from a single 10-mg/kg dose (on the assumption that this would be 1/10th the rate observed after 10 such doses) would be only 75% of the spontaneous mutation rate. Mouse spermatogonia apparently have an efficient repair system that is effective even against a potent mutagen.

Russell, W. L., L. B. Russell, and E. M. Kelly. 1958. Radiation dose rate and mutation frequency. Science 128(3338): 1546-1550.

New data have clearly confirmed the earlier finding that specific locus mutation rates obtained with chronic gamma irradiation of spermatogonia are lower than those obtained with acute x-rays. Since this result is in contrast to classical findings for Drosophila spermatozoa, and apparently contradicts one of the basic tenets of radiation genetics, it was important to determine what factors were responsible for it. Experiments undertaken for this purpose reveal the following: (i) the lower mutation frequency is due mainly to difference in dose rate of radiation, rather than quality; (ii) a dose-rate effect is not obtained in experiments with mouse spermatozoa, confirming classical findings for spermatozoa, and indicating that the explanation for intensity dependence in spermatogonia resides in some characteristic of gametogenic stage; and (iii) a dose-rate effect is found not only in spermatogonia but also in oocytes, where cell selection is improbable, indicating that the radiation intensity effect is on the mutation process itself. A threshold response for all mutations in spermatogonia and oocytes is not a necessary consequence of the findings. Plausible hypotheses consistent with the present results can lead to other predictions. From a practical point of view, the results indicate that the genetic hazards, at least under some radiation conditions, may not be as great as those estimated from the mutation rates obtained with acute irradiation. However, it should not be forgotten that even the lower mutation rates obtained with the present intensity levels are still appreciable (16).

Genome-enabled Selection Doubles the Accuracy of Predicted Breeding Values for Bacterial Cold Water Disease Resistance Compared to Traditional Family-based Selection in Rainbow Trout Aquaculture

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Keywords: aquaculture; genomic selection; disease resistance; bacterial cold water disease; rainbow trout

ABSTRACT

We have shown previously that bacterial cold water disease (BCWD) resistance in rainbow trout can be improved using traditional family-based selection, but progress has been limited to exploiting only between-family genetic variation. Genomic selection (GS) is a new alternative enabling exploitation of within-family genetic variation. We compared three GS models to predict genomic-enabled breeding values (GEBVs) for BCWD resistance in a commercial rainbow trout population and compared the accuracy of GEBVs to traditional breeding values (EBVs) estimated with a pedigree-based BLUP model. For these comparisons, we used BCWD survival phenotypes recorded on 7893 training fish from 102 families, from which 1473 fish from 50 families had genotypes (57K SNP array). Naïve siblings of the training fish (n = 930 testing fish) were genotyped to predict their GEBVs, of which 193 were mated to produce 138 progeny testing families (PTFs). In the following generation, 9968 progeny from the PTFs were BCWD phenotyped to empirically assess the accuracy of GEBV predictions made on their non-phenotyped parents. The accuracy of GEBVs from all three GS models were substantially higher than the BLUP model EBVs, with the increase in accuracy ranging from 83.3% to 108.8% depending on the GS model and survival phenotype used. Reducing the training sample size to n = ~1000 had no negative impact on the accuracy (0.67–0.72), but with n = ~500, the accuracy dropped to 0.53–0.61, if the training and testing fish were full-sibs, and even substantially lower to 0.22–0.25 when they were not full-sibs. Thus, using progeny performance data, we have shown that the accuracy of genomic predictions with GS models (0.63–0.71) is substantially higher than the traditional pedigree-based BLUP model (0.34–0.36). We also found that using a much smaller training sample size compared to similar studies in livestock, GS can substantially improve the selection accuracy and genetic gains for BCWD resistance in commercial rainbow trout aquaculture.

INTRODUCTION

Bacterial cold water disease (BCWD) causes significant mortality and economic losses in salmonid aquaculture, and methods to control outbreaks are limited (Nematollahi et al. 2003; Barnes and Brown 2011). We previously reported a family-based, selective breeding program (Silverstein et al. 2009), with the objective of increasing rainbow trout resistance against *Flavobacterium psychrophilum* (*Fp*), the etiological agent of BCWD. Resistance to laboratory injection challenge with *Fp* strain CSF259-93 is a moderately heritable trait that responds to selection (Leeds et al. 2010). The genetic architecture of resistance is complex (Vallejo et al. 2010) and we previously identified several major resistance QTL in the NCCCWA odd- and even-year rainbow trout selective-breeding populations (Wiens et al. 2013; Vallejo et al. 2014; Liu et al. 2015a; Palti et al. 2015b). While those loci can be evaluated for marker assisted selection (MAS) following fine-mapping, the complex genetic architecture of BCWD resistance and high genetic variation we discovered in past studies (Leeds et al. 2010; Vallejo et al. 2014; Liu et al. 2015b; Palti et al. 2015b) led us to hypothesize that a genome-enabled selection approach would be a more efficient strategy for improving rainbow trout genetic resistance against BCWD.

Genomic selection is a methodology (Meuwissen et al. 2001) that is revolutionizing animal and plant breeding. This method uses dense marker genotypes that cover the genome, combined with phenotypic data to predict breeding values of all genotyped individuals. In GS, a reference population is genotyped and recorded for the trait to train the GS model and estimate the effects of each single nucleotide polymorphism (SNP). Selection candidates are also genotyped, and by combining their genotypes with the estimated SNP effects, genomic-enabled breeding value (GEBV) are estimated for the selection candidates. The GS approach does not necessarily require pedigree recording and the

selection candidates do not need phenotypes. Thus, the GS methodology is particularly relevant for traits that cannot be measured directly on selection candidates, including carcass traits, sex-limited traits, and disease resistance, and has been demonstrated to be very effective in commercial dairy cattle (Hayes et al. 2009a; VanRaden et al. 2009; Goddard et al. 2011; Wiggans et al. 2011). For aquaculture species, the main advantage of GS is that it enables exploitation of within-family genetic variation for traits that cannot be measured directly on selection candidates. In addition to increasing accuracy of selection, GS is expected to reduce rates of inbreeding because the increased accuracy of Mendelian sampling terms in GS allows for identification and selection of elite breeding candidates from more families, with lower co-selection of sibs (Daetwyler et al. 2007; Dekkers 2007).

The genomic BLUP (GBLUP) method assumes a polygenic architecture of the trait and uses all the markers data in estimating the genomic relationship G matrix; in contrast, the Bayesian variable selection methods assume that the genetic variance is explained by a reduced number of markers with small-moderate or large effects (Habier et al. 2007; Hayes et al. 2009b; de los Campos et al. 2013; Fernando and Garrick 2013; Tiezzi and Maltecca 2015). Based on this assumption, GBLUP is not expected to perform as well as Bayesian variable selection models when the trait is not polygenic and it is controlled by several moderate-to-large effect QTL. The GBLUP method was modified into the single-step GBLUP method which allows the combination of the pedigree (A) and genomic-derived relationships (G) into a combined relationship matrix (H) (Aguilar et al. 2010; Legarra et al. 2014) and to the weighted single-step GBLUP method which emulates the Bayesian variable selection models by fitting in the model selected SNPs that explain moderate-large fraction of the genetic variance (Wang et al. 2012). Therefore, when evaluating a trait for the first time in a population, it is important to compare the accuracy of GEBV predictions from several GS models to those obtained with pedigree-based BLUP.

This study was conducted to assess the potential impact of GS on commercial breeding in rainbow trout aquaculture, as well as the feasibility of its real-time implementation into current commercial breeding schemes. Thus, the objectives of this study were to (1) predict GEBV for BCWD resistance in a commercial breeding population that has been selected primarily on growth; (2) compare the accuracy of pedigree-based EBV with that of GEBV from three GS models using progeny performance data; and (3) assess the impact of the study design on the accuracy of genomic predictions using different sampling schemes.

MATERIALS AND METHODS

Fish Rearing and Disease Challenge

All fish work was conducted in accordance with national and international guidelines. The protocol for this study was approved by the Institutional Animal Care and Use Committee (IACUC; Protocol # 053) of the U.S. Department of Agriculture, Agricultural Research Service, the National Center for Cool and Cold Water Aquaculture. All efforts were made to ensure fish welfare and to minimize suffering.

Details of the fish rearing conditions and the 21-day survival trial following intraperitoneal injection with *Flavobacterium psychrophilum* (*Fp*), the causative agent of bacterial cold water disease (BCWD), have been reported elsewhere (Silverstein et al. 2009; Leeds et al. 2010). Mortalities were removed and recorded daily and fin clipped. Fish that survived to day 21 post-infection were euthanized in 200 mg L^{-1} of tricaine methanesulfonate, MS 222 (Sigma-Aldrich, St. Louis, MO, USA) for at least 10 min prior to sampling of fin clips. Fin clips from all mortalities and survivors were individually kept in 95% ethanol until DNA was extracted using established protocols (Palti et al. 2006).

Training and Testing Data

The training sample included 102 pedigreed full-sib (FS) families from year-class (YC) 2013 of the Troutlodge, Inc., all-female, May-spawning population (Fig. 1). The 102 YC 2013 families represented a nucleus breeding population undergoing selection for growth, and thus had not previously been selected for BCWD resistance. The fish from YC 2013 families were evaluated in the laboratory BCWD challenge in two tanks per family, with an initial stocking of 40 fish per tank (total phenotyped fish $n = 7893$). The original study design was to sample $n = 1500$ fish with phenotypes and genotypes from 50 FS families. Of the 50 FS families, 25 were full-sibs of the testing sample and 25 were least related to the testing sample families based on pedigree records. We sampled ~40 fish from the 25 FS families that were closely related to the testing sample and ~20 fish per family from the other less related 25 families. In practice, we sampled $n = 1473$ fish with phenotypes and genotypes from those 50 families ($n = 17$-40 per family). Thus, from the 7893 BCWD evaluated fish, 1473 fish had genotype data.

The testing sample included 930 potential breeders or selection candidates (sires and dams) that were disease naïve fish sampled from 25 families ($n = 31$ to 44 testing fish per family). The testing fish had family-based EBV for survival days (DAYS) and survival

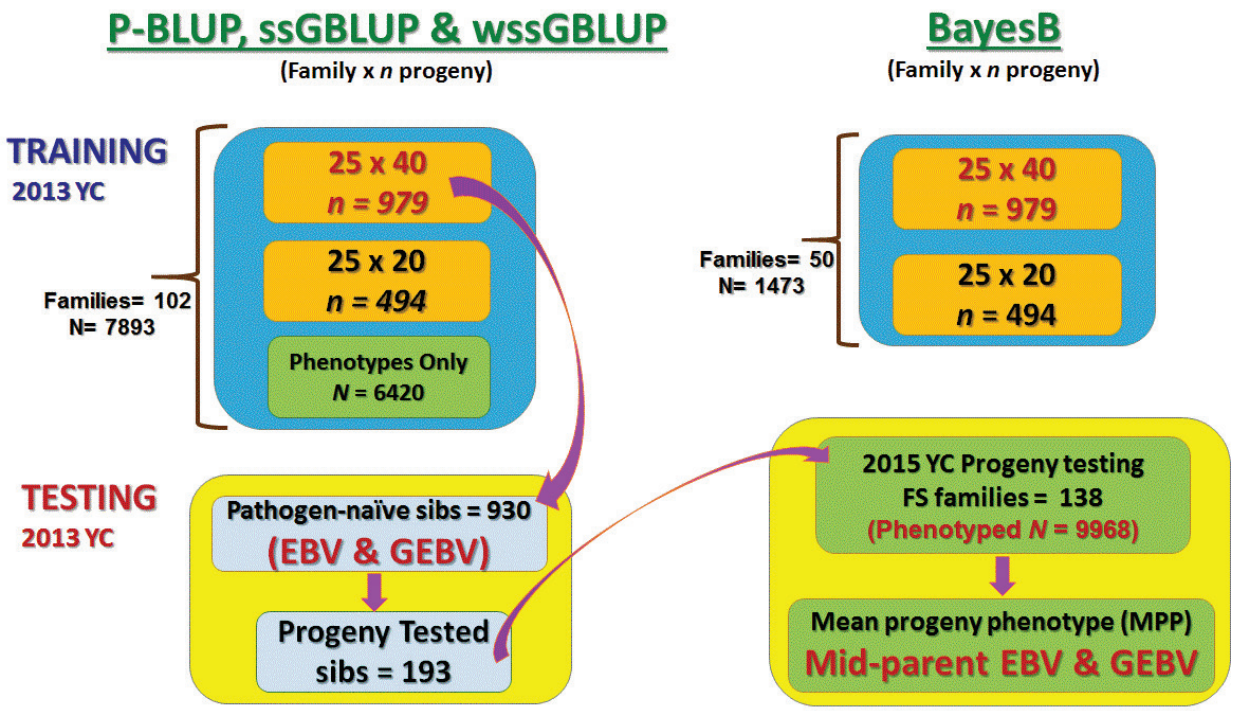

Figure 1. Scheme of genomic selection for BCWD resistance in rainbow trout used in this study.

status (STATUS) that were estimated with a pedigree-based BLUP model (described below) using BCWD survival records measured on their siblings and any collateral relatives among the 102 FS families (n = 7893). Each of these testing fish also had predicted GEBV from GS models (also described below).

To assess the accuracy of the GEBV, we generated 138 next-generation YC 2015 FS progeny testing families (PTF) from crosses that involved 193 of the YC 2013 testing fish (Fig. 1). These 138 YC 2015 PTF were phenotyped in 2015 for BCWD survival (n = 9968) to calculate the mean progeny phenotype (MPP) per PTF.

BCWD Resistance Phenotypes

Survival DAYS, the number of days to death post-challenge, were recorded for a total of 21 days post-challenge, with survivors being assigned a value of 21 d post-challenge. Each fish also had a binary survival STATUS record. The binary STATUS had two classes: two for fish that were alive on day 21 post-challenge and one for fish that died during the 21 d post challenge evaluation period. In the GS analysis, we used DAYS and STATUS records from training sample fish to estimate marker effects to then predict GEBV for DAYS and STATUS for each of the testing sample fish.

SNP Genotyping Platform

Genotyping was performed by a commercial genotyping service provider (Neogen, Inc., Lincoln, NE) using the Rainbow Trout Axiom® 57K SNP array, as previously described in (Palti et al. 2015a). Our quality control (QC) bioinformatics pipeline filtered out SNPs with significant distortion from the expected Mendelian segregation in each FS family (Bonferroni adjusted to $P < 0.10$) and also removed two training fish that did not have genotypes that matched the parents based on the pedigree records. After genotype data QC, a total of 41,868 SNPs were included in the genotype dataset.

Before training the GS models, all genotyped SNPs were further filtered using QC algorithms that are implemented in the computer program BLUPF90 (Misztal et al. 2015). The QC retained SNPs with a genotype calling rate higher than 0.90, minor allele frequency higher than 0.05, and departures from Hardy-Weinberg equilibrium less than 0.15 (difference between expected and observed frequency of heterozygotes). Parent-progeny pairs were tested for discrepant homozygous SNPs, those SNPs with a conflict rate of more than 1% were discarded. After this final QC step, 35,636 SNPs remained for the GS analysis.

Estimation of Pedigree-based EBV

For the testing fish, we estimated EBV for BCWD resistance phenotypes using a pedigree-based BLUP (P-BLUP) model. Family-based EBV were estimated using BCWD survival records measured on siblings of the testing fish and any collateral relatives. The phenotypic dataset included records from n = 7893 fish from 102 FS families. The pedigree dataset included 32,279 fish from seven generations.

Based on past genetic analyses for estimating EBV for BCWD resistance in rainbow trout (Leeds et al. 2010; Vallejo et al. 2016), we decided to use an animal model that included a population mean, random animal genetic and random residual effects. The records of the BCWD survival phenotypes DAYS and STATUS were fit into P-BLUP linear and threshold models, respectively, using the computer application BLUPF90 (Misztal et al. 2015).

Estimation of GEBV with Bayesian Variable Selection Models

The SNP genotype data from the training fish (YC 2013 families), with their corresponding BCWD phenotypic records, were used to train the prediction model and estimate marker effects using the Bayesian variable selection model BayesB implemented in the software GENSEL (Fernando and Garrick 2009) as previously described in (Vallejo et al. 2016); an animal model was used that included a population mean, random marker and random error effects. The mixture parameter was assumed to be known and defined to meet the condition , where is the number of training fish. After testing by performing 5-fold cross-validation analyses (results not presented), we decided to use in the final GS analysis with BayesB because it yielded the best accuracy predictions.

The software GENSEL uses a Gibbs sampling approach in the BayesB analysis (Garrick and Fernando 2013). In this study, DAYS and STATUS were analyzed using 210,000 Markov chain Monte Carlo (MCMC) iterations, of which the first 10,000 samples were discarded as burn-in. From the remaining 200,000 samples, we saved one from every 40 samples, thus a total of 5,000 samples were used in the analysis. The proper mixing and convergence of the MCMC iterations were assessed using the R package CODA (Plummer et al. 2006).

Estimation of GEBV with Single-step GBLUP Models

The SNP genotype data from training fish and pedigree information on all fish included in this GS study were used to estimate GEBV for the testing sample fish (n = 930 full-sibs of training fish that were not disease challenged) using two methods: (i) single-step genomic BLUP (ssGBLUP) (Aguilar et al. 2010; Christensen and Lund 2010) and (ii) weighted ssGBLUP (wssGBLUP), as previously described (Vallejo et al. 2016). The linear and threshold models to estimate GEBV for DAYS and STATUS, respectively, included a population mean, random animal genetic effects, and random error effects and were fitted as previously described in Vallejo et al. (2016) using the software BLUPF90 (Misztal et al. 2015).

Before performing the GS analysis with ssGBLUP and wssGBLUP, we estimated genetic parameters to use as priors in the Bayesian analysis of the binary trait STATUS as previously described in (Vallejo et al. 2016). The MCMC Gibbs sampling scheme included a total of 210,000 iterations; the first 10,000 iterations were discarded as burn-in iterations. Then, from the remaining 200,000 samples, one from every 40 samples were saved for analysis. This Gibbs sampling scheme collected 5000 independent samples for analysis. The proper mixing and convergence of these MCMC iterations were also assessed using the R package CODA (Plummer et al. 2006).

Predictive Ability and Bias of EBV and GEBV

The predictive ability (PA) of EBV and GEBV, which are both estimates of additive genetic effects, was estimated under the assumption that the correlation of mid-parent EBV or GEBV with the mean progeny performance (MPP) for each PTF is an estimate of the accuracy of the estimated breeding values (Odegard et al. 2007; Cheng et al. 2015; Vallejo et al. 2016). We used the mid-parent EBV or GEBV instead of the individual EBV or GEBV of each parent because the testing fish were mated to each other to generate the 138 PTF, rather than mating each testing fish to a large random sample of fish from a common genetic background, as is often done in GS studies with terrestrial agricultural animals and birds.

Bias of the EBV was estimated as the regression coefficient of MPP on predicted mid-parent EBV ($\beta_{MPP,EBV}$). Similarly, bias of the GEBV was estimated as the regression coefficient of MPP on predicted mid-parent GEBV ($\beta_{MPP,GEBV}$). A value of 1.0 for the regression of true breeding value, performance phenotype or MPP on predicted EBV or GEBV is theoretically expected for unbiased estimates of BV, and a deviation from 1.0 can be interpreted as prediction bias (Saatchi et al. 2013).

Impact of GS Study Design on Accuracy of GEBV

To evaluate the impact of sample size and relatedness between the training and testing fish on the accuracy of GEBV predictions, we used five GS schemes that were developed using the genotype and phenotype records collected in this study, as outlined in Table 3. The following study design variables were evaluated: size of the training data (~500, ~1000 or ~1500 fish); number of training families (25 or 50 families); size of the training families (20 or 40 fish per family); and proportion of fish in the training data that were full-sibs (FS) of the testing fish (Table 3). For the latter variable, scheme 1 = 0.66 means that 66% of the fish in the training data were FS of fish in the testing data; scheme 2 = 0.50 means that 50% of the fish in the training data were FS of fish in the testing data;

Table 1. Accuracy of genomic prediction for BCWD survival DAYS in rainbow trout.

Model[a]	Training sample				Testing sample		
	Phenotyped fish	Genotyped fish	Effective SNPs	h^2 [b]	Genotyped fish	Predictive ability[c]	Bias[d]
P-BLUP	7893	0	0	0.37	0	0.34	0.86
ssGBLUP	7893	1473	35,636	0.33	930	0.63	0.99
wssGBLUP2	7893	1473	35,623	0.33	930	0.67	0.71
wssGBLUP3	7893	1473	35,623	0.33	930	0.65	0.65
BayesB	1473	1473	35,636	0.23	930	0.71	1.16

[a] The estimated breeding values (EBVs) were estimated with a pedigree-based animal model (P-BLUP); and the genomic EBVs (GEBVs) were estimated with three genomic selection models: single-step GBLUP (ssGBLUP), weighted ssGBLUP (wssGBLUP), and Bayesian method (BayesB). The wssGBLUP2 and wssGBLUP3 correspond to iterations 2 and 3, respectively.
[b] For the GS models, h^2 is the proportion of phenotypic variance explained by the markers. For the P-BLUP model, h^2 is the trait narrow-sense heritability estimated from pedigree and phenotypic records.
[c] The predictive ability of EBV (PA_{EBV}) or GEBV (PA_{GEBV}) was defined as the correlation of midparent EBV or GEBV with MPP from each PTF: $PA_{EBV} = CORR(MPP, Midparent\ EBV)$; $PA_{GEBV} = CORR(MPP, Midparent\ GEBV)$.
[d] The bias of EBV ($Bias_{EBV}$) or GEBV ($Bias_{GEBV}$) was defined as the regression coefficient of performance MPP on predicted midparent EBV or GEBV: $Bias_{EBV} = REGRES(MPP, Midparent\ EBV)$; $Bias_{GEBV} = REGRES(MPP, Midparent\ GEBV)$.

Table 2. Accuracy of genomic prediction for BCWD survival STATUS in rainbow trout.

Model[a]	Training sample				Testing sample		
	Phenotyped fish	Genotyped fish	Effective SNPs	h^2 [b]	Genotyped fish	Predictive ability[c]	Bias[d]
P-BLUP	7893	0	0	0.35	0	0.36	0.67
ssGBLUP	7893	1473	35,636	0.35	930	0.66	0.86
wssGBLUP2	7893	1473	35,623	0.35	930	0.70	0.68
wssGBLUP3	7893	1473	35,623	0.35	930	0.68	0.64
BayesB	1473	1473	35,636	0.25	930	0.71	1.01

[a] The estimated breeding values (EBVs) were estimated with a pedigree-based animal model (P-BLUP); and the genomic EBVs (GEBVs) were estimated with three genomic selection models: single-step GBLUP (ssGBLUP), weighted ssGBLUP (wssGBLUP), and Bayesian method (BayesB). The wssGBLUP2 and wssGBLUP3 correspond to iterations 2 and 3, respectively.
[b] For the GS models, h^2 is the proportion of phenotypic variance explained by the markers. For the P-BLUP model, h^2 is the trait narrow-sense heritability estimated from pedigree and phenotypic records. The heritability estimated on the underlying scale of liability was transformed to the observed scale of survival STATUS.
[c] The predictive ability of EBV (PA_{EBV}) or GEBV (PA_{GEBV}) was defined as the correlation of midparent EBV or GEBV with MPP from each PTF: $PA_{EBV} = CORR(MPP, Midparent\ EBV)$; $PA_{GEBV} = CORR(MPP, Midparent\ GEBV)$.
[d] The bias of EBV ($Bias_{EBV}$) or GEBV ($Bias_{GEBV}$) was defined as the regression coefficient of performance MPP on predicted midparent EBV or GEBV: $Bias_{EBV} = REGRES(MPP, Midparent\ EBV)$; $Bias_{GEBV} = REGRES(MPP, Midparent\ GEBV)$. The predicted EBV and GEBV for STATUS estimated on the underlying scale of liability were transformed to the observed scale (probability of survival).

Table 3. Accuracy of genomic prediction for BCWD resistance with BayesB using progeny testing families[a] in five GS schemes.

GS	Family			Training–testing			DAYS[f]		STATUS[f]	
scheme	Number	Size	Training size	relationship[c]	π[d]	SNPs[e]	PA_{GEBV}[g]	$Bias_{GEBV}$[h]	PA_{GEBV}[g]	$Bias_{GEBV}$[h]
1	50	20–40[b]	1473	0.66	0.97	1069	0.71	1.16	0.71	1.01
2	50	20	991	0.50	0.98	713	0.67	1.55	0.67	1.51
3	25	40	979	1.00	0.98	713	0.69	1.26	0.72	1.23
4	25	20	497	1.00	0.987	463	0.53	1.37	0.61	1.66
5	25	20	494	0.00	0.987	463	0.25	3.33	0.22	5.08

[a] A sample of 193 testing fish (from total n = 930 testing fish) were inter-mated to develop 138 progeny testing families (PTFs). After disease evaluation of progeny from the 138 PTFs (n = 9968), we estimated the mean progeny phenotype (MPP) for each PTF.
[b] In scheme 1, there were two groups of training families: (i) A set of 25 families with 40 offspring each that contributed fish to the testing sample; and (ii) A set of 25 families with 20 offspring each that did not contribute fish to the testing sample.
[c] Proportion of training fish that were full-sibs (FS) of testing fish: scheme 1= 0.66 indicates that 66% of training fish were FS of testing fish; scheme 2 = 0.50 indicates that 50% of training fish were FS of testing fish; schemes 3 and 4 = 1.0 indicates that ALL training fish were FS of testing fish; and scheme 5 = 0.0 indicates that NONE of training fish were FS of testing fish (i.e., training and testing fish were sampled from different families).
[d] BayesB method uses a mixture parameter (π) that specifies the proportion of loci with zero effect, and the analyses included 35,636 effective SNPs.
[e] Number of SNPs that are sampled as having non-zero effect ($1 - \pi$) and fitted simultaneously in the multiple regression model.
[f] Bacterial cold water disease (BCWD) resistance phenotypes: BCWD survival days (DAYS) and survival status (STATUS).
[g] The predictive ability of GEBV (PA_{GEBV}) was defined as the correlation of MPP with midparent GEBV from each PTF: $PA_{GEBV} = CORR(MPP, Midparent\ GEBV)$.
[h] Bias of GEBV ($Bias_{GEBV}$) was defined as the regression coefficient of performance MPP on predicted midparent GEBV: $Bias_{EBV} = REGRES(MPP, Midparent\ EBV)$; $Bias_{GEBV} = REGRES(MPP, Midparent\ GEBV)$. The predicted GEBV for STATUS estimated on the underlying scale of liability was transformed to the observed scale (probability of survival).

schemes 3–4 = 1.0 means that all fish in the training data were FS of fish in the testing data; and scheme 5 = 0.0 means that none of the fish in the training data were FS of fish in the testing data (i.e., fish in the training and testing data were sampled from different families from the same breeding population). In scheme 1, there were two distinct groups of training families: (i) a set of 25 families with ~40 progeny each (n= 979) that also contributed fish to the testing data and (ii) a set of 25 families with ~20 progeny each (n= 494) that did not contribute fish to the testing data (Fig. 2). In scheme 2, we used both groups again but reduced the number of fish sampled per family in group (i) to ~20 (n = 497). In scheme 3, we only sampled group (i) (n = 979). In scheme 4, we only sampled group (i) again, but reduced the number of

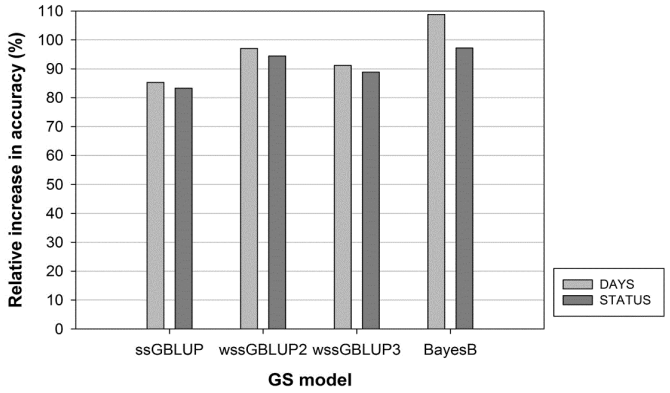

Figure 2. Relative increase in accuracy of GEBV from GS models over those estimated with pedigree-based BLUP model.

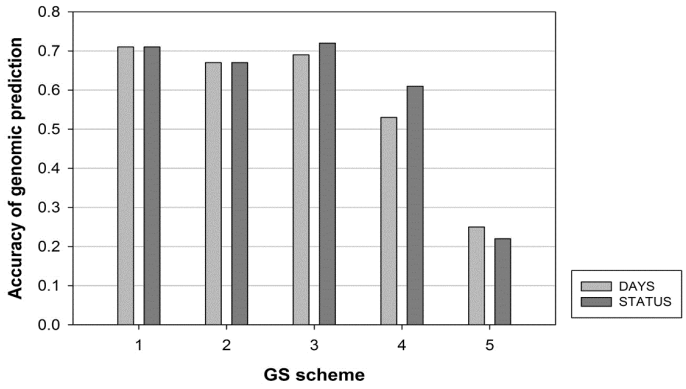

Figure 3. Accuracy of GEBV for BCWD resistance estimated with BayesB in five GS schemes.

fish sampled per family to ~20 (n = 497). In scheme 5, we only sampled group (ii). The accuracy of predicted GEBV for each tested GS scheme was estimated using the BayesB method.

RESULTS

Heritability of BCWD Resistance

Estimates of the narrow-sense heritability for DAYS and STATUS were equal to 0.37 and 0.35, respectively, using the BLUP model without genomics data (Tables 1 and 2). Similarly, the proportion of phenotypic variance explained by the markers for DAYS and STATUS ranged from 0.23 to 0.33 and from 0.25 to 0.35, respectively, using the GS models.

Accuracy and Bias of EBV

The prediction accuracy (PA) of EBV for DAYS was marginally lower than the PA of EBV for STATUS (Tables 1 and 2). The bias of EBV for DAYS deviated less from 1.0 than the bias of EBV for STATUS.

Accuracy and Bias of GEBV

The PA of GEBV for DAYS ranged from 0.63 to 0.71 and the BayesB model had genomic predictions with the highest accuracy (Table 1). The bias of the GEBV for DAYS ranged from 0.65 to 1.16 and the predictions obtained with ssGBLUP were the least biased. The for STATUS ranged from 0.66 to 0.71 and BayesB resulted in genomic predictions with the highest accuracy (Table 2). The bias of GEBV for STATUS, ranged from 0.64 to 1.01 and the predictions obtained with BayesB were the least biased.

Comparing Accuracy of EBV and GEBV

The relative increase in accuracy of GEBV from GS models over those estimated with the classical P-BLUP model is shown in Fig. 3. Overall, the GS models substantially outperformed the P-BLUP model. The highest increase in accuracy of prediction was achieved with BayesB (DAYS = 108.8%; STATUS = 97.2%) followed by wssGBLUP at iteration 2 (wssGBLUP2) (DAYS = 97.1%; STATUS = 94.4%). The wssGBLUP2 outperformed the wssGBLUP at iteration 3 (wssGBLUP3) (DAYS = 91.2%; STATUS = 88.9%). The lowest increase in accuracy of prediction was achieved with ssGBLUP (DAYS = 85.3%; STATUS = 83.3%).

Accuracy of GEBV in the Five GS Schemes

The accuracies of GEBV for DAYS and STATUS obtained with BayesB using the five GS schemes are in Table 3. Schemes 1 and 3 had the highest prediction accuracies (0.69 to 0.72), followed by scheme 2 (0.67). Scheme 4 GEBV had moderate accuracies (0.53 to 0.61) that were substantially lower than those for schemes 1 to 3. Scheme 5 had the lowest accuracies (0.22 to 0.25) among the tested GS schemes. The accuracies of GEBV from scheme 5 were even lower than the classical pedigree-based BLUP model accuracies (0.34 to 0.36) (Tables 1 and 2).

DISCUSSION

The accuracy of GEBV for DAYS and STATUS were similar when using the Bayesian method BayesB and higher than those estimated with ssGBLUP and wssGBLUP (Table 1 and 2). However, the accuracies of GEBV for STATUS were slightly higher than those estimated for DAYS when using ssGBLUP and wssGBLUP methods, which may be due to (1) a better

fit of the binary trait STATUS with a threshold model than the discrete data DAYS with a linear model, (2) our imprecise measure of DAYS for fish that survived the challenge (arbitrarily assigned 21 days of survival), and (3) the resulting slightly higher heritability of STATUS compared to DAYS.

In this study, the accuracy of genomic predictions for BCWD resistance ranged from 0.63 to 0.72, which is substantially higher than accuracies of EBV estimated with the classical P-BLUP model and is also significantly higher than the 0.55 maximum realized accuracy of EBV prediction using pedigree and phenotype data with a P-BLUP model given a heritability of 0.30 for BCWD resistance (Van Vleck et al. 1987).

The accuracy of genomic prediction in dairy cattle exceeded 0.8 for milk production traits and 0.7 for health-related traits using large reference populations that included progeny-tested bulls with highly accurate phenotypes based on average daughter performance (Lund et al. 2011; Wiggans et al. 2011). In this study, it was remarkable to have genomic evaluations with an accuracy of 0.71 using a relatively small training dataset (n = 1473) in comparison to those used in dairy cattle. We hypothesize that the relatively high accuracy achieved in the current study was due to the high relationship between the training and testing fish, the small effective population size of this farmed rainbow trout population, which leads to extensive linkage disequilibrium (LD) and a substantially smaller number of effective chromosome segment effects to be estimated, hence better predictions and higher accuracies (Daetwyler et al. 2010), and the high extent of long-range LD observed in admixed salmonid populations (Rexroad and Vallejo 2009; Odegard et al. 2014).

Comparing GS Models

The differences in accuracy between the GS models that we tested here were small and all of them outperformed the classical P-BLUP model. For DAYS and STATUS, the GEBV obtained with BayesB had the highest accuracy (0.71), and the GEBV obtained with ssGBLUP had the lowest accuracy of predictions (0.63 to 0.66). The wssGBLUP2 model outperformed ssGBLUP by 0.04 units of accuracy for both BCWD phenotypes. The Bayesian method BayesB outperformed wssGBLUP2 marginally by 0.01 and 0.04 units of accuracy for STATUS and DAYS, respectively.

Previously, in a different rainbow trout population, we showed that BCWD resistance is controlled by oligogenic inheritance of a few moderate to large effect QTL and many genes/loci each with a small effect (Vallejo et al. 2010; Vallejo et al. 2014; Palti et al. 2015b). Thus, given this genetic architecture, variable selection models (Meuwissen et al. 2001; Fernando and Garrick 2013; Garrick and Fernando 2013) that fit markers with mostly moderate to large effects can yield GEBV with higher accuracy than GS models that use pedigree and phenotype records with marker genotype data in a single-step GS BLUP analysis (Aguilar et al. 2010; Christensen and Lund 2010; Legarra et al. 2014). Thus, our finding that wssGBLUP2, which fits only SNPs with an effect different from zero and weighted by their genetic variance (Wang et al. 2012) to emulate Bayesian variable selection models, predicts GEBV with higher accuracy than ssGBLUP and remarkably close to BayesB was largely expected.

Comparing GS Study Designs

The most interesting result from comparing the different GS sampling schemes was that the accuracy of scheme 3 was similar to that of scheme 1, in spite of the smaller training data size of scheme 3 (n = 979 vs. n = 1473), which is likely due to the higher relationship between the training and testing fish in scheme 3 (1.0 vs. 0.66) and also because the average relationship among the fish in the training data was higher in scheme 3 than in scheme 1 (Fig. 2; Table 3). These results validate the notion that if the main breeding objective is to obtain high accuracy GEBV only for selection candidates (not for the entire population), then we should design GS studies that ensure a high genetic relationship between the training and testing fish, and also a high average relationship among the training fish (Pszczola et al. 2012; Wu et al. 2015). Likewise, the prediction accuracy of scheme 3 was better than that of scheme 2 (0.69 to 0.72 vs. 0.67) because of the following two design characteristics: (i) a higher relationship between training and testing fish in scheme 3 than in scheme 2 (1.0 vs. 0.5) and (ii) a higher average relationship among training fish in scheme 3 than in scheme 2 due to the larger family size of scheme 3. The substantial superiority of scheme 3 over scheme 4 on accuracy of predictions (0.69 to 0.72 vs. 0.53 to 0.61), in spite of the same level of relationships between the training and testing fish in those two schemes, was due to the overall larger sample size and larger family size in scheme 3. Scheme 5 had genomic predictions with the lowest accuracy (0.22 to 0.25) because the relationship between training and testing fish was the lowest and the overall sample size was the lowest of the five GS training fish sampling schemes.

These results have important implications on the design of effective GS studies in finfish aquaculture using similar SNP array densities for genotyping, because they highlight the following: (1) the importance of a high relationship between training and testing fish for genomic prediction, i.e. the accuracy of predictions will drastically drop if the training and testing fish are sampled from different families within a population, and (2) the accuracy of GEBV from GS across populations will be relatively low, i.e. training sample from one population and testing from another.

Additional Remarks

In comparison to dairy cattle and other livestock species, one of the main challenges of implementing GS in traditional family-based breeding programs with salmonid species is the large number of selection candidates and the limited value of the individual candidates compared to the genotyping cost. Nevertheless, the classical sib-testing scheme used in disease resistance breeding programs with salmonids can be redesigned to capitalize on the ability of GS to increase the accuracy of breeding value prediction and rate of genetic progress. To this end, for implementing GS for BCWD resistance in sib-selection schemes in the rainbow trout industry, we suggest combining a first step of sib-testing disease challenge evaluations to pre-select families for disease resistance, as suggested elsewhere (Sonesson and Meuwissen 2009; Lillehammer et al. 2013; Odegard and Meuwissen 2014), with a second step of selective genotyping individuals from the disease resistance pre-selected families to reduce genotyping costs. In this GS scheme, the disease phenotype and marker genotype records from the pre-selected families can be used to train the prediction model and then, in a third step, to predict GEBV for each genotyped selection candidate from families that were pre-selected at the first step.

So far, to the best of our knowledge, this is the first study that assesses the accuracy of genomic predictions for BCWD resistance using progeny performance data and empirically tests the potential of GS to exploit within-family genetic variation in sib-selection breeding schemes in the rainbow trout industry. In this study, we have shown that (i) the accuracy of genomic predictions is substantially higher than those from a classical P-BLUP model; (ii) high and near-optimal accuracy of genomic predictions for BCWD resistance can be obtained in the rainbow trout commercial population that was evaluated in this study using a relatively small training sample size of $n = 1000$; and (iii) the accuracy of GEBV estimated with BayesB is higher than those from wssGBLUP3 and ssGBLUP, followed by the accuracy of wssGBLUP2. Finally, this study provides guidelines for the implementation of GS in the rainbow trout industry.

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ANNOTATED BIBLIOGRAPHY OF KEY WORKS

Meuwissen, T. H. E., B. J. Hayes, and M. E. Goddard. 2001. Prediction of total genetic value using genome-wide dense marker maps. Genetics 157: 1819-1829.

This is a landmark publication on whole genome-enabled selection. Using computer simulated data, the authors proposed the method of genomic selection (GS) when high density SNP chips were not yet available for any plant and livestock species. The authors attempted to estimate the effects of ~50,000 marker haplotypes simultaneously from a limited number of phenotypic records. A genome of 1000 cM was simulated with a marker spacing of 1 cM. The markers surrounding every 1-cM region were combined into marker haplotypes. Due to finite population size ($N_e = 100$), the marker haplotypes were in linkage disequilibrium with the QTL located between the markers. Then using least squares, all haplotype effects could not be estimated simultaneously. When only the biggest effects were included, they were overestimated and the accuracy of predicting genetic values of the offspring of the recorded animals was only 0.32. Next, with best linear unbiased prediction of haplotype effects assuming equal variances associated to each 1-cM chromosomal segment, the accuracy of genomic predictions increased to 0.73. They also used Bayesian methods that assumed a prior distribution of the variance associated with each chromosome segment and increased the accuracy to 0.85. The authors concluded that selection on genetic values predicted from high density marker maps could substantially increase the rate of genetic gain in animals and plants. With this publication, the authors proposed a GS method that today has revolutionized traditional animal and plant breeding programs. The authors laid out concepts and methods that are used in our GS research in rainbow trout aquaculture.

Odegard, J., T. Moen, N. Santi, S. A. Korsvoll, S. Kjoglum, and T. H. Meuwissen. 2014. Genomic prediction in an admixed population of Atlantic salmon (*Salmo salar*). Frontiers in Genetics 5: 402.

The authors for the first time tested the reliability of genomic selection (GS) models in an admixed population of Atlantic salmon. The models included ordinary genomic BLUP models (GBLUP), using genome-wide SNP markers of varying densities(1–220K), a genomic identity-by-descent model (IBD-GS), using linkage analysis of sparse genome-wide markers, as well as a classical pedigree-based model. The authors compared the reliability of the models using 5-fold cross-validation, and the studied traits were salmon lice resistance (LR) and fillet color (FC) with heritability of 0.14 and 0.43, respectively. Overall, the authors found that all genomic models outperformed the classical pedigree-based model for both traits and at all marker densities. However, the relative improvement differed considerably between traits, models and marker densities. For the highly heritable FC, the IBD-GS had similar reliability as GBLUP at high marker densities (>22K). In contrast, for the lowly heritable LR, IBD-GS was clearly inferior to GBLUP, irrespective of marker density. Hence, GBLUP was robust to marker density for the lowly heritable LR, but sensitive to marker density for the highly heritable FC. The authors hypothesized that this phenomenon may be explained by historical admixture of different founder populations, expected to reduce short-range linkage disequilibrium (LD) and induce long-range LD. The

authors also highlighted that using ordinary GBLUP, the typical long-range LD of an admixed population may be effectively captured by sparse marker density, while efficient utilization of relationship information may require denser markers (e.g., >22K).

Vallejo, R. L., T. D. Leeds, B. O. Fragomeni, G. Gao, A. G. Hernandez, I. Misztal, T. J. Welch, G. D. Wiens, and Y. Palti. 2016. Evaluation of genome-enabled selection for bacterial cold water disease resistance using progeny performance data in rainbowtrout: insights on genotyping methods and genomic prediction models. Frontiers in Genetics 7: 96.

The authors evaluated the potential utility of implementing GS in traditional family-based selective breeding programs using training and testing fish sampled from the first generation of the NCCCWA's BCWD resistance breeding line. The authors used GS models to predict genomic breeding values (GEBVs) for BCWD resistance, compared the predictive ability (PA) of GEBVs to pedigree-based estimated breeding values (EBVs), and compared the impact of two SNP genotyping methods on the accuracy of GEBV predictions. The best GEBV predictions were similar to EBV with PA values of 0.49 and 0.46 vs. 0.50 and 0.41 for DAYS and STATUS, respectively. Among the GEBV prediction models, ssGBLUP consistently had the highest PA. The RAD genotyping platform had GEBVs with similar PA to those of GEBVs from the Chip platform. The overall GEBV accuracy in this study was low to moderate, likely due to the small training sample used. In this study, the authors explored the potential of GS for improving resistance to BCWD in rainbow trout using, for the first time, progeny testing data to assess the accuracy of GEBVs, and it provided the basis for further investigation on the implementation of GS in commercial rainbow trout populations.

Atlantic Salmon and Eastern Oyster Breeding Programs at the National Cold Water Marine Aquaculture Center

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ABSTRACT

The USDA-ARS National Cold Water Marine Aquaculture Center (NCWMAC) focuses on the coldwater marine aquaculture industry's highest priority research needs including development of improved genetic stocks. Coldwater aquaculture production has potential for expansion, and both Atlantic salmon and Eastern oysters are widely accepted by American consumers. Commercial salmon and oyster producers predominantly utilize stocks that are not many generations removed from wild, unselected strains. North America salmon producers are legally required to culture certified stocks of North American salmon. The NCWMAC is the USDA-ARS facility supporting the U.S. coldwater marine aquaculture industry by developing genetically improved salmon stocks for the past thirteen years. Aquaculture of the Eastern oyster is a large segment of shellfish aquaculture in the U.S. and minimal selective breeding has been accomplished. In both species, there is a need to improve the performance of existing stocks. Our research meets this need through the following objectives: 1) define phenotypes, estimate genetic and phenotypic parameters, and develop a selection index in Atlantic salmon for important traits such as carcass weight, cold tolerance, fillet color, fat content, and sea lice resistance; 2) evaluate and validate the usefulness of incorporating genomic information into the salmon breeding program; and 3) establish links between disease susceptible and resistant phenotypes and genotype for the Eastern oyster. Identification of genes associated with oyster disease will provide markers that can be used to enhance and accelerate the development of high-performing oyster lines through selective breeding and will support the East Coast shellfish aquaculture industry. Research accomplished with salmon will result in the development of genetically improved Atlantic salmon for release to U.S. producers and consumers.

INTRODUCTION

Selective breeding is a powerful tool in animal husbandry. The USDA-ARS started the National Cold Water Marine Aquaculture Center (NCWMAC) in 2003 to begin a genetic selection and improvement program for Atlantic salmon (*Salmo salar* L.) of North American origin. The program was started in response to producer requests for assistance when U.S. regulations were established to prohibit the culture of Atlantic salmon with European genes as a mechanism to protect populations of wild Atlantic salmon federally listed as endangered. This regulation essentially prohibited culture of existing commercially improved stains as they are all based on European genetic stocks. In 2013, requests from East Coast U.S. oyster producers resulted in the expansion of the program to include selection of Eastern oysters (*Crassostrea virginica* Gmelin 1791).

Selective breeding programs for Atlantic salmon began over 40 years ago in Norway, and currently, various programs exist in all of the major salmon producing regions (Gjedrem and Baranski 2009). The primary focus of most of these programs, including the one at NCWMAC, is improved growth. The response in salmon to selection for growth or weight is robust with a 10-15% gain per generation (Gjedrem and Baranski 2009), which has resulted in the growth rate for salmon doubling in 6-7 generations of selection (Gjedrem and Baranski 2009). Despite achieving a doubling in growth rate, selective breeding programs have not been able to manifest this in halving the production time as might be expected. The NCWMAC has been focusing on how to realize and characterize the response to selection for growth in Atlantic salmon. More recently, resistance to sea lice infection has been added as an important selection criteria.

Sea lice (*Lepeophtheirus salmonis* Krøyer 1837) was estimated to cause the global salmon farming industry approximately $742 million USD in damage in 2012

(Roth 2015). Traditionally, control of sea lice has relied on the use of various chemotherapeutics; however, resistance to many of these has recently led to a host of non-chemotherapeutic control strategies including selective breeding (Aaen et al. 2015; McNair 2015; Roth 2015; Gjerde et al. 2011). The NCWMAC began evaluating their pedigreed families for sea lice resistance in 2015 for inclusion into the breeding selection program. Concurrently, the program is exploring approaches to estimate genotypic breeding values in addition to the experimentally derived phenotypic breeding values in order to improve overall trait selection.

There are currently three breeding programs for the Eastern oyster in the U.S. in Maine, New Jersey and Virginia. Each of these programs has selected for improved oyster growth and survival to regionally important conditions and pathogens. The NCWMAC has established a program in Rhode Island with the goal of working across the different strains produced by these programs to characterize and enhance resistance to Dermo, which is caused by the parasite *Perkinsus marinus* (Mackin, Owen and Collier) Levine 1978. The disease is spread from Massachusetts through the Gulf of Mexico and can cause > 50% annual crop loss on infected farms. The program is currently working to identify genes associated with parasite resistance, avoidance and susceptibility with an ultimate goal of developing genetic markers for selection. In addition, the program is collaborating on the Eastern Oyster Genome Project to facilitate future selection efforts.

In summary, the NCWMAC is focused on utilizing genetic selection and various genetic tools to improve strains of the commercially important species Atlantic salmon and Eastern oyster. Here, we discuss examples of current efforts including characterizing the response to selection for improved growth, selecting for resistance and developing genetic tools to improve the selection for sea lice and *P. marinus* resistance.

METHODS

Atlantic Salmon Fish Culture

Originally, individual families of Atlantic salmon were obtained from the existing industry in Maine. Additional strains of Atlantic salmon were obtained: a wild non selected strain from the U.S. Fish and Wildlife Service (Green Lake and Craig Brook National Fish Hatcheries) and a landlocked strain from Grand Lake, Maine. Fry were transferred after hatching but prior to first feeding, into individual 0.15-m^3 tanks from a recirculating biological filtration system. At approximately 30 days after initiation of feeding and a fish weight of 1-2 g, fish densities were equalized by stocking 250 fish at random into each tank. At approximately 15 months after hatching, fish from each family were pit tagged. Each tank was supplied with 2.0 ppt salinity water from a recirculating biological filtration system and illuminated on a natural photoperiod. Fish were fed a commercial diet (45%P, 25%F) from Arvotec® automatic feeders at a rate determined by fish size and temperature from experimental growth models (Ursin 1967; Ruohonen and Markinen 1992).

Salmon Sea Lice Challenges

One fish from each family was stocked into 12 1-m^3 meter tanks. Each tank contained 100-120 fish (one fish per family). In order to make sea lice counting manageable, fish in a single tank were exposed to 100 copepodid per fish for two hours to allow the sea lice to settle on the fish. After 12 to 15 days, depending on the temperature, the sea lice were allowed to develop to the chalamus stage (an attached sessile stage) and then counted. This procedure was repeated for each tank to stagger the collection of sea lice settlement data. This procedure has also been done for every year sea lice settlement data have been collected.

Oyster Breeding Program

The oyster breeding program at the NCWMAC obtained family stocks from the Virginia Institute of Marine Science (VIMS), Rutgers University and the University of Maine. Oysters are cultured in tanks containing seawater. Eastern oysters are exposed to Dermo and survival, parasite load, feeding behavior and gene expression profiles are measured.

Table 1. Estimates of heritabilities (h^2±SE) for total sea lice count (TC), sea lice density (LD), and body weight (BW), and the estimated phenotypic (r_p±SE above diagonal) and genetic correlations (r_g±SE below diagonal) among the three traits.

Trait	h^2±SE	r_p±SE	r_g±SE	
		TC	LD	BW
TC	0.11±0.05	—	0.92*	0.10*
LD[a]	0.14±0.06	0.87±0.08	—	-0.14*
BW	0.30±0.09	-0.03±0.27	-0.60±0.20	—

[a] Unitless calculated index based on body weight and lice counts.

Table 2. Estimates from heritabilities (h^2±SE) for lice density.

	2015	2016
Infection Dates	Jun 26-Nov 8	Jun 15-Aug 22
Tanks Infected	9	15
Avg # Fish/Family	8.3 (5-10)	13.8 (10-15)
Avg Weight	295.1	210.3
Avg # Lice/Fish	21.1 (8.1-60)	52.4 (32.4-114.6)
Lice/cm2	0.05	0.22
Lice Density	0.52	1.55
Heritability	0.19 (0.108)	0.17 (0.098)

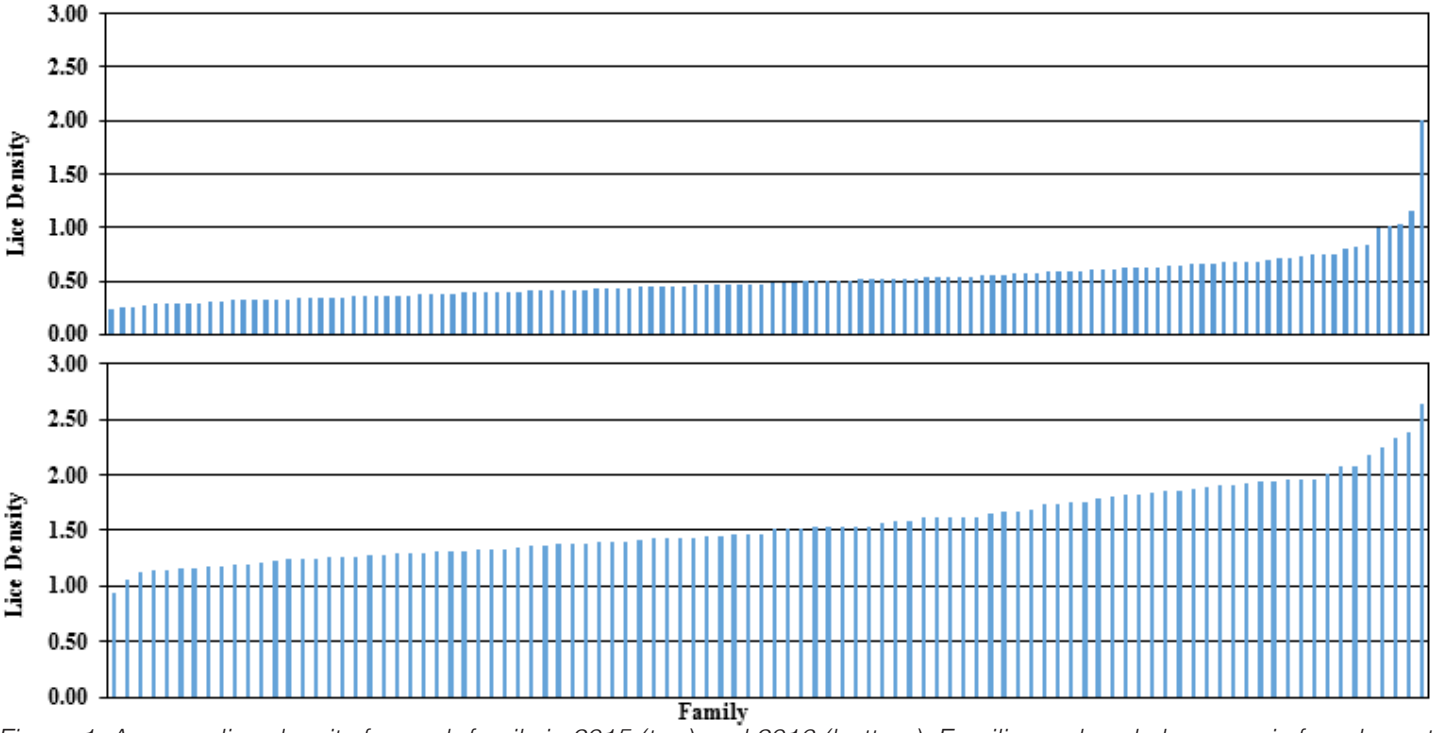


Figure 1. Average lice density for each family in 2015 (top) and 2016 (bottom). Families ordered along x-axis from lowest to highest lice density.

Statistical Analysis of Phenotypes

Three body size traits (total, carcass, and fillet weights) and sea lice data (lice density) were analyzed with an animal model including fixed effects (stock, sex, stage of sexual maturity, and replicate culture tank), random animal genetic, and residual effects. Estimates of variance components that maximize the restricted likelihood (REML) were obtained with a derivative-free algorithm using the MTDFREML programs (Boldman and Van Vleck 1991; Boldman et al. 1995). The parents of the families were assumed to be unrelated. Heritabilities, genetic and environmental correlations were estimated from the components of variance and covariance. The (co)variances were estimated from bivariate analyses of pairs of traits. The REML estimates were used to obtain Best Linear Unbiased Predictors (BLUP) of individual breeding values for carcass weight.

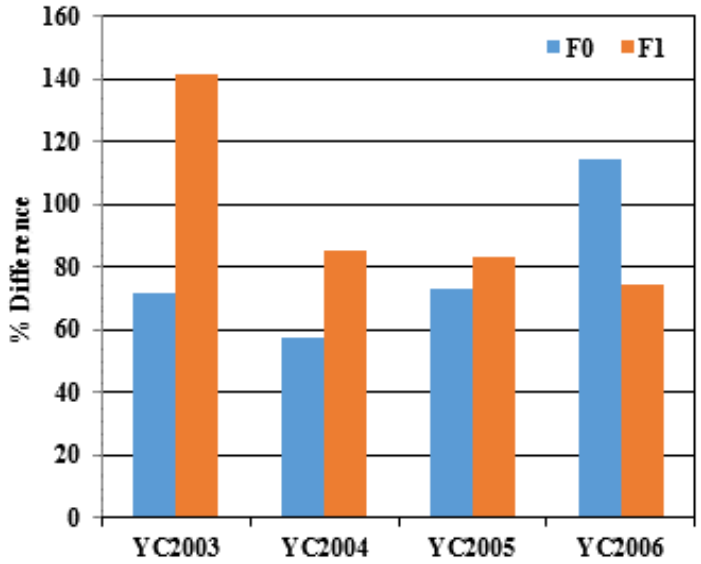
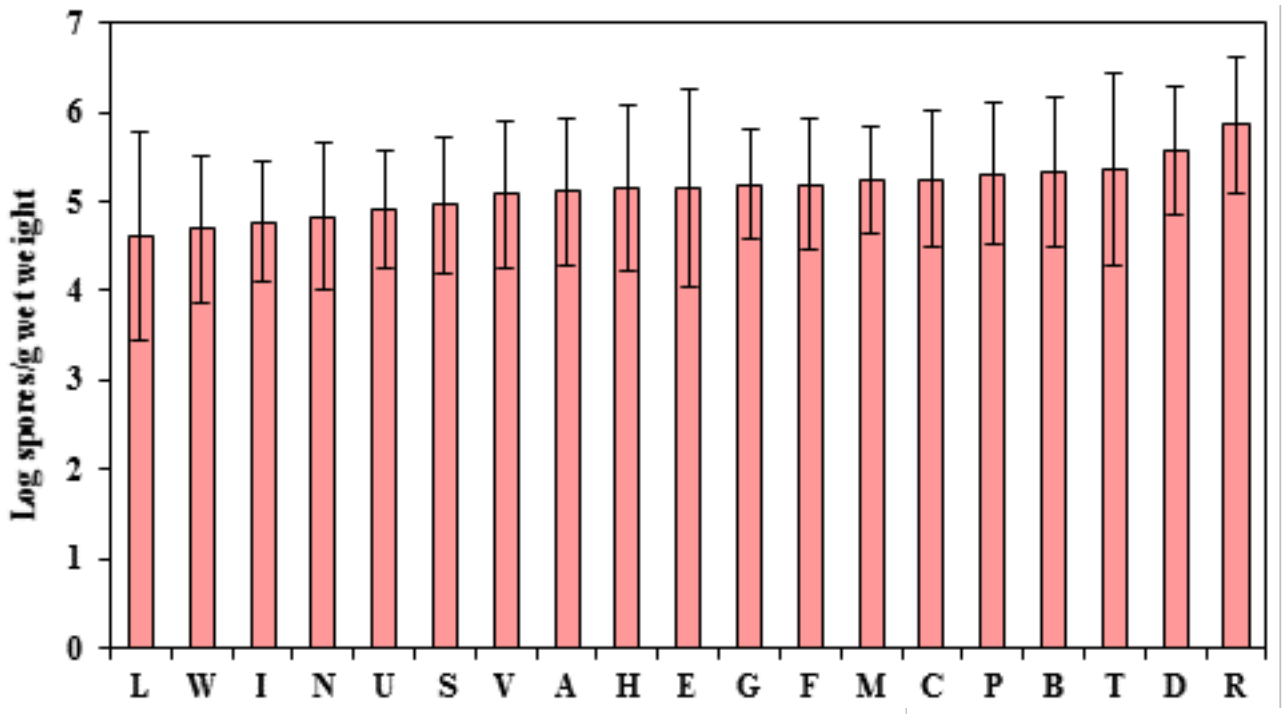


Figure 2. Percent difference in weight between the selected strain.

RESULTS AND DISCUSSION

Sea lice challenges were added to the breeding program at the request of industry. The average sea lice density was highly correlated with total sea lice count and negatively correlated with body weight. Total lice count were positively correlated with lice density and body weight (Table 1). The estimates of heritabilities for sea lice resistance ranged from 0.11 ± 0.05 to 0.14 ± 0.06, which is similar to what has previously been observed for natural infection challenges (Glover et al. 2005; Kolstad et al. 2005) but lower than observed for tank infections (Kolstad et al. 2005; Gjerde et al. 2011). The natural infection challenges recorded both motile and sessile stages which could result is different heritability estimates (Glover et al. 2005; Kolstad et al. 2005). Gjerde et al. (2011) only counted sessile sea lice and had a lower initial infection rate compared to our studies (74 or 36 compared to 100 for our trails). In later trials (Table 2, Fig. 1), we observed more uniform infection of the fish and a higher estimated heritabilities. The calculated heritabilities were 0.17 ± 0.11 in 2015 and 0.19 ± 0.10 in 2016. We will continue to evaluate sea lice resistance for families in each year class.



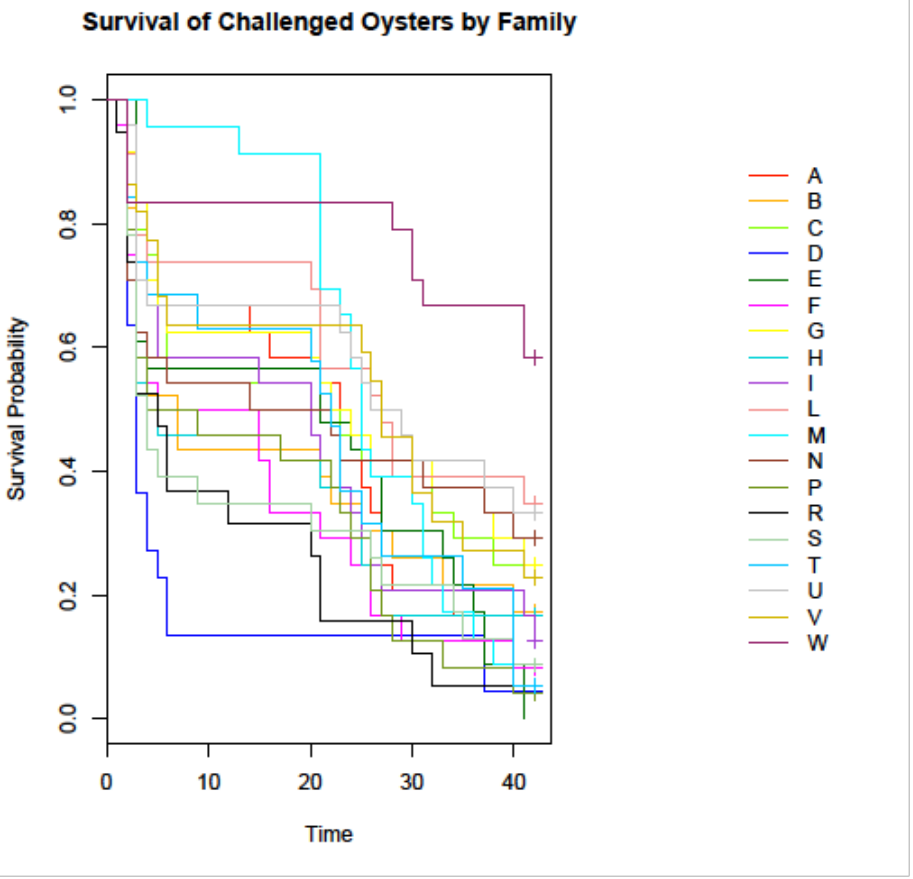


Figure 3. Dermo mean parasite load by family (top) and survival over time (days) of challenged oysters by family (bottom).

Growth rates are also being evaluated in the selected strain of North American Atlantic salmon with assistance of industry partners. Currently, we are using a wild unselected strain as a control, and we are detecting increases in the selected strain ranging from 57% to 141% over the unselected strain grown under the same conditions (Fig. 2). Gjedrem (2005) and Gjedrem and Baranski (2009) estimated that an Atlantic salmon breeding program should be able to produce fish with increased growth by around 15% each generation. We are able to obtain similar gain per generation (Bill Wolters personal communication). We are currently adding fillet pigment content (color), fillet fatty acid content (EPA and DHA levels), and fillet lipid levels to the breeding program. The breeding program at the NCWMAC will soon be adding genomic breeding values to the EBVs that are currently in use to further enhance selection for desirable traits.

The NCWMAC oyster breeding program is currently evaluating five different oyster stocks for resistance to Dermo. Oysters that were susceptible to the disease had the highest parasite load, while resistant families had the lowest parasite load (Fig. 3). Oysters appear to be able to control their exposure to the parasite by reducing feeding rates (Fig. 4). However, oysters that are able to reduce their exposure to the parasite are not the best families at resisting or tolerating the infection. In the future, oysters that are resistant to disease through phenotypic trials will be used to identify genes associated with resistance, tolerance

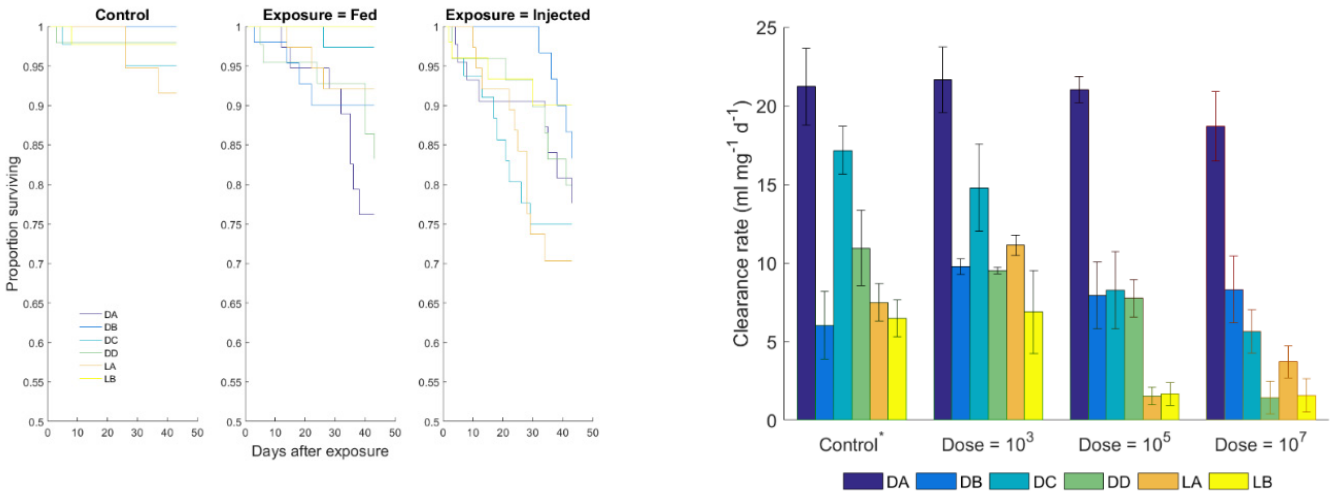


Figure 4. Modes of Dermo resistance by families in the Eastern oyster breeding program.

and susceptibility to Dermo. This should allow the development of genetic markers to assist with selection similar to the Atlantic salmon breeding program.

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Gjedrem, T. (ed.). 2005. Selection and breeding programs in Aquaculture. Springer, Dordecht, The Netherlands, 364 pp.

Gjedrem, T., and M. Baranski. 2009. Selective breeding in aquaculture: an introduction. Reviews: Methods and Technologies in Fish Biology and Fisheries 10, Springer, Dordecht, The Netherlands.

The books referenced above describe selective breeding programs for a wide range of cultured species. The editor and many authors are from Norway, which has

the longest running Atlantic salmon selective breeding program in the world.

Gjerde, B., J. Odegard, and I. Thorland. 2011. Estimates of genetic variation in the susceptibility of Atlantic salmon (*Salmo salar*) to the salmon louse *Lepeophtheirus salmonis*. Aquaculture 314: 66-72.

The paper above provides a brief overview of previous research looking at the heritability of sea lice resistance, while importantly demonstrating the need to normalize lice infection counts to a factor, such as lice density, that accounts for fish size at the time of infection.

Proestou, D.A., B.T. Vinyard, R.J. Corbett, J. Piesz, S.K. Allen, J.M. Small, C. Li, M. Liu, G. DeBrosse, X. Guo, and P. Rawson. 2016. Performance of selectively-bred lines of eastern oyster, *Crassostrea virginica*, across eastern U.S. estuaries. Aquaculture 464: 17-27.

The article referenced above looked at the performance of selectively-bred lines of eastern oysters in five representative grow-out sites and detected significant effects of selection and large and significant genotype by environment interactions. This information will be useful in evaluating current breeding strategies for the eastern oyster.

Ermgassen, P. S. E., M. D. Spalding, B. Blake, L. D. Coen, B. Dumbauld, S. Geiger, J. H. Grabowski, R. Grizzle, M. Luckenbach, K. McGraw, W. Rodney, J. L. Ruesink, S. P. Powers, and R. Brumbaugh. 2012. Historical ecology with real numbers: past and present extent and biomass of an imperiled estuarine habitat. Proceedings of the Royal Society of London B: Biological Sciences. 279: 3393-3400.

The article referenced above analyzed historical shellfish landing and biomass data along the Eastern U.S. coast and correlated it to various anthropogenic stressors including overexploitation, habitat degradation, climate change and the resultant spread of disease.

The Molluscan Breeding Program: 20 Years of Selective Breeding of the Pacific Oyster, *Crassostrea gigas*, on the West Coast of the U.S.

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Corresponding author: Not available.

Keywords: Not available.

ABSTRACT

The Molluscan Broodstock Program (MBP) was initiated in 1996 by Oregon State University (OSU) and the U.S. Dept. of Agriculture with the goal of increasing the performance of Pacific oysters (*Crassostrea gigas*) for use in aquaculture on the West Coast, USA. MBP utilizes a family-based selection breeding design and was founded by broodstock collected from wild populations in Washington State and British Columbia, Canada. Breeding design and hatchery production of larvae and spat is undertaken by OSU at the Hatfield Marine Science Center in Newport, Oregon and families are planted out for field trait estimates at industry sponsored field sites along the West Coast. The selection process utilizes an index based on family performances for three traits of interest: family yield, average individual harvest weight and survival. Results of five generations of selective breeding show that substantial genetic gains have been attained. Realized gains are lower than expected, however, and we suspect this might be caused by competition for food in dense culture conditions and a changing climate, including intensified upwelling events, which bring deep, acidified, and nutrient-rich water to the surface. Heritability estimates have declined in the last two generations of the program, which corroborate these results and further suggest a changing environment. Significant genetic by environment (GxE) interactions have been detected between Puget Sound and coastal estuarine plant-out sites, indicating major environmental differences between the two types of sites. Taken together, these results are being used to develop modified breeding approaches for MBP, including a more careful indexing approach to take into account GxE interactions. Further considerations for MBP include breeding for new traits in response to emerging issues such as ocean acidification and diseases.

ANNOTATED BIBLIOGRAPHY OF KEY WORKS

Langdon, C., F. Evans, D. Jacobson, and M. Blouin. 2003. Yields of cultured Pacific oysters *Crassostrea gigas* Thunberg improved after one generation of selection. Aquaculture 220(1): 227-244.

This publication marks the first evaluation of the genetic gain obtained by the MBP after one generation of selection. The authors demonstrate significant improvement in yields of selected lines compared to unselected controls. However, they found only weak correlation between family performances in intertidal and subtidal environments, which indicated possible interactions between family performance and environment.

Evans, F., S. Matson, J. Brake, and C. Langdon. 2004. The effects of inbreeding on performance traits of adult Pacific oysters (*Crassostrea gigas*). Aquaculture 230(1): 89-98.

The effect of inbreeding was evaluated in the context of a commercial growing operation by measuring survival, family yield, and individual growth after one or two growing season. Results showed that families with a high (F = 0.2) inbreeding coefficient performed worse than families with a low (F = 0.006) inbreeding coefficient, and this effect was stronger after two growing seasons. The result called for a strict monitoring of inbreeding in a selective broodstock program, such as the MBP.

Melo, C. M. R., E. Durland, and C. Langdon. 2016. Improvements in desirable traits of the Pacific oyster, *Crassostrea gigas*, as a result of five generations of selection on the West Coast, USA. Aquaculture 460: 105-115.

Authors evaluated the heritability, genetic correlations, and genetic gain for survival, individual weight, and yield over five generations of selective breeding in the MBP. Substantial genetic gain was observed throughout generations except for individual weight, where gains were limited after the second generation. Realized gains were less than expected, however, given the genetic gain, perhaps due to poor control over the selection of broodstock for controlled crosses. Heritabilities seemed to decline in the 4th and 5th generations, and the authors suspect this was caused by changing environmental conditions, such as the intensification of upwelling events due to climate change.

Seriola Genomics and the Knowledge Repository serioladb.org

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Keywords: aquaculture, *Seriola dorsalis*, genome browser, sex determination

ABSTRACT

Due to the high value of *Seriola* in the seafood industry, *Seriola* aquaculture is of great interest in the U.S. and elsewhere. Hatchery production of *Seriola* species is accelerating but has been hindered by a propensity for deformities and growth heterogeneity developed during larval and early juvenile stages that limits production capacity and efficiency.

To advance *Seriola* aquaculture, we are developing genomic resources for *Seriola dorsalis* and *Seriola rivoliana*. The starting point for this resource includes a draft genome assembly with gene annotations for *S. dorsalis.* In addition, we have resequenced 90 fish from three locations (Cedros Island, La Paz, and San Diego) and generated an F2 population to explore the genetic component in malformations in juvenile fish (swim bladder inflation, several head and jaw deformities and malformations in the operculum and branchiostegal structures).

To consolidate this data into a single knowledge repository, we are creating an international web resource, serioladb.org. As yellowtail culture grows, the genetic tools on the website will be made available to producers and other groups working to improve culture for this and other *Seriola* species. This research may also serve as a model for genetic resource development for aquaculture species or conservation dependent species with limited genomic resources and may help reveal genomic regions and genes contributing to trait variation in wild populations.

INTRODUCTION

Aquaculture production is becoming increasingly important to global food supplies. Amberjacks or yellowtail are fishes of the genus *Seriola* (*S. dorsalis, S. dumerili, S. lalandi, S. rivoliana, S. quinqueradiata*). Japan has been very successful with the culture and production of *S. quinqueradiata* and *S. dumerili* as part of the high valued sashimi industry prominent in that region and globally. More recently, *S. dorsalis* and *S. rivoliana* have been considered prime candidates for aquaculture development in Southern California and Hawaii, respectively.

Agriculture and livestock production went through a genomic revolution in the first decade of the new century shortly after the sequencing of the human genome in 2001 (Lander et al. 2001) as observed by the sequencing of 40 or so agriculture species (Michael and VanBuren 2015) and the five major livestock species (cow, pig, sheep, horse, and chicken) by 2012 (Bai et al. 2012). Aquaculture is now similarly going through its own genomic revolution with the sequencing of (rainbow trout, sea bass, Atlantic salmon, tilapia, catfish, flounder, and Atlantic cod) (Palti et al. 2011; Terova et al. 2014; Spivakov et al. 2014). It is now possible for small groups as opposed to large consortia to create genomic resources to improve production of a species in agriculture, livestock and aquaculture. These resources can be used to gain an understanding of the genetic basis of traits that currently limit/enhance development of domestic aquaculture (Rondeau et al. 2013). These resources can be used to identify the genetic element responsible for traits of economic importance such as disease resistance, growth rate, tolerance of environmental stressors, diet/nutrition, reproduction, and general health (Fuji et al. 2010; Huete-Pérez and Quezada 2013; Ozaki et al. 2013; Spivakov et al. 2014). High throughput sequencing that includes a combination of second generation Illumina/PacBio, third generation NanoPore technologies and super-long distance information library preparation methods (Dovetail Chicago and HiC) makes it possible for rapid application of these sequencing technologies and provides advancements in broodstock selection and sustainable *Seriola* aquaculture.

In this study, we describe preliminary data from the genome assembly project of *Seriola dorsalis* and the application of resequencing of 90 *Seriola dorsalis* fish to identify the Sex Determining Region (SDR). We

compare our progress with preliminary data from ongoing assembly projects of *Seriola quinqueradiata* and *Seriola dumerili* with our Japanese collaborators. Given the relative ease at which genomic data can now be generated, we also describe the need for and implementation of a better genomic resource to manage these data by groups collaborating across the world.

METHODS

DNA from a juvenile *S. dorsalis* (50 days post-hatching) was extracted from an individual from Hubbs-SeaWorld Research Institute (San Diego, CA), which was humanely euthanized by overdose with MS-222. The tissue was placed immediately into Invitrogen™ RNA*later*® Stabilization Solution (ThermoFisher Scientific, Waltham, MA, USA), stored for 24 hours at 4°C and then frozen at -20°C until DNA extraction.

Several hundred wild *Seriola dorsalis* fish were collected via hook and line by either private sport anglers or commercial/subsistence fishers aboard various fishing vessels from San Diego, California; Cedros Island, Mexico; and La Paz, Mexico. Sex phenotype was determined by scientific observers examining gonadal tissue. Muscle, fin, or gonadal tissue was placed in 100% ethanol and DNA from 90 samples was extracted (15 of each sex from each of the three locations for a total of 45 male and 45 female specimens).

The DNeasy Blood and Tissue Kit (Qiagen, Hilden, GER) was used to extract DNA from all samples following the manufacturer's protocol. Heart and spleen tissue was used from the specimen undergoing genomic sequencing. Muscle, fin, or gonadal tissue was used for the 90 specimens undergoing resequencing. Samples prepared for the genomic and re-sequencing applications were sent to the DNA Sequencing Facility at Iowa State University (Ames, Iowa) for library preparation and sequencing.

MaSuRCA assembler (version 2.3.2) (Zimin et al. 2013) was used to assemble the raw data. Scaffolds were filtered with the following parameters: scaffolds were removed when they contained fewer than 800 bases or when 90% of the total scaffold length was contained in a different larger scaffold. The scaffolds must also contain a gene or be larger than 10,000 bases. BRAKER (Nanda et al. 2002; Hoff et al. 2015)England was used to annotate the genome using 547 million 50 bp PE raw RNA-Seq data from a related project.

Raw DNA resequencing reads were aligned to the assembled genome using BWA-MEM (Li 2013). SNPs and InDels were called using GATK (McKenna et al. 2010). A genome-wide association study (GWAS) using a Generalized Linear Model (GLM) was performed in TASSEL (version 5) (Bradbury et al. 2007). TASSEL identified the sex-determining region (scaffold 22 between 231,000 and 420,000bp).

All insertions/deletions (InDels) greater than 40 nucleotides in the SDR were identified. One 61-nucleotide deletion on scaffold 22, starting at base position 246,495 on scaffold 22, was identified as heterozygous in females and homozygous in males. Two sets of primers, SdorDel01 and SdorDel02, were designed to span across the deletion using Primer3 (Untergasser et al. 2012). PCR products were visualized using a 2% agarose gel electrophoresis with 100bp size standard markers (ThermoFisher Scientific, Waltham, MA, USA) run at 78V for 40 and 60 minutes, for SDorDel02 and SDorDel01, respectively.

Genome statistics from draft genome assemblies for *Seriola quinqeradiata* and *Seriola dumerili* were obtained through a personal communication with Ozaki. Genome papers for these species are currently in progress; however, a draft of the *S. dumerili* genome was used in an initial exploration of synteny between *S. dorsalis* and *S. dumerili*. Opscan (0.1) (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3961402/), which uses fastp alignments and the Needleman-Wunsch-Sellers Algorithm to calculate orthologous gene families was used to determine the synteny. All alternatively spliced variants and all possible multi-family genes were considered. To infer synteny, iAdHoRe (3.0.01) (https://www.ncbi.nlm.nih.gov/pubmed/17947255?dopt=Abstract) was used with a prob_cutoff=0.001, level two multiplicons only, gap_size=15, cluster_gap=20, q_value=0.9, and a minimum of three anchor points. Syntenic regions are displayed using Circos (0.69.2) (http://genome.cshlp.org/content/early/2009/06/15/gr.092759.109.abstract).

We are in the process of creating a genomics toolbox and web resource that will facilitate the exploration and integration of large sequencing data sets. Website content will be generated using the Drupal Luggage Suite, which has form-based entry and was developed here at ISU. The website will include JBrowse (Skinner et al. 2009), a Genome Browser for visualization of data aligned to the *Seriola* genome and InterMine (Smith et al. 2012; Lyne et al. 2013), an open source data warehouse that enables researchers to explore biological data.

Table 1. Comparison of assembly quality statistics for three Seriola *species.*

	S. dorsalis	***S. dumerili***	***S. quinqeradiata***
Scaffolds	4,439	1,341	3,902
N50	1.5 Mb	5.8 Mb	0.8 Mb
Nucleotide content	653 Mb	671 Mb	844 Mb
Genes	45,581	47,313	in progress

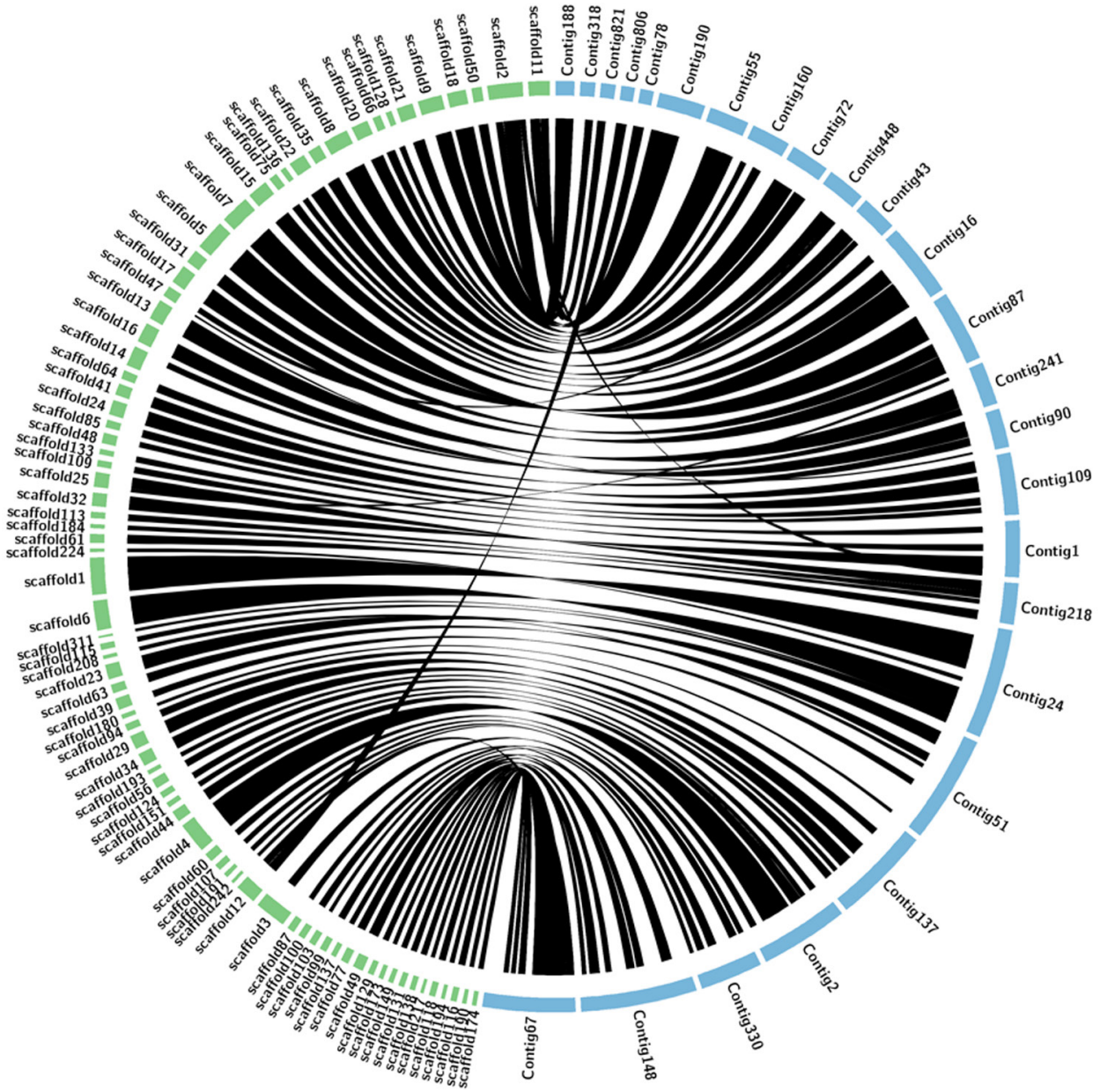

Figure 1. Circos plot comparing the largest scaffolds of Seriola dorsalis *(green, on the left) with the corresponding syntenic scaffolds in* Seriola dumerili *(blue, on the right). Significant synteny is observed with only a couple potential chromosomal rearrangements.*

RESULTS AND DISCUSSION

High throughput sequencing has become a powerful tool in developing genomic resources (Taylor 2014) (Dunham et al. 2014), and these data are being used to begin developing genomic resources for several *Seriola* species. Together, we and our collaborators at the Japan Fisheries Research and Education Agency have three *Seriola* genomes in varying stages of assembly and publication (Table 1). We can see that the quality of these preliminary assemblies based on the number of scaffolds between these species are very similar 4,439; 1,341; and 3,902 for *S. dorsalis, S. dumerili* and *S. quinqeradiata*, respectively. N50 is another measure of the assembly quality. The sum of all scaffolds N50 size and smaller equals half the total nucleotides in the assembly. N50 for *S. dorsalis, S. dumerili* and *S. quinqeradiata* is 1.5 Mb, 5.8 Mb and 0.8 Mb, respectively. The *S. dumerili* preliminary assembly is better due to additional long read data that was collected for that assembly from PacBio (Menlo Park, CA, USA). All future assemblies will include both PacBio and super long-range information from either Dovetail Chicago libraries or genetic maps that should bring these assemblies close to complete chromosomal level assemblies. Comparisons between *S. dorsalis* and *S. dumerili* reveal a great deal of synteny between these species. As more *Seriola* species genomes become available and researchers perform Genome Wide Association Studies for specific traits of interest (disease resistance, growth rate, tolerance of environmental stressors, diet/nutrition, reproduction,

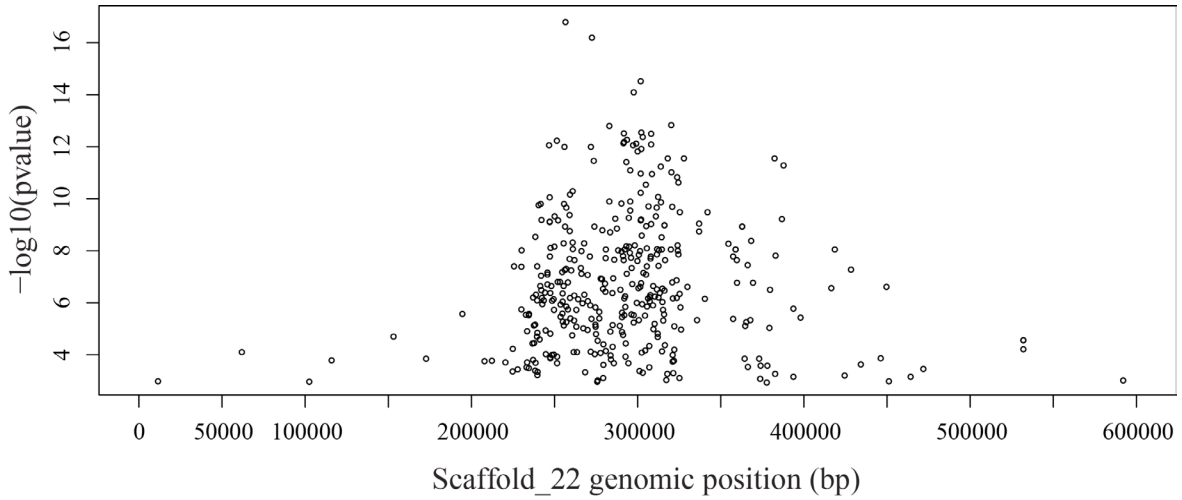

Figure 2. Graph of negative log 10 p-values GWAS output from TASSEL plotted across a portion of scaffold 22 in the Seriola dorsalis genome. The figure shows many SNPs in linkage disequilibrium with significant correlation to the sex phenotype.

and general health), there will be great potential to translate these findings among species (Fig. 1).

The assembled *S. dorsalis* genome was used to align resequencing data from the sexed 90 wild-caught fish. The Sex Determining Region (SDR) was identified along with a sex-specific marker based on a 61 nucleotide deletion (Fig. 2). The identified SDR in *S. dorsalis* is consistent with the known sex marker in *S. quinqueradiata (*ssr263g21) (Koyama et al. 2015), which maps just upstream of this region: scaffold 22 at 194817-194840 base pairs (linkage group 12; Fuji *et al.* 2010). However, the marker reported in that study does not distinguish sex in other *Seriola* species (Ozaki, pers. com) and lies just outside of the SDR. Interestingly, markers we have identified for sex determination did not work in a small number of *S. rivoliana* specimens, although the effectiveness in other *Seriola* species has not yet been tested.

With the collection of vast quantities of sequencing data comes the responsibility to make this data available to the public. This poses challenges since funding and personnel to curate genomic web

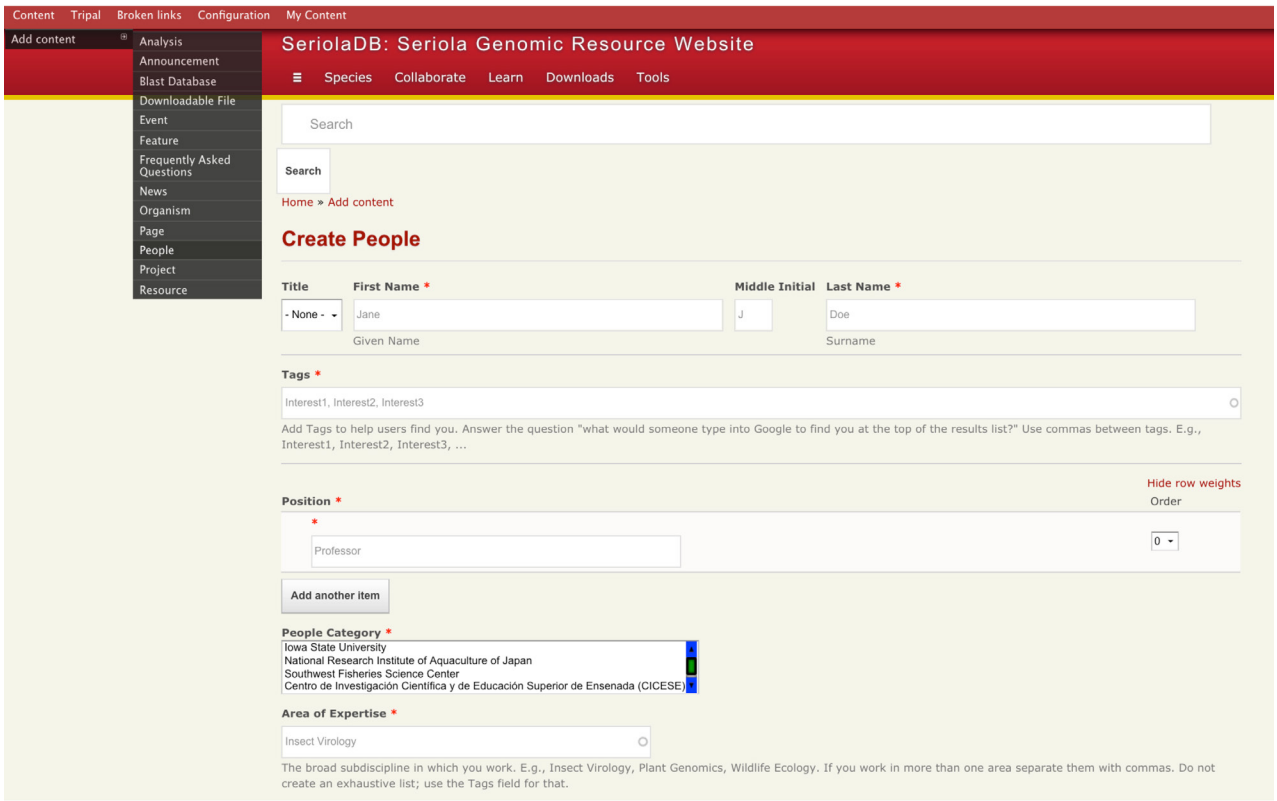

Figure 3. Form-based page creation and data deposition ensures the website is easy to maintain, build and search.

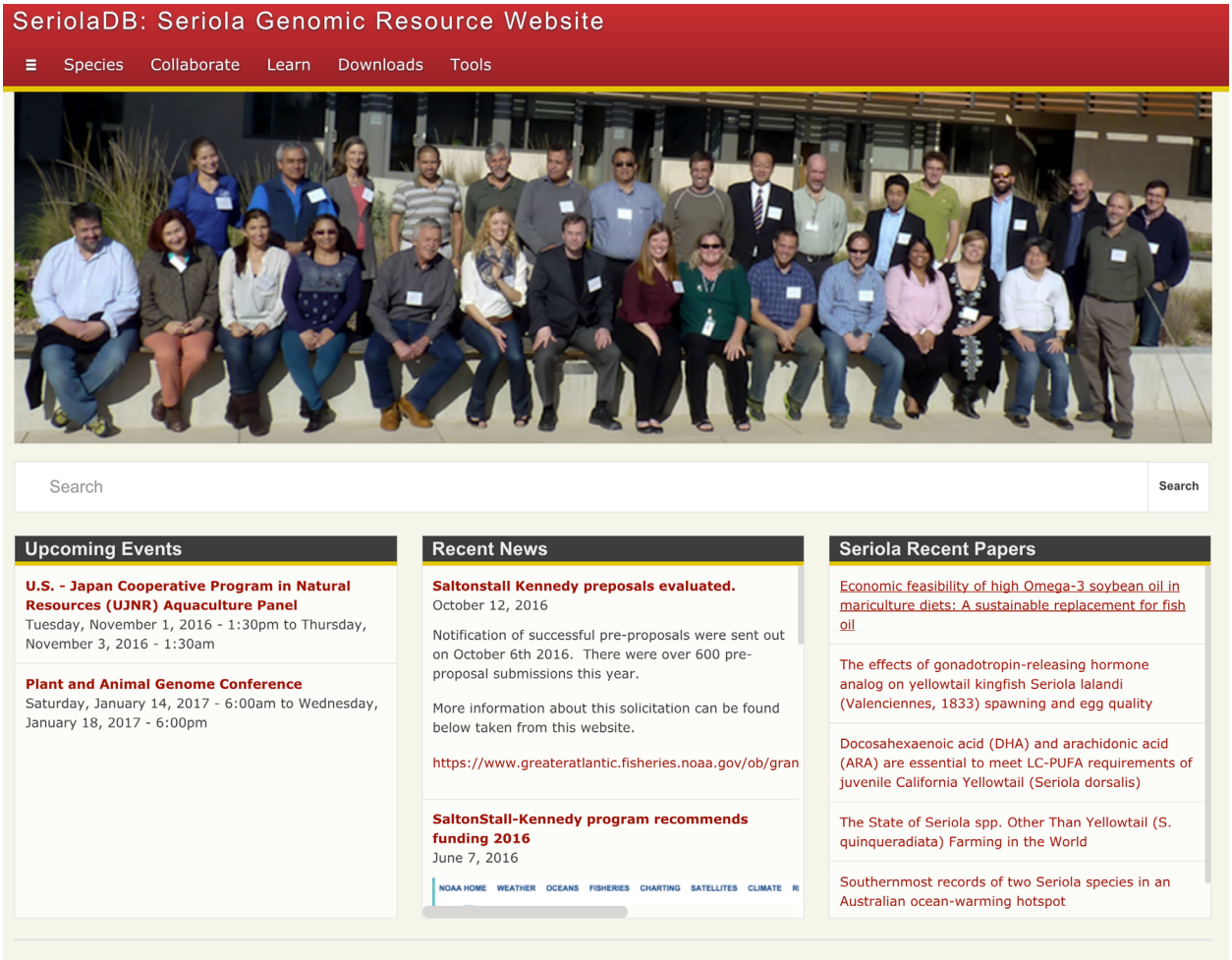

Figure 4. Professional looking main page of Serioladb.that was created using the Drupal Luggage suite.

resources are limited and the ability to sequence a species increasingly becomes easier. In an attempt to overcome these challenges, a form-based web-content generating approach will be adopted for Serioladb.org where multiple groups working on *Seriola* species from across the world can collaboratively curate the web resource to add their data and knowledge. By using forms (Fig. 3), web content creation will be user friendly with a uniform format for generated content that looks professional (Fig. 4). The content and organization of the website will be specific to each genomic resource and the collaborating group maintaining it. However, there are several curation tasks that can and should be automated such as the creation of files for download and tracks to be visualized in the genome browser. While we are still in the process of figuring out how to make it easier to collaborate on a shared genomic resource, we are excited about how Serioladb.org and similar genomic resources will advance sustainable aquaculture.

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Authors sampled 42 *Seriola lalandi* from South Africa, Japan, New Zealand, California, Mexico Pacific, Mexico Gulf and Chile to better understand regional populations and evolutionary patterns. Two mitochondrial genes (CR and COI) and four nuclear genes (RAG2, EHHADH, UBE3A, and MLL) were used to generate a phylogenetic tree with *Seriola dumerili* as an outgroup. Based on this data, it was concluded that fish currently named as *Seriola lalandi* in fact comprise three species. The authors propose that *Seriola lalandi* in the Northwest Pacific revert to the name *Seriola aureovittata* and the Northeast pacific fish should revert to the name *Seriola dorsalis* while the fish in the southern hemisphere should remain as *Seriola lalandi*.

Westesson, O., M. Skinner, and I. Holmes. 2012. Visualizing next-generation sequencing data with JBrowse. Briefings in Bioinformatics 14(2): 172-177.

The authors describe a web-based browser for displaying genetic sequencing data at the nucleotide level. JBrowse is the successor to the very successful GBrowse genome browser. The most significant advance is the user-side processing of tracks for visualization, which allows for greater scalability without significant investment on the server side. Other improvements include Google maps-style browser interface with smooth transitions. JBrowse is actively maintained and can be downloaded from https://github.com/GMOD/jbrowse. JBrowse is an important tool for the visualization of high throughput sequencing data and should be included in any aquaculture genomic resources toolkit.

Ficklin, S. P., L. A. Sanderson, C. H. Cheng, M. E. Staton, T. Lee, I. H. Cho, S. Jung, K. E. Bett, and D. Main. 2011. Tripal: a construction toolkit for online genome databases. Database 2011: bar044.

In this work, the authors describe web based content management system named Tripal. Once a research group starts to accumulate high throughput sequencing data such as a genome assembly, annotation, SNPs, a genetic map, and epigenetic data etc, Tripal provides a web interface that organizes this data in a database for easy display on web pages. It is widely used by model organisms (FlyBase, WormBase, Genome Database for Rosaceae, TreeGenes and so on). The Chado database that serves to store and distribute the data to the webpages can also be used by JBrowse to visualize genomic data.

Uses of Genetic Parentage Analysis in Cultured California Yellowtail (*Seriola dorsalis*)

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Keywords: microsatellites, egg quality, aquaculture, yellowtail, *Seriola*

ABSTRACT

The genus *Seriola* is an important group of cultured species representing a more than USD$1.5 million industry. With the demand for seafood continually rising, this popular group of fishes is a prime candidate for culture, thus supplementing supply and relieving harvest pressure on wild populations. In a culture setting, we have the ability to obtain a complete set of genetic markers for the brood stock, allowing for determination of the parental contribution to each spawning event. In order to maximize output and quality of offspring, we explored the various usages of genetic parentage data in a cultured population of the California yellowtail, *Seriola dorsalis*. It was determined that parentage was informative in not only allowing for an estimate of parental contribution numerically but was also able to provide linkages to egg quality and may provide linkages to the presence/absence of deformities in offspring.

INTRODUCTION

As harvest rates from capture fisheries have begun level off, the demand for seafood and fishery products continues to increase (FAO 2012). To satisfy this demand, aquaculture has become increasingly important on a global scale, making it one of the fastest-growing animal food-producing sectors, increasing at an average annual rate of 8.8 % since the 1980s (FAO 2012). The number of species in culture and the number of locations using culture production has risen substantially in the past two decades (Fraser et al. 2010; Lorenzen et al. 2012). Although aquaculture is seen as a way to reduce fishing pressure on natural populations, it also allows for the introduction of novel species to the market.

Among the numerous species of finfish that are produced at scales that are relevant to market demand are jacks of the genus *Seriola* (Carangidae). Among all sectors of the fishery, the estimated global market value of *Seriola* is $1.5 billion USD. *Seriola* is a coastal pelagic genus with species ranging globally in all but Polar Regions. All species of *Seriola* are important components of coastal ecosystems and are favored species for recreational and commercial fishing. With the rising practice of aquaculture, members of the genus *Seriola* have also become a practical and cost-effective culture species. Three species of *Seriola* are primarily cultured for large-scale production: *Seriola dumerili*, *Seriola lalandi sensu lato*, and *Seriola quinquradiata*.

The yellowtail jack (*Seriola lalandi sensu lato*; Fig. 1) was thought to be a single, cosmopolitan species with a disjunct distribution. Yellowtail jacks are now considered to be a complex of at least three morphologically similar species: *S. aureovittata* (western Pacific), *S. dorsalis* (eastern Pacific), and *S. lalandi* (southern hemisphere; Martinez-Takeshita et al., 2015). These species have been commercially cultured in Japan for decades with production having recently spread to Australia, New Zealand, Chile, and Mexico. Yellowtail are also likely to become an

Figure 1. Photograph of Seriola dorsalis *taken in California. (Photograph by B. Gratwicke. Reproduced without changes under Creative Commons License 2.0 https://creativecommons.org/licenses/by/2.0/legalcode. Source: https://www.flickr.com/photos/briangratwicke/5625034070/in/photostream/, 14 December 2016.)*

important aquaculture fish in Southern California within the next few years, where it is also the target of commercial and recreational fisheries.

Since 2013, researchers at the NOAA NMFS Southwest Fisheries Science Center (SWFSC) have been working in collaboration with researchers at the Hubbs Sea World Research Institute (HSWRI) to evaluate the genetic architecture of the HSWRI California yellowtail production system. In particular, we were interested in determining the spawning dynamics of this system. That is, we questioned how successful spawning events were distributed among the broodstock population. We therefore embarked on a study of parentage, examining the genetic signatures of larvae and matching them to the most likely female and male brood fish. With this information, important lessons could be learned about spawning dynamics and quality of production runs from individual parent pairs. This report documents some of the uses of genetic parentage analysis in California yellowtail (CYT).

METHODS

The HSWRI CYT Production System

Hubbs Sea World Research Institute is located on Mission Bay in San Diego, California. The primary CYT broodstock were collected from the wild off the coast of Southern California and are housed in a single, 140 m^3 flow-through tank. Ambient water is extracted from the adjacent Mission Bay, filtered, and the flow rate allows for 3-6 turnovers per day. The tank is open to natural light cycling. More information on the CYT production system may be found in Stuart and Drawbridge (2013). Spawning occurs during the normal spawning season for wild fish and the natural broadcast spawning of CYT occurs several times from March through September. The CYT broodstock has consisted of varying numbers of male and females from 2013-2016 (Table 1). Fertilized eggs are collected and reared under controlled conditions.

Table 1. Number of male and female California yellowtail broodstock in the Hubbs-Sea World Research Institute culture facility, 2013-2016.

Sex	2013	2014	2015	2016
Male	11	19	19	18
Female	8	18	18	13
Total	19	37	37	31

Genetic Methods

From each spawning event, a random sample of 47 yolk-sac larvae zero days post hatch were sampled for genetic analysis. DNA was extracted from whole larvae using a 10% Chelex resin solution incubated at 60°C for 20 minutes followed by 103°C for 25 minutes. Nine microsatellite loci (Table 2) were used to create a genetic profile for each larva that could then be matched to previously determined genetic profiles of the broodstock (Smith, 2015; Craig, M. unpublished data). The polymerase chain reaction (PCR) was used to amplify the nine loci. PCR reactions consisted of the following in 11 µl volumes: 10X PCR Buffer (670mM Tris, 166mM $(NH_4)_2SO_4$, 20mM $MgCl_2$, 100mM 2-mercaptoethanol, pH=8.8), 2 mM each dNTP, 5 mg bovine serum albumin (BSA), 10 mM of each primer, 1 unit of *Taq* polymerase, and ~10 µg sample DNA. Thermal cycling conditions for each reaction were: 94°C for 4 minutes, followed by 35 cycles of 94°C for 30 seconds, annealing (53-59°C; Table 2) 35 seconds, 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. Two multiplex reactions (four loci each) were performed for eight of the loci and the ninth was run independently (Table 2). Forward primers for all PCRs were marked with fluorescent labels for genotyping on an ABI 3730 genetic analyzer using 1/200 diluted PCR products and HiDi/Rox size standard.

Genotypes were scored using GeneMapper v5.0 (company). Allele calls were standardized to whole numbers and parentage was assessed using Cervus v. 3.0.6. The most likely parent pair was determined using combined LOD scores at 80 % (relaxed) and 95% (strict) confidence intervals with a minimum of five loci scored per individual.

Table 2. PCR primers used for California yellowtail microsatellite parentage analysis.

Locus	Forward	Reverse	Annealing Temp (C)	Approx. Fragment Length (bp)
SEQU38F	/56-TAMN/CCATTACAATTTGTCTCTC	CTTATCAACACACGAGCG	53	100-145
SEQU77F	/5hex/CCTACACATGCACATGAA	CAAGGCTGATACGTCATG	55	135-190
SDUGA3DF	/56-fam/CTCAACATGAGAGGCAACG	GCATGGCTTCATGGGAAGG	55	140-180
SDU46F	/56-TAMN/GCAGTGTGAGCCATACATTAC	CTACAGGACAAAAGCCATT	55	220-260
SDU4F	/56-fam/GGAAATAGTTTGGATCACGCTGG	GGATGCTCAGTGAAGTTGTGC	55	270-310
SEQU320F	/5hex/GACAGGGTAAGAAACGAAAC	GACAATGACCAAAGCTGCC	59	90-140
SEQU230F	/56-fam/CTCCAGAAACGCCACATAAC	AAGCAAACCGCACAAGTAGG	59	150-165
SDU10F	/56-TAMN/CCAAGTCCTCCGCTACTACCAT	CCTTGTGGATGACCTGTTTG	59	250-310
SDN06F	/56-fam/GGGTTTGTGCTGTGAGTG	TCCGTCTGTCTTTTCCTGT	59	300-330

RESULTS

From 2013-2015, we genotyped 3170 from 68 spawns, 2789 from 50 spawns, and 3275 larvae from 70 spawns, respectively. Parentage was assigned for 100% of all larvae at the strict (95%) confidence level. In all years, one large female (tag number 083-027-609) contributed disproportionally high numbers of offspring for each spawning event, with additional females contributing far less until 2015. In all three years, males contributed roughly equally per spawning event.

DISCUSSION

The parentage analyses allowed for a careful evaluation of several important factors in the spawning dynamics for captive CYT at HSWRI. In particular, we were able to assess proportional contributions by individual broodstock fish. This allowed for comparisons of two key factors in the production system: egg quality for each female and proportion of deformities resulting from individual parents and parental pairs.

Individual Parental Contribution

In 2013, the broodstock population consisted of eight females and 11 males (Table 1). In 2014, an additional 10 females and eight males were added to the population. In all three years, one large female produced ~40% of all eggs produced, far out producing other females in the system. This may be problematic in culture systems as any dominant producer may not be producing the highest quality offspring and a lack of genetic diversity in offspring may result in deleterious effects on future generations through inbreeding depression.

Egg Quality

A central concept in fisheries biology is that larger females produce more and better offspring. Often termed the BOFFF (Big, Old, Fat, Fecund, Female) hypothesis, this idea hinges on size as being a limiting factor for absolute number of eggs and female can produce, and that younger, smaller females may pay high energetic costs to produce high-quality eggs. Few studies have evaluated how this hypothesis for individuals reaching the end of their lifespan. Our results challenge this hypothesis and suggest that with senescence comes a reduction in egg quality.

In all three years examined, one female dominated the spawning events in terms of the number of larvae produced. While the number of offspring produced was proportionally higher than any other female, the quality of her eggs was among the lowest and declined each year. In 2013, the total annual fecundity for this individual was 1,181,577 eggs/kg/year, while in 2015 it had dropped to 423,157 egg/kg/year. More importantly, the viability of her eggs was alarmingly lower in 2015 than in 2013, dropping from 59.9 % to just 9.6%. The female was near the maximum size and age known for CYT, which may suggest that age is playing a large role in determining the quality of the eggs produced.

Offspring Deformities and Growth Rates

Another important aspect of culture production is ensuring that naturally occurring offspring deformities are not propagated in a system. Careful manipulation of broodstock populations can prevent this, and our assessment of parentage yielded some interesting results. Growth rates of larvae are often variable for each batch with larvae of the same age being quite different in size (Fig. 2). There are also several known deformities in CYT larvae produced in culture facilities, many of which include incomplete or abnormal formation of the operculum (Fig. 3).

We compared the proportion of abnormally large and small larvae produced by several female CYT in the HSWRI system to a random sample of all larvae. We found that two females produced abnormally high proportions of undersized offspring when compared with the overall population.

We also compared the frequency of offspring deformities from male broodstock and from various parental pairs. In the male population, two individuals produced disproportionately higher frequencies of all deformities relative to a random sample. We also found that, when paired with any female, the pair produced disproportionately high numbers of deformities.

Figure 2. Growth differences in captive bred California yellowtail at 31 days post hatch.

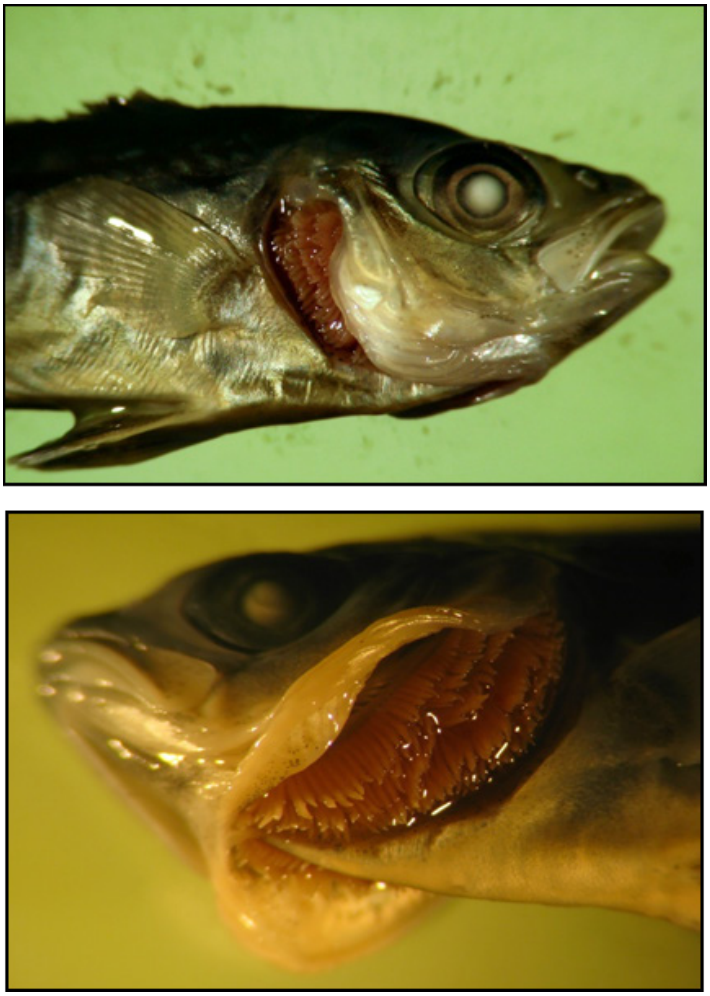

Figure 3. Opercular deformities in captive bred California yellowtail.

CONCLUSION

Knowledge of the dynamics of captive broodstock populations can inform culture practices in several ways. We showed that spawning by individual members with the CYT culture population at HSWRI is uneven. We also demonstrated that individual members of the broodstock population can contribute differentially to the number of offspring with sub-optimal growth rates or deformities. In response to this work, the female that was producing most of the eggs, although at low quality, was removed from the broodstock population. This manipulation of the broodstock based on examination of parental contribution to the offspring produced will ensure that quantity and quality are maximized in the HSWRI production system.

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Purcell, C., C. Chabot, M. Craig, N. Martinez-Takeshita, L. Allen, and J. Hyde. 2015. Developing a genetic baseline for the yellowtail amberjack species complex, *Seriola lalandi sensu lato*, to assess and preserve variation in wild populations of these globally important aquaculture species. Conservation Genetics 16(6): 1475-1488.

The authors present a global description of genetic variation in the *Seriola lalandi* species complex. Overall genetic differentiation among locations, as revealed by microsatellite data, was highly significant (FST = 0.085, DEST = 0.382, P < 0.001), and pairwise estimates of divergence derived from mitochondrial and microsatellite data support the presence of four significantly differentiated populations corresponding to the N.E. Pacific, N.W. Pacific, S. Pacific, and South Atlantic. Based on the genetic differentiation detected in this study, and recently published sequence data, these populations more accurately reflect the presence of at least three cryptic species of *Seriola*. Especially strong genetic differentiation between hemispheres indicates that the equatorial region is a significant dispersal barrier for yellowtail. This study represents the broadest geographic investigation of genetic population structure conducted, to date, for specimens of the *S. lalandi* complex.

Baskett, M., and R. Waples. 2013. Evaluating alternative strategies for minimizing unintended fitness consequences of cultured individuals on wild populations. Conservation Biology 27(1): 83-94.

The authors discuss important considerations for the maintenance of adaptively positive traits in cultured populations in order to minimize impact on wild populations due to accidental or intentional release into the wild. The authors quantitatively assessed two strategies: 1. Reduce selection in captivity or 2. Maintaining a second population to minimize captive/

wild interactions. They concluded that the appropriate approach depends on the goals of the culture activity.

Martinez-Takashita, N., C. Purcell, C. Chabot, M. Craig, C. Paterson, J. Hyde, and L. Allen, 2015. A tale of three tails: cryptic speciation in a globally distributed marine fish of the genus *Seriola*. Copeia 103(2): 357-368.

This paper demonstrates that the nominal species *Seriola lalandi* is actually comprised of three distinct species. The authors present compelling evidence for genetic separation of populations. This information is critical in culture applications for this species in that what were once considered populations of the same species will now be separated in new broodstock groups.

Past, Present, and Future Research on Ostreid Herpesvirus 1 Infections of the Pacific Oyster in Tomales Bay, California

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Corresponding author: Not available.

Keywords: Not available.

ABSTRACT

Large-scale sporadic mortalities of juvenile Pacific oyster, *Crassostrea gigas* have occurred in Tomales Bay, California, for 20 years. The Ostreid herpesvirus 1 (OsHV-1) is the identified causative agent consistently associated with mortalities, with a confirmed infectious etiology. OsHV-1 is a global pathogen of bivalve molluscs, although detection in the United States is limited to Tomales Bay and nearby Drakes Bay. OsHV-1 is a member of the Order *Herpesvirales*, sharing common morphological criteria with vertebrate herpesviruses, although sequence data indicates a tenuous relationship. OsHV-1 was classified as the first member of an invertebrate herpesvirus family, Family *Malacoherpesviridae.* Sequence data indicate that multiple global variants of OsHV-1 exist, and the virus detected in Tomales Bay is not identical to any one variant. Elevated water temperatures are consistently associated with oyster mortalities in Tomales Bay and may trigger viral replication and/or transmission of OsHV-1 to naïve juvenile oysters. Laboratory trials indicate qPCR and RT qPCR can be used to demonstrate virus replication and gene expression. Survival of young Pacific oysters in Tomales Bay is dependent on outplant time, size, and oyster stock, indicating genetic improvement and development of biomarkers for improved survival of Pacific oysters infected with OsHV-1 is possible. Since 2008, an economically devastating increase in *C. gigas* mortality in France has been associated with a new genetic variant OsHV-1 μvar, which is lethal to all life history stages. OsHV-1 μvar continues to spread in Europe, and a similar variant causes losses in Australia, New Zealand, and Asia. OsHV-1 μvar's ability to kill seed and adults heightens concern over this variant relative to its progenitor, OsHV-1, which is lethal to larvae and seed only. OsHV-1 resistance has been shown to confer resistance to μvar. Recent studies demonstrated the ability to select for resistance to OsHV-1, and we propose studies to evaluate selection of resistance to both OsHV-1 μvars and their progenitor in U.S. oyster lines.

Type 1 Ostreid Herpesvirus (OsHV-1) Variants in Japan

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Keywords: OsHV-1, *Crassostrea gigas,* Japan

ABSTRACT

Ostreid herpesvirus 1 (OsHV-1) µVar is a variant of OsHV-1 newly reported from France (GenBank accession no. HQ842610), and it is suspected of being the causative agent of acute mass mortality events of Pacific oysters *Crassostrea gigas* during summer in Europe since 2008. OsHV-1 µVar differed from reference OsHV-1 by nucleotide mutations in the C2/C6 fragment, including ORF4, and in the IA2/IA1 fragment, including ORF42/43 (Segarra et al. 2010). Japan is one of the major producers of Pacific oyster. Our previous study indicated that 23 types of OsHV-1 variant, showing 96% to 99% similarity to the reference OsHV-1, were obtained from Pacific oyster, kumamoto oyster (*C. sikamea*) and suminoe oyster (*C. ariakensis*) collected in 2007 and from Pacific oyster collected in 2011 in Japan (Shimahara et al. 2012). Although 18 variants among the obtained 23 possessed a microsatellite deletion in C2/C6 unique to OsHV-1 µVar, the nucleotide sequence was not identical to the OsHV-1 µVar. In this study, further surveillance of OsHV-1 variants was conducted for Pacific oysters in 2012. Nine hundred spat, or juveniles, of Pacific oyster collected in the four main oyster producing areas were subjected to PCR using the primer pairs C2 and C6 (Renault and Arzul 2001). PCR products were amplified from 40 out of 900 oysters and 11 different nucleotide sequences, showing 96% to 99% similarity to the reference OsHV-1, were obtained. Although 11 sequences among the 13 possessed a microsatellite deletion unique to OsHV-1 µVar, all PCR products contained two conserved nucleotides that were shared with the reference OsHV-1 and not with OsHV-1 µVar. Here, we found variable types of OsHV-1 in oysters in Japan, which had different nucleotide sequences from that of OsHV-1 µVar in France.

INTRODUCTION

Ostreid herpesvirus 1 (OsHV-1) µVar is a variant of OsHV-1 newly reported from France (GenBank accession no. HQ842610), and it is suspected of being the causative agent of acute mass mortality events of Pacific oysters *Crassostrea gigas* during summer in Europe since 2008. OsHV-1 µVar differed from reference OsHV-1 by nucleotide mutations in the C2/C6 fragment, including ORF4, and in the IA2/IA1 fragment, including ORF42/43 (Segarra et al. 2010).

Our previous study indicated that 23 types of OsHV-1 variant showing 96% to 99% similarity to the reference OsHV-1 were obtained from Pacific oyster, kumamoto oyster (*C. sikamea*) and suminoe oyster (*C. ariakensis*) collected in 2007 and from Pacific oyster collected in 2011 in Japan (Shimahara et al. 2012). Although 18 variants among the obtained 23 possessed a microsatellite deletion in C2/C6 unique to OsHV-1 µVar, the nucleotide sequence was not identical to the OsHV-1 µVar. Although large-scale mortality outbreaks, such as those seen recently in Europe, have not occurred in the shellfish farms where OsHV-1 positive samples were collected, further epidemiological study must be undertaken to collect more information. In this study, further surveillance of OsHV-1 variants was conducted for Pacific oysters in 2012.

MATERIALS AND METHODS

Nine hundred spat, or juveniles, of Pacific oyster were collected from four main oyster producing areas, including Miyagi, Hiroshima, Mie and Okayama (Fig. 1), and subjected to DNA extraction with Maxwell 16 Tissue DNA Purification Kit (Promega, Madison, WI, USA) following the manufacturer's instructions. PCR targeting the C2/C6 fragment including ORF4 was

Figure 1. Map of Japan showing the four Prefectures where oyster samples were collected.

performed using the primer pairs C2: 5'-CTC TTT ACC ATG AAG ATA CCC ACC-3' and C6: 5'-GTG CAC GGC TTA CCA TTT TT-3' in a final reaction volume of 20 µL, as described by Renault and Arzul (2001). The PCR reaction mixture contained 0.5 U of *TaKaRa Ex Taq* Hot Start Version (TaKaRa, Shiga, Japan), 2 µL of 10x reaction buffer, 0.5 pmol of dNTP's and 20 pmol of each primer. One microliter of template DNA was added to each mixture. The program consisted of one cycle of denaturation at 94°C for 10 min, followed by 40 amplification cycles at 94°C for 30 sec, 63°C for 30 sec, and 72°C for 30 sec, with a final extension step of 72°C for 7 min. PCR products were purified using Agencourt AMpure (Beckman Coulter, Danvers, MA, USA) following the manufacturer's instructions and sequenced directly using the BigDye Terminator Kit v3.1 (Applied Biosystems, CA, USA). Sequencing reactions were purified with Agencourt CleanSEQ (Beckman Coulter, Danvers, MA, USA) following the manufacturer's instructions and loaded into an ABI PRISM 3130 *xl* Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Basic alignments of nucleotide sequences were made using GENETYX-MAC software (GENETYX, Tokyo, Japan). The sequences obtained were compared with those of variants recently reported by Renault et al. (2012), reference OsHV-1 (AY509253) (Davidson et al. 2005), and OsHV-1 µVar (HQ842610) (Segarra et al. 2010).

RESULTS AND DISCUSSION

The C2/C6 product of approximately 700 bp was amplified from 40 out of 900 samples in 2012 (Table 1). Although the detection rate was lower than those investigated previously, the C2/C6 product was detected in DNA extracted from spat taken from all sampling areas. The nucleotide sequences of the C2/C6 products were polymorphic and 11 different nucleotide sequences, JPType 1, 2, 6, 9, 27, 28, 29, 30, 31, 32 and 33 (Fig. 2), were obtained. Two types of OsHV-1 were simultaneously present in two animals collected in Miyagi. One animal contained JPType 2 and 9, while the other contained JPType 6 and 33. The 11 sequences were 96% to 99% homologous to the reference OsHV-1 (AY509253). Although 10 sequences among the obtained 11 possessed a microsatellite deletion unique to OsHV-1 µVar, all PCR products contained two conserved nucleotides that were shared with the reference OsHV-1 and not with OsHV-1 µVar. OsHV-1 µVar, which is one of the major concerns for oyster producers in France at present, has not yet been found in Japan. Furthermore, the nucleotide sequences obtained in this study were not identical to those previously found in France, Ireland, the United States, New Zealand, and China (Renault et al. 2012; Martenot et al. 2011, 2012).

Of the 23 variants of OsHV-1 previously detected in Japan (Shimahara et al. 2012), four variants, JPType 1, 2, 6, and 9, were persistently present in 2012 (Table 2). The four variants were present in at least two

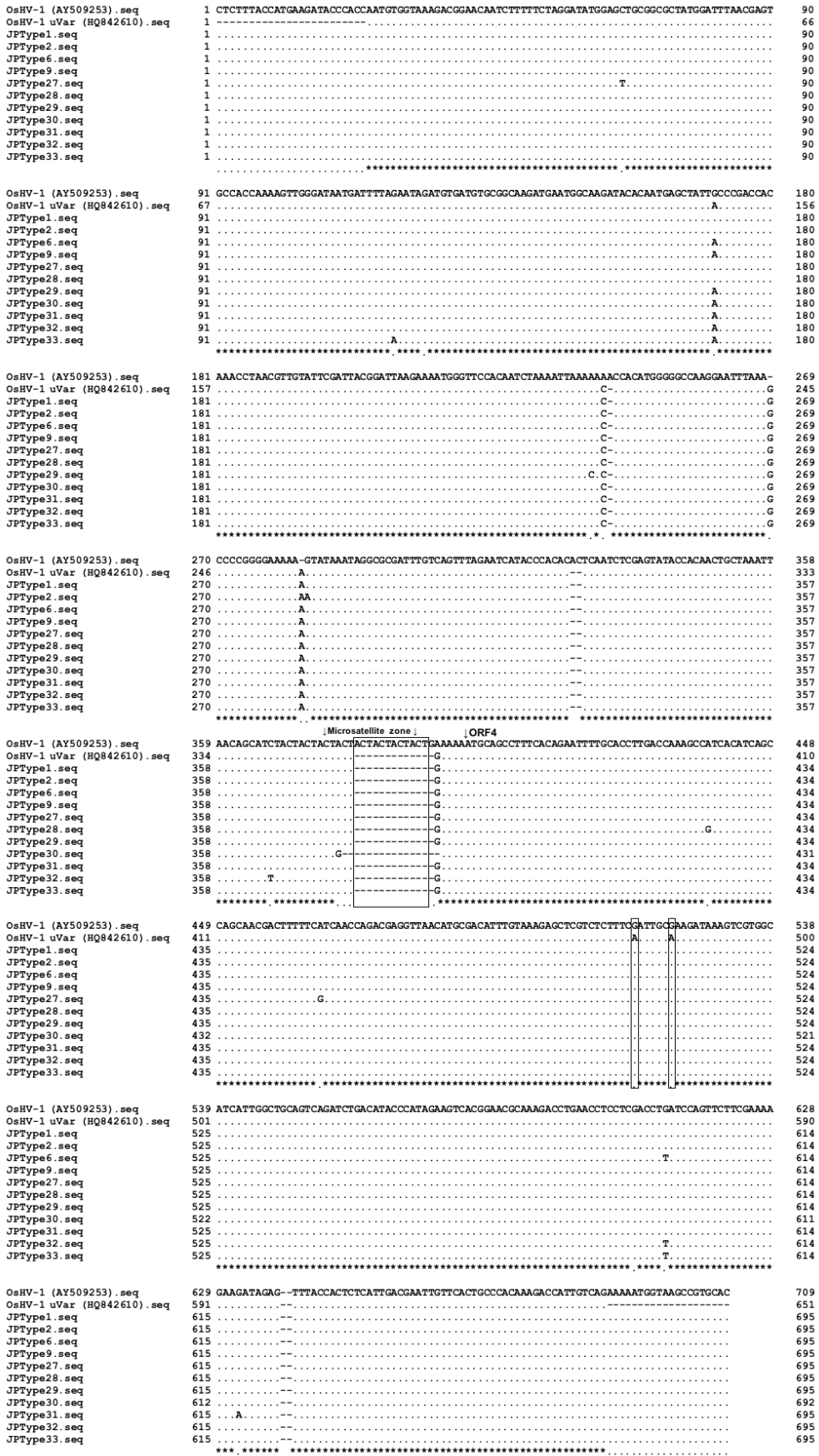

Figure 2. C2/C6 sequence alignment of OsHV-1 variants obtained in this study (JPType 1, 2, 6, 9, 27, 28, 29, 30, 31, 32 and 33) compared with those of reference OsHV-1 (AY509253) and OsHV-1 µVar (HQ842610). The locations of the microsatellite zone and the initiation codon for ORF 4 are indicated. OsHV-1 µVar is characterized by a deletion of 12 consecutive nucleotides in the microsatellite zone, two non-synonymous substitutions, and the other mutations in this C2/C6 fragment. The major deletion in the microsatellite zone, and the substitutions of the two nucleotides in ORF 4, are framed. The substitutions of two nucleotides in ORF4 are conserved in all JPTypes found in the present study.

geographic areas in a series of observations in 2007, 2011 and 2012. It was considered that JPType 1, 2, 6, and 9 had spread to different areas by translocation of infected spat and continuously infect oysters in each area, probably due to the slightly higher infectivity than the other variants. However, infections by these variants do not seem to be expanding either temporally or geographically. The distribution of the OsHV-1 variants were different from that in France, where OsHV-1 µVar has been rapidly increased and dominant after the outbreaks (Segarra et al. 2010). In addition, large-scale mortality outbreaks, such as those seen recently in Europe, have not occurred in the shellfish farms where OsHV-1 positive samples were collected. It is considered that the OsHV-1 variants in Japan may not be as pathogenic as OsHV-1 µVar in France.

Since OsHV-1 µVar had a high impact on the oyster aquaculture industry in France, OsHV-1 microvariants, including OsHV-1 µVar, were listed in aquatic animal diseases by the World Organisation for Animal Health (OIE). OsHV-1 microvariants are termed as "OsHV-1, which have sequence variations in a microsatellite locus upstream of the ORF4 and in ORF4 itself and ORF42/43 when compared with the reference type (AY509253)" (OIE 2017). It is uncertain if the Japanese variants are included in OsHV-1 microvariants, since ORF42/43 were not analyzed in this study. However, the functions of the ORF4 and ORF42/43 are not well determined, and it is unknown if they reflect pathogenicity of OsHV-1. Although isolation and cultivation of OsHV-1 has not been successful due to the lack of suitable cell lines (Renault and Novoa, 2004), pathogenicity of OsHV-1 µVar was determined by using the infected animals (Segarra et al. 2010). Experimental infection with OsHV-1 will be conducted to determine the pathogenicity of the Japanese variants in a future study.

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ANNOTATED BIBLIOGRAPHY OF KEY WORKS

Shimahara, Y., J. Kurita, I. Kiryu, T. Nishioka, K. Yuasa, M. Kawana, T. Kamaishi and N. Oseko. 2012. Surveillance of type 1 ostreid herpesvirus (OsHV-1) variations in Japan. Fish Pathology 47: 129-136.

Ostreid herpesvirus 1 (OsHV-1) µVar is a variant of OsHV-1 that is suspected of being the causative agent of acute mass mortality events of Pacific oysters during summers in Europe since 2008. The authors investigated a distribution of OsHV-1 variants in six main oyster-producing areas in Japan by PCR to determine whether OsHV-1 µVar was present in Japan. PCR products were amplified from 123 out of 1,714 oysters, and 23 different nucleotide sequences were obtained. Although 18 sequences among the 23 obtained possessed a microsatellite deletion unique to OsHV-1 µVar, all PCR products contained two conserved nucleotides that were shared with reference OsHV-1 and not OsHV-1 µVar in ORF4. It was found that variable types of OsHV-1 distribute throughout Japan, but their nucleotide sequences were not completely identical to OsHV-1 µVar.

Epigenetic Considerations in Aquaculture

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Keywords: epigenetics, DNA methylation, aquaculture, shellfish

ABSTRACT

Epigenetics has garnered considerable attention in many aspects of agricultural production, particularly where environmental conditions can be manipulated or natural variation exists. In the past decade, technology and our fundamental knowledge of transcriptional regulation has dramatically increased to where we are now able to characterize numerous aspects of epigenetic variation in aquaculture species. This review will introduce key concepts and definitions of epigenetic mechanisms including DNA methylation, histone modifications and non-coding RNA, review the current understanding of epigenetics in both fish and shellfish and propose key areas of aquaculture where epigenetic knowledge could be applied. Based on aspects of life history and husbandry practices in aquaculture species, application of epigenetic knowledge could have a significant impact on productivity and sustainability of aquaculture practices. Conversely, there is the possibility that elucidating the role of epigenetic mechanisms in aquaculture species may upend traditional assumptions about selection practice. Ultimately, there are still many unanswered questions regarding how epigenetic mechanisms might be leveraged in aquaculture.

INTRODUCTION

Maintaining and improving aquaculture production requires an understanding of genetic and physiological mechanisms that control desired traits. An understanding of these mechanisms has led to the development of pioneering biotechnological methods that have important applications. For example, molecular markers are used in broodstock selection and transcriptomic studies have been used to improve environmental conditions to decrease physiological stress in animals. Recently, interest in epigenetics has surged within the agricultural community as it becomes clearer that epigenetic mechanisms can provide a measurable link between environment and phenotype.

Epigenetics refers to heritable processes that alter gene activity without manipulating the underlying DNA sequence (Jablonka and Lamb 2002). Epigenetic mechanisms (or 'marks'), including DNA methylation, histone modifications and non-coding RNA activity, influence gene expression primarily through local modification of chromatin. Unlike DNA, epigenetic marks can be directly influenced by the environment, and because of this, have been shown to be important mediators of phenotypic responses to environmental signals (Fig. 1). For example, in mammals, nutrition (Weaver et al. 2004), exposure to toxins (Dolinoy et al. 2006), and photoperiod (Azzi et al. 2014) have all been associated with changes in DNA methylation and concomitant changes in phenotype. Fish, while less studied, show similar environmental sensitivity in DNA methylation patterns (Wang et al. 2009; Strömqvist et al. 2010; Campos et al. 2013). Many environmentally-induced epigenetic changes are transient, while some may persist over the course of an organism's lifetime (Weaver et al. 2004; Dolinoy et al. 2006; Heijmans et al. 2008). In certain cases, epigenetic changes can be transgenerationally inherited (Guerrero-Bosagna et al. 2010; Manikkam et al. 2012). As such, it is important to understand the nature and function of these mechanisms and their influence on phenotype in fish and shellfish.

Interest in epigenetics has been gaining ground in agricultural science for crops (Ong-Abdullah et al. 2015; Álvarez-Venegas and De-la-Peña 2016) and, more recently, livestock (Goddard and Whitelaw 2014; González-Recio 2015), but less is known about epigenetic mechanisms in economically valuable aquaculture species. Considering that a majority of aquaculture takes place in open or natural systems subject to environmental change, it is important to consider the role of epigenetics. This is particularly the case now that tools and resources are available to study these important molecular mechanisms. In recent years, studies in species ranging from salmonids to sea bass to oysters and mussels are providing the first evidence that epigenetics is associated with commercially important traits in aquaculture species. In sea bass and half smooth tongue sole, temperature induced sex-determination has been associated with changes in DNA methylation (Navarro-Martin 2011; Shao et al. 2014). In salmonids, there is some evidence that changes in DNA methylation are associated with variation in life history phenotypes including early male maturation (Morán et al. 2011), smoltification (Morán et al. 2013), and anadromy (Baerwald et al.

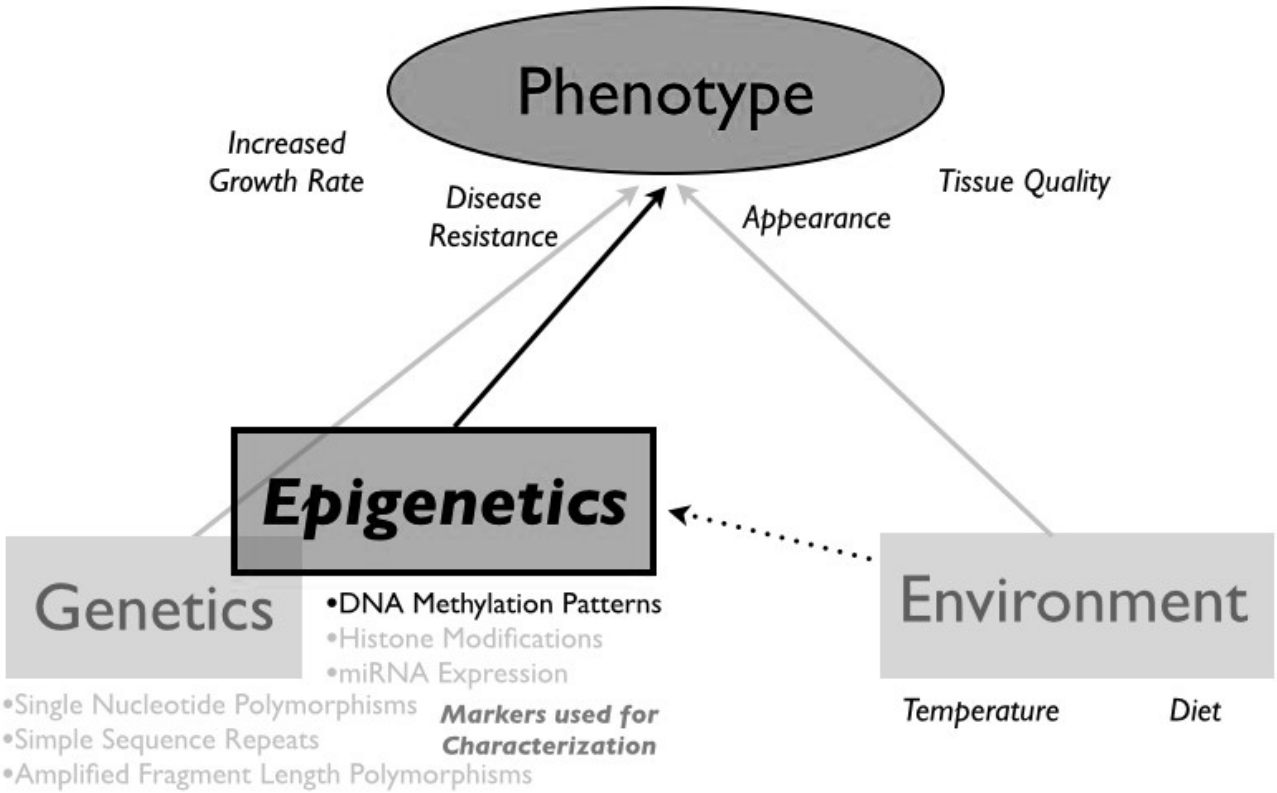

Figure 1. Schematic representation of epigenetic influence on phenotype. Markers used for characterizing organismal variation are listed.

2016). Recent studies in European sea bass and rainbow trout examined the role of epigenetics in mediating phenotypic responses to various aspects of diet (Terova et al. 2016; Marandel et al. 2016; Panserat et al. 2017). In Pacific oysters, the role of epigenetics in mediating effects of temperature on oyster physiology has been investigated (Fellous et al. 2015). As is described below, the relationship between epigenetics and phenotype is less clear in shellfish as opposed to finfish.

This mini-review will introduce key concepts and definitions of epigenetic mechanisms, briefly review the literature as it pertains to the nascent field of epigenetics in aquatic species and highlight key aspects of aquaculture that would benefit from a deeper understanding of the role of epigenetics. There have been excellent reviews published recently regarding epigenetics, primarily DNA methylation, and various aspects of finfish aquaculture (e.g. Li and Leatherland 2013; Moghadam 2016) and those will be highlighted where appropriate.

WHAT IS EPIGENETICS?

The following section will briefly describe specific epigenetic marks and review where we stand in terms of understanding (or lack of understanding) the relationship between epigenetics, environment and phenotypes in aquaculture species.

DNA Methylation

DNA methylation refers to the enzymatic addition of a methyl group to a cytosine residue in DNA, which occurs almost exclusively at CpG dinucleotides (i.e. a cytosine located 5' of a guanine) in animals. The enzymatic machinery supporting DNA methylation includes a family of DNA methyltransferases (DNMTs) including the maintenance methyltransferase DNMT1 (responsible for copying pre-existing DNA methylation patterns to the new strand during mitosis) and the *de novo* methyltransferases DNMT3A/3B. DNA methylation is known to be repressive when located in promoters of genes through associations with other DNA binding proteins or through physical blocking of transcription factors (Bell and Felsenfeld 2000); however, DNA methylation located in gene bodies is associated with high levels of expression (Jones 1999). Therefore, although typically associated with silencing, the regulatory role of DNA methylation is specific to the genomic context. In mammals, DNA methylation plays important roles in providing genomic stability through repression of transposable elements (Maloisel and Rossignol 1998), genomic imprinting (Bell and Felsenfeld 2000), and dosage compensation (Csankovszki et al. 2001). DNA methylation is also important for cell-type differentiation and embryonic development (Li et al. 1992). DNA methylation is the most well studied epigenetic mechanism, where

a majority of studies have been done in plants and mammals. For instance, in these systems, it has been shown DNA methylation is sensitive to external factors including nutrition (Dolinoy et al. 2006) exposure to toxins (Dolinoy et al. 2007), and photoperiod (Azzi et al. 2014). It is important to note that the meiotic transmission of DNA methylation patterns, thus the opportunity for transgenerational epigenetic inheritance through DNA methylation, is rare in mammals, which undergo extensive DNA methylation reprogramming in early embryos (Daxinger and Whitelaw 2012). As you will see in the following sections, it is unclear if and to what extent DNA methylation resetting occurs in fish and shellfish.

Histone Modifications

Chromatin is a dynamic structure that supports both the packaging of the genome into the nucleus, and importantly, the regulation of genes and other genomic regions via changes in DNA accessibility (Cheung et al. 2000). The basic repeating structure of chromatin is the nucleosome, which consists of DNA wrapped around histone proteins. These histones can be post-translationally modified at their N-terminal tails altering the degree of which DNA can be wrapped around them resulting in either euchromatin (referring to open chromatin that is accessible to transcription factors, RNA polymerase II (Pol II) and other DNA binding proteins that support gene expression) or heterochromatin (referring to tightly packed DNA associated with transcriptional silencing). These states are dependent on the type (i.e. acetylation, methylation, phosphorylation, ubiquitylation) and location (e.g. various lysine or arginine residues) of the modification (for a complete list of modifications, see review by Lawrence et al. 2016). These various modifications can exist in bivalent and multivalent states with each other and have led to the much debated existence of the 'histone code' (Jenuwein and Allis 2001). These modifications are enabled by various families of enzymes including histone acetylases (HATs), histone deacetylases (HDACs), histone methyltransferases (e.g. HMT) and histone demethylases (e.g. Jumonji and Lys-specific demethylase). Modifications are important for regulation of gene activity but also have roles in DNA repair, replication, and cell fate/determination (see reviews by Lawrence et al. 2016; Martin and Zhang 2005; Eberharter and Becker 2002). The enzymatic machinery responsible for these modifications are highly regulated during embryonic development (Lin and Dent 2006), and like DNA methylation, can be altered by various environmental conditions (Chinnusamy and Zhu 2009). Less is known about the mitotic and meiotic persistence of histone modifications, but interestingly, it has been shown in both mammals and zebrafish that certain modified histones are non-randomly retained during spermatogenesis when the majority of these proteins are replaced with protamines, suggesting that these marks may have a role transferring epigenetic information to the embryo (Brykczynska et al. 2010; Wu et al. 2011).

Non-coding RNA

Although a large majority of the genome is transcribed, only a small portion of these transcripts code for protein. These non-coding transcripts, originally regarded as 'junk', are now recognized for their role in modulating gene expression and are categorized broadly as non-coding RNA (ncRNA). There are two major classes of ncRNA: long ncRNA (> 200 nt) and small ncRNA (< 200 nt), which includes micro RNA (miRNA), short interfering RNA (siRNA), and piwi-interacting RNA (piRNA). Small ncRNAs are highly conserved and their major mechanism of action is to inhibit protein synthesis by blocking or degrading primary transcripts (see review by Castel and Martienssen 2013). Long ncRNAs (lncRNA) by contrast are less conserved and have complex mechanisms of action that may work either in *cis* or *trans* (see review by Wang and Chang 2011). Non-coding RNAs have important functions in gene expression and have demonstrated to be important regulators of genome stability, environmental plasticity and embryonic development (Mercer et al. 2009; Bizuayehu et al. 2014). Generally speaking, ncRNA molecules are considered 'epigenetic' in the traditional sense because they interact with other epigenetic mechanisms, such as DNA methylation and histone modifications, to silence or activate various parts of the genome (Peschansky and Wahlestedt 2014).

TAXA SPECIFIC PATTERNS

Epigenetic mechanisms and particularly DNA methylation have been the focus of numerous studies in both fish and shellfish in recent years. However, a majority of what we know about epigenetics in animals comes from studies done in mammals and care should be taken when generalizing functions from mammals to fish and shellfish. Although there are certainly similarities (e.g. DNA methylation patterns are very similar across all vertebrates), there are also important differences (e.g. invertebrate DNA methylation patterns are very different from vertebrates). This section will focus on foundational information about epigenetic marks in fish and shellfish and highlight both significant gaps in our understanding as well as noting differences from well-studied mammalian systems.

DNA Methylation in Fish and Shellfish

DNA methylation is the most well-studied epigenetic mark among fish and shellfish. Both fish and shellfish have genes present that encode basic methylation machinery (e.g. DNMTs and MBDs) and DNA

methylation is present in all species examined to date. However, there are striking differences in DNA methylation patterns between vertebrates and invertebrates as well as significant unknowns in terms of DNA methylation resetting in both fish and shellfish.

Considerable work has been done on understanding patterns and functions of DNA methylation in model fish species such as zebrafish and medaka, with increasing information on DNA methylation in non-model species. For example, a recent review by Metzger and Schulte (2016) extensively covers the current state of knowledge of DNA methylation patterns and functions in marine fish. Generally speaking, DNA methylation patterns are similar across all vertebrates which exhibit a 'global' DNA methylation pattern, meaning majority of CpGs are methylated with the exception of regions of DNA with high CpG content referred to as CpG islands. In comparison to mammals, however, global DNA methylation levels are higher in fish though the significance of this remains unclear (Jabbari 1997; Zhang et al. 2016). The function of DNA methylation also appears to be similar across vertebrates with the exception of a role in genomic imprinting which is unique to mammals (Potok et al. 2013). One outstanding question concerns the extent of DNA methylation resetting in fish. While mammals undergo extensive DNA methylation reprogramming in the early embryo (Daxinger and Whitelaw 2012), it is unclear to what extent DNA methylation reprogramming occurs in fish (Potok et al. 2013; Jiang et al. 2013). A recent study, discussed in more detail in the following section, shows clear evidence of transgenerational inheritance of environmentally-induced DNA methylation patterns in a fish, suggesting at least some of the genome escapes putative resetting between generations (Shao et al. 2014). Certainly there is a need for more detailed studies on the extent of DNA methylation resetting in fish, particularly in cultured aquatic species. In addition, more studies should examine the potential meiotic inheritance of environmentally-induced epigenetic changes.

Invertebrate DNA methylation patterns are strikingly different from vertebrates. Whereas vertebrates exhibit a global pattern of DNA methylation, invertebrates show a 'mosaic' pattern with stretches of methylated DNA punctuating regions of unmethylated DNA (Tweedie et al. 1997; Simmen et al. 1999). DNA methylation was examined genome-wide in the Pacific oyster where it was reported that 15% of CpGs were methylated in a somatic tissue, whereas 60-70% of CpGs are methylated in mammals (Gavery and Roberts 2013). In oysters, as in other invertebrates, the methylated fraction tends to consist of gene bodies, while other genomic regions exhibit less methylation. Unlike vertebrate species, transposable elements show surprisingly little methylation in oysters and other invertebrate species (Simmen et al. 1999; Feng et al. 2010; Zemach et al. 2010). Functionally, DNA methylation does appear to be associated with gene regulation in shellfish. In the Pacific oyster, high levels of methylation in gene bodies (and putative promoter regions) were associated with high levels of expression (Gavery and Roberts 2013; Olson and Roberts 2014). Interestingly, genes with limited methylation in oysters have variability in exon-specific expression across tissue types, indicating that hypomethylation allows increased plasticity (Gavery and Roberts 2013). While more studies are needed to quantify this relationship, there are significant implications for improving resilience in shellfish - particularly if DNA methylation patterns are heritable. There are few studies on heritability of DNA methylation patterns in shellfish, however, a small study looking at methylation states in parents and larvae found significant clustering of methylation patterns within families, indicating that methylation patterns differ significantly depending on the male parent (Olson and Roberts 2015). More recently, Rondon et al. (2017) have shown parental herbicide exposure influences progeny DNA methylation patterns in oysters.

Histone Modifications in Fish and Shellfish

Histone modifications and their dynamics have been studied in zebrafish where evidence indicates modifications are conserved among vertebrates. Functional analysis of histone acetylation in zebrafish confirms its role in embryogenesis (Vastenhouw and Schier 2012) and in tissue regeneration (Stewart et al 2009). In terms of meiotic inheritance, zebrafish show multivalent modified histone retention in sperm similar to mammals (Wu et al. 2011). Studies examining histone modification in non-model fish are rare, though recent studies in rainbow trout and European sea bass indicate that diet influences bulk histone modification levels and can regulate the expression of associated enzymes (Marandel et al. 2016; Terova et al. 2016; Panserat et al. 2017).

Histone modifications are less studied in shellfish, but work by Fellous et al. (2014) identified homologs of Jumonji histone demethylase genes (Jmj) in Pacific oysters that, similar to vertebrates, were regulated during embryonic development. A subsequent study showed that both bulk histone methylation levels and the expression of histone demethylases were responsive to temperature during development, suggesting a role for histone modifications in mediating the physiological responses of oysters to temperature (Fellous et al., 2015). Histones are not replaced with protamines in bivalve sperm as they are in mammals, rather they are replaced with various sperm nuclear basic proteins that may be protmaine- or histone-like (Ausio 2006). Additional research will be required to determine to what extent histones and their modifications are retained in bivalve sperm, shedding light on the potential transmission of epigenetic information from parent to offspring in bivalves.

Non-coding RNA in Fish and Shellfish

A majority of studies on non-coding RNAs in fish and shellfish focused on miRNAs, including important aquaculture species [e.g. Atlantic salmon (Bekaert et al. 2013; Andreassen et al. 2013) and rainbow trout (Juanchich et al. 2016)]. There are several examples of examining miRNAs in a physiological context including: maternal transcripts in the egg (e.g. Ma et al. 2012), immune function (e.g Andreassen et al. 2017) and embryonic development (Bizuayehu et al. 2015). There is less information about other types of small ncRNA, except in zebrafish where, for example, piRNA have been shown to silence transposable elements in gametes, functioning similarly as in mammals (Houwing et al. 2007). Recently there have been several descriptions of long non-coding RNAs in salmonids including associations between lncRNA expression and disease in both Atlantic salmon and rainbow trout (Boltaña et al. 2016; Valenzuela-Miranda et al. 2016; Paneru et al. 2016).

Predictably, non-coding RNAs are less investigated in shellfish, though generally speaking, miRNAs and their biogenesis are highly conserved over evolutionary scales (Wheeler et al. 2009). As such, genes for miRNA biogenesis have been detected in bivalve species (Rosani et al. 2016). With respect to long non-coding RNAs, researchers have reported an association with larval development in the Pacific oyster (Yu et al. 2016).

POTENTIAL AQUACULTURE APPLICATIONS

Environmental Manipulation

Given what we know about environmental influences on epigenetic mechanisms in fish and shellfish and the relationship with phenotype, one avenue where epigenetics and aquaculture could intersect is environmental manipulation. In addition to mechanisms described in the previous section, there is also the fundamental concept of "developmental programming". Developmental programming suggests that environmental conditions experienced in early-life influence phenotypes later in life and this has gained momentum in human research (e.g. Gluckman et al. 2008). In other words, developmental programming offers an environmental memory that could be beneficial in controlled aquaculture settings. However, in some cases embryos and juveniles are not raised in the same environmental conditions as the adults. For example, hatchery-reared salmon or hatchery-reared bivalve juveniles placed into a natural setting for grow out. Identifying sensitive periods for environmental memory could offer a "programming window" that could be leveraged in husbandry practices.

There are several lines of evidence for developmental programming in fish, and for an excellent review see Jonnson and Jonnson (2014). Traits associated with early environmental conditions include metabolism, growth, sex determination, fecundity, and behavior (Jonnson and Jonnson 2014). There are also several examples where environmental memory has been described in shellfish. In some instances, this occurs within a generation and in other cases there is transmission of information from parents to offspring. Within generation environmental memory has been described in Olympia oysters where early larval exposure to ocean acidification impacted juvenile traits (Hettinger et al. 2013). Adult Manila clams exposed to low pH during gonadal maturation have faster growing offspring compared to controls (Zhao et al. 2017). In the Sydney rock oyster, larvae produced by parents incubated under low pH conditions are larger and develop faster in low pH conditions and also have higher fitness as adults (Parker et al. 2012, 2015). In addition to water chemistry, disease is another significant concern in shellfish aquaculture. There is increasing evidence to suggest prior exposure to immune challenge can increase response later in life and that this environmental memory can be transmitted to offspring. Green et al. (2016) demonstrated offspring of Pacific oyster parents treated with poly(I:C) possess enhanced protection against Ostreid herpesvirus type I infection. The mechanism(s) responsible for providing this memory are not fully understood in cultured species and arguably not required to improve aquaculture production, though we would suggest that elucidating the epigenetic mechanisms involved could increase the degree and breadth of improvement.

Two relevant cases for aquaculture where the epigenetic mechanisms have been described involve sex determination in fish. In European sea bass (*Dicentrarchus labrax*), exposure to high temperature in early development is associated with a higher proportion of phenotypic males (Navarro-Martin et al. 2009). In work done by Navarro-Martin et al. (2011) this early high temperature exposure was associated with increased DNA methylation in the promoter of the aromatase gene (*cyp19a1a*) in adults. Furthermore, the authors showed that *in vitro* methylation of the aromatase promoter was sufficient to suppress transcription of the gene. More recently, the commercially important half-smooth tongue sole (*Cynoglossus semilaevis*) was used as a model to investigate the role of epigenetic regulation in environmental sex determination. Using genome-wide DNA methylation profiling, the investigators showed that pseudomales (generated by exposing genetic females to high temperature during a sensitive developmental window) exhibit methylation patterns consistent with genetic males, both of which differ from the methylome of normal females. Excitingly,

it was reported that global methylation patterns are inherited by F1 pseudomale offspring generated by crosses between pseudomales and normal females, suggesting transgenerational epigenetic inheritance of environmentally-induced sex reversal in this species (Shao et al. 2014). Controlling sex in fish broodstock is certainly a priority for aquaculture and these studies shed light on the epigenetic mechanisms that could be leveraged in future work. More work would be needed to establish the degree of mechanism conservation across species exhibiting environmental sex determination.

Nutrition and feeding are important aspects of aquaculture production, and research has shown memory of early-life nutritional conditions influences key phenotypic traits later in life. In mammals, nutritional status of the mother can predispose offspring to adult onset metabolic disease and mounting evidence suggests that epigenetic mechanisms are involved (reviewed by Vickers et al. 2014). In fish, rainbow trout swim-up fry fed a plant based diet for three weeks showed higher growth rates, feed intakes, and feed efficiencies when challenged again with a plant based diet after seven months of grow out on a fishmeal/ fish oil diet (Geurden et al. 2013). Interestingly, in a follow up study, transcriptomic analyses suggest that epigenetic mechanisms may be involved in this response (Balasubramanian et al. 2016). In addition, a study looking at vitamin supplementation at first feeding in rainbow trout identified changes in global methylation and histone modification four months after the supplementation had ended, despite no observed phenotypic responses (Panserat et al. 2017). Collectively, these studies provide the first link between early-environmental exposures and epigenetic mechanisms in aquaculture species.

Epigenetic Selection

Beyond understanding how early environmental exposure can be used for improving aquaculture, there is the possibility epigenetic markers could be integrated into broodstock selection. This is plausible as in one important agriculture commodity, oil palms, it has been shown that a critical trait, oil content, can be epigenetically selected for (Ong-Abdullah et al. 2015). While there is much more we need to learn with regard to desired phenotypes and epialleles, work such as this demonstrates value in including epigenetics in association studies. Furthermore, Patel et al. (2013) have shown in a clinical study that integrating both genetic (SNP) and epigenetic (DNA methylation) markers in genome wide association studies improved associations with a phenotype (i.e. diabetes). The influence of epigenetics, specifically DNA methylation, on estimating breeding values for quantitative traits has been considered recently for finfish aquaculture in a review by Moghadam et al. (2015).

It should also be noted that epigenetics might make genetic selection more challenging. Many organisms have the potential to generate new genetic variation in response to stressful conditions through modulation of epigenetic marks associated with transposable elements (Dowen et al. 2012; Yu et al. 2013; reviewed by Rey et al. 2016). Transposable elements, or "jumping genes", are regions of repetitive DNA that can move and amplify their copy number in the host genome. In the model plant *Arabidopsis*, the genomic response to bacterial challenge was a global reduction of DNA methylation and reactivation of previously silent TEs associated with defense genes (Yu et al. 2013). It is interesting to consider that in invertebrates, shown specifically in *C. gigas,* that TEs are not preferentially methylated (Gavery and Roberts 2013; Olson and Roberts 2015). It has been hypothesized that the lack of TE silencing by DNA methylation may indicate pressure to generate and maintain genetic diversity in a species that inhabits heterogeneous environments (Gavery and Roberts 2014). This means that in theory, if culture conditions become stressful, shellfish could respond by modulating transposable element expression to create new genetic variation (Rey et al. 2016), thereby having the unintended consequences of "erasing" phenotypic gains made through selective breeding.

CONCLUSIONS

Epigenetics has the potential to change the way we think about how a phenotype is generated and maintained. Through a greater understanding of DNA methylation, histone modifications and ncRNAs we can functionally annotate genomes, better predict phenotypic outcomes of early environmental exposures, and possibly select on epigenetic markers. With careful experimental design and special considerations for epigenetic differences between taxa (see Lea et al. 2016), the aquaculture community is primed to begin to integrate epigenetics into husbandry practices. The concepts and ideas of epigenetics provide an attractive lens through which to consider manipulation of traits through environmental memory or selection of beneficial traits based on epigenetic markers. It is also important to consider that epigenetics may also function to disrupt predictable, robust phenotypes through the creation of new, unexpected variation.

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ANNOTATED BIBLIOGRAPHY OF KEY WORKS

Baerwald, M. R., M. H. Meek, M. R. Stephens, R. P. Nagarajan, et al. 2015. Migration-related phenotypic divergence is associated with epigenetic modifications in rainbow trout. Molecular Ecology 25: 1785-1800.

This study investigates the role of epigenetics (DNA methylation) in migration-related life history traits in *Oncorhynchus mykiss*. The authors used reduced representation bisulfite sequencing to perform comparative DNA methylation analysis between juvenile resident and smolt F2 siblings generated from a cross between steelhead (migratory) and rainbow trout (nonmigratory). Fifty-seven differentially methylated regions, many of which were in gene regulatory regions, were identified between residents and smolts, suggesting a relationship between epigenetic variation and variation in migration-related phenotypes.

Gavery, M.R., and S.B. Roberts. 2013. Predominant intragenic methylation is associated with gene expression characteristics in a bivalve mollusc. PeerJ 1: e215.

This dataset, generated using methylation-enriched high-throughput bisulfite sequencing, represents the first high-resolution methylome in any mollusc. DNA methylation data were compared to gene expression datasets and a positive relationship between intragenic methylation and gene expression levels was identified. These data suggest that DNA methylation patterns may play a role in regulating gene expression in molluscs.

Navarro-Martín, L., J. Viñas, L. Ribas, N. Díaz, A. Gutiérrez, et al. 2011. DNA methylation of the gonadal aromatase (*cyp19a*) promoter is involved in temperature-dependent sex ratio shifts in the European sea bass. PLoS Genetics 7: e1002447.

The authors report that in European sea bass (*Dicentrarchus labrax*), which exhibits temperature-dependent sex determination, exposure to high temperature in early development was associated with increased DNA methylation in the promoter of the aromatase gene (*cyp19a1a*) and a higher proportion of phenotypic males. Furthermore, *in vitro* methylation of the aromatase promoter was sufficient to suppress transcription of the gene, supporting a role for DNA methylation as a mechanistic link between temperature and sex ratios in species exhibiting temperature-dependent sex determination.

Potok, M. E., D. A. Nix, T. J. Parnell, B. R. Cairns. 2013. Reprogramming the maternal zebrafish genome after fertilization to match the paternal methylation pattern. Cell 153: 759-772.

Genome-wide DNA methylation patterns in zebrafish gametes, various stages of embryos and a somatic tissue (muscle) were analyzed using whole genome bisulfite sequencing. This high-resolution approach identified dynamic and unique patterns of DNA methylation during development in zebrafish. Results suggest that the functional significance of sperm DNA methylation patterns in fish is to provide transcriptional competency to the early embryo, which 'inherits' the DNA methylation pattern in the sperm.

Shao, C., Q. Li, S. Chen, P. Zhang, J. Lian, et al. 2014. Epigenetic modification and inheritance in sexual reversal of fish. Genome Research 24: 604-615.

The half-smooth tongue sole (*Cynoglossus semilaevis*) was used as a model to investigate the role of epigenetic regulation in species with environmental sex determination. Using genome-wide bisulfite sequencing of normal male, female and pseudomale fish (generated by exposing genetic females to high temperature during a sensitive developmental window), the authors showed that sex-reversed genetic females exhibit methylation patterns consistent with genetic males, both of which differ from the methylome of normal females. Furthermore, it was reported that global methylation patterns are inherited by F1 pseudomale offspring generated by crosses between temperature-induced sex-reversed pseudomales and normal females, suggesting transgenerational epigenetic inheritance of sex reversal in this species.

Gavery, M.R., and S.B. Roberts. 2014. A context specific role for DNA methylation in bivalves. Briefings in Functional Genomics doi:10.1093/bfgp/elt054.

A review of current knowledge of DNA methylation in bivalves. A primary conclusion is that the functional role of the gene could influence the role of DNA methylation in influencing expression.

Olson, C.E., and S.B. Roberts. 2014. Indication of family-specific DNA methylation patterns in developing oysters. bioRxiv doi: http://dx.doi.org/10.1101/012831

This study provides the first single-base pair resolution DNA methylomes for both oyster sperm and larval samples from multiple crosses. While sample sizes are very low, this work suggests DNA methylation patterns could be inherited.

Roberts, S. 2015. Compilation of DNA Methylation Genome Feature Tracks (*Crassostrea gigas*). figshare https://dx.doi.org/10.6084/m9.figshare.1456226.v2

Genome feature tracks and accompanying IGV session file to visualize DNA methylation data for the Pacific oyster (*Crassostrea gigas*).