# Preface

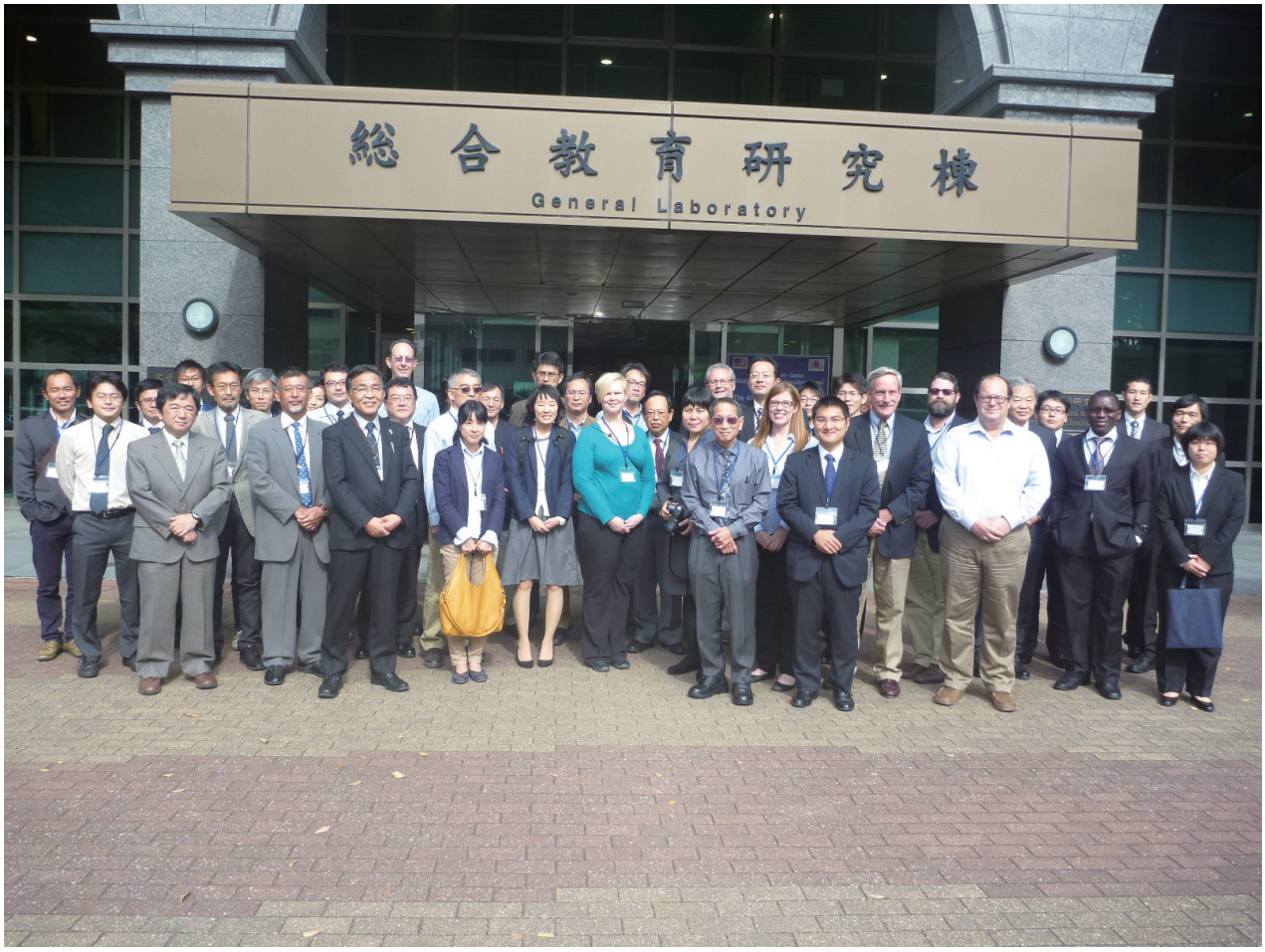
The UJNR (The U.S.-Japan Cooperative Program in Natural Resources) Aquaculture Panel was established in 1968, and the business meeting and symposium have been held every year since 1971. Through the long history of UJNR, Aquaculture Panel has contributed to the development of aquaculture researches of both countries by means of various cooperative activities, such as the exchange of scientists and literatures, and the promotion of joint research projects. The Aquaculture Panel is highly appraised as one of the most active UJNR panels in both countries.

The 43rd Joint Meeting of the UJNR Aquaculture Panel was conducted in Nagasaki, Saga and Oita Prefectures, the northern Kyushu Island, Japan from November 9-14, 2015; while the Scientific Mini-Symposium was held in Nagasaki University from November 10-11. The symposium theme was "Evaluation of the impact of breeding organisms on the ecosystem and aquaculture industry", which was under the 9th Three-Year Plan, "Genetics and Breeding Studies in Aquaculture Industry", commenced in 2014. Sixteen oral presentations were made on topics such as the impact of breeding organisms on the ecosystem and aquaculture industry, selective breeding techniques, and bioinformatics, and twenty poster presentations were also made on broader topics related to aquaculture during the two-day symposium.

The proceedings of the 43rd UJNR Aquaculture Panel Scientific Mini Symposium "Evaluation of the impact of breeding organisms on the ecosystem and aquaculture industry" is published as the special issue of the Bulletin of Japan Fisheries Research and Education Agency. With great pleasure, this UJNR proceedings containing high quality papers authored by selected American and Japanese aquaculture scientists will hopefully help in the improvement of genetic/breeding programs, which is expected to contribute to the development of the aquaculture industry in both the United States and Japan.

Finally, I would like to express my sincere gratitude to the staff at Nagasaki University and colleagues involved in the UJNR Aquaculture Panel for their efforts in the preparation and organization of the symposium. I would also like to deeply thank the editorial board members for publishing the proceedings.

Fuminari Ito
Chair of UJNR Aquaculture Panel
Executive Director
Japan Fisheries Research and Education Agency




Participants in 43rd UJNR Aquaculture Panel Symposium, held in Nagasaki University, Nagasaki, Japan, November 10 – 11, 2015

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## Abstracts of Poster Presentations

# Program

## The 43rd Scientific Symposium of UJNR Aquaculture Panel

## Evaluation of the impact of breeding organisms on the ecosystem and aquaculture industry

Date:

| | | |
|---|---|---|
| November 10 | 15：00–17：30 | Poster Session |
| November 11 | 9 ：30–17：30 | Oral Session, Poster Session during lunch break |

Venue:

Lecture room, 2F General education and research building, Bunkyo Campus, Nagasaki University

## Aim of the Symposium

Breeding research will be of great help with the improvement of aquaculture technology. The utilization of wild broodstock / seeds does not result in genetically improved stocks, and the importance of genetics and breeding studies has increased to meet various needs. Although genetic improvement is common in agriculture and the resulting benefits unquestioned, there are few selectively bred commercially available strains in the aquaculture industry except in inland aquaculture.

Moreover, we should pay attention to the ecological impacts of breeding technology. At the same time selection may increase risks when selected animals escape and breed with wild stocks unless proper safeguards are put into place. Integration of scientific knowledge of the benefits and risks can help contribute to genetic improvement programs. Our UJNR activity should deepen discussions on the issue of genetics/breeding studies, which is expected to contribute to the development of the aquaculture industry in both countries of Japan and United States.

This Symposium consists of oral session and poster session. In oral session, we will deal with the subjects relating to ‘genetics and breeding’ such as: the impact of breeding organisms on the ecosystem and aquaculture industry; selective breeding techniques; bioinformatics, etc. In poster session, we will present and discuss broader topics relating to Aquaculture.

**Tuesday, November 10th, 2015**

Registration: 14 : 00–17 : 30

## Poster Session

Poster presentation: 15 : 00–17 : 30

**Wednesday, November 11th, 2015**

Registration: 9 : 00-12 : 00

## Oral Session

### Opening Session

(Moderators: J. Higano & M. Rust)

**Welcome to Nagasaki University**

Atsushi Hagiwara (Dean of the Graduate School of Fisheries and Environmental Sciences, Nagasaki University) ····· 9 : 30– 9 : 35

Aim of the Symposium

Fuminari Ito (Japan Chair, Fisheries Research Agency) ····· 9 : 35– 9 : 50

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(Moderators: S. Watanabe & A. Fuller)

1. Appropriate conditions for the production of triploidy induced by cold shock in yellowtail *Seriola quinqueradiata*
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2. Potential Application of Germplasm Preservation in Breeding Programs for Molluscan Shellfish Aquaculture and Restoration
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3. Improving Aquaculture Production in Haliotis Species Through the Development of a Genomic Toolkit
   Catherine Purcell (NOAA Fisheries) ····· 10 : 35–10 : 55
4. Culture Protocols and Production of Triploid Purple-Hinge Rock Scallops
   Paul Olin (California SeaGrant) ····· 10 : 55–11 : 15

### Session II. Genetic improvement 1

(Moderators: A. Ozaki & B. Bosworth)

5. Big Data in Agriculture and the USDA/ARS Initiative
   Jeffrey Silverstein (USDA Agricultural Research Service) ····· 11 : 15–11 : 35
6. Signature of artificial selection in a breed of coho salmon Oncorhynchus kisutch
   Sho Hosoya (Fisheries Laboratory, University of Tokyo) ····· 11 : 35–12 : 05

Group Photo …… 12 : 05–12 : 10
Lunch Break …… 12 : 10–13 : 10

7. Genetic Selection in Animals Using Pedigree, Phenotypic, and Genomic Information
   Shogo Tsuruta (University of Georgia) …… 13 : 10–13 : 40
8. Exploring Transcriptomic Patterns in Slow- and Fast-Growing Seriola dorsalis Larvae
   Catherine Purcell (NOAA Fisheries) …… 13 : 40–14 : 00

## Session III. Risk evaluation of escaped fish

(Moderators: K. Ikuta & B. Iwamoto)

9. Modeling the Variable Effects of Using Wild and Cultured Broodstock on the Fitness Risk Due to Escaped Farmed Fish
   Kristen Gruenthal (NOAA Fisheries) …… 14 : 00–14 : 20
10. Did farmed Coho salmon Oncorhynchus kisutch that escaped during the earthquake and tsunami disaster of 2011 interbreed with native Masu salmon Oncorhynchus masou?
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11. Evaluation of the tsunami impact on the genetic diversity of the marbled flounder *Pseudopleuronectes yokohamae* in Sendai Bay, Miyagi, Japan
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15. Coho Salmon Broodstock Development: A Case Study of the Domsea Coho (1977 to 2015)
    Bob Iwamoto (Spring Salmon LP) …… 16 : 35–16 : 55
16. Development of Improved Catfish Germplasm at the Warmwater Aquaculture Research Unit, USDA-ARS
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Discussion …… 17 : 20–17 : 50

(Moderators: F. Ito & M. Rust)

Closing remarks …… 17 : 50–18 : 00

(Michael B. Rust, U.S.A. Panel Chair)

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# Preliminary Study on Triploid of Yellowtail

Yukinori SHIMADA[*1], Hiroyuki NAGOYA[*2], Hiroyuki OKAMOTO[*2], Toshiya YAMAGUCHI[*1], Nariaki INOUE[*1], Takashi ISHIKAWA[*2], Kazuhisa HAMADA[*3], Kazuharu NOMURA[*2], Kazunori YOSHIDA[*4], and Hironori USUKI[*5]

**Abstract**: The possibility of a large-scaled cold shock induction of triploidy, viability of triploid fish and growth performance were investigated in yellowtail *Seriola quinqueradiata*. Induction of triploidy by a large-scaled cold shock (approximately 0.1 million fertilized eggs) was successful in our preliminary test, and approximately 40 thousand hatched larvae were obtained. Some of the hatched larvae were tested for polyploidy, the results of which indicated that fish treated with two different cold shocks (two trials) were 100% triploid. Then, the growth performance of the triploid and diploid fish was investigated in both mixed and separate rearing. At the age of 100 and 122 days, the body weights of the triploid and untreated control fish were not significantly different in both mixed and separate rearing in two trials. However, as they grew larger, the growth rate of the untreated control was significantly higher than that of the triploid fish in both rearing conditions. Thus, the triploid fish at early development (at least half a year) showed a delay in growth. Also, the triploid fish showed longer term viability. In future studies, we will investigate growth performance at larger size and infertility (gonadal developmental stage) at the age of maturation.

**Key words**: *Seriora quinqueradiata*, triploid, cold shock, growth performance

## Introduction

Yellowtail (*Seriola quinqueradiata*) is one of the most important species for aquaculture in Japan. Under culture conditions, however, mature yellowtail are remarkably reduced in body weight after the spawning season (mainly summer). This is a serious concern for the market. Thus, there is an interest in the production of sterile yellowtail. This can be achieved by chromosome set manipulation techniques (Thorgaard, 1983; Felip *et al*., 2001), including the production of triploids (e.g. Purdom, 1972; Garrido-Ramos *et al*., 1996; Holmefjord and Refstie, 1997; Felip *et al*., 1997; Piferrer *et al*., 2000). Recently, the basis for triploid induction by a cold shock treatment was established for yellowtail at the small-scale (Shimada unpublished data; Nagoya *et al*., unpublished data). It showed that cold shocks for 5 – 20 min duration at 0 – 5 °C within 5 min after fertilization resulted in approximately > 80% triploid rate.

Our goal is to achieve industrial use of triploidy in yellowtail, but there remain several issues to resolve, such as developing a large-scaled cold shock technique and determining viability, growth performance and infertility of triploid fish, and also making a basic law system for triploid fish culture in

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a sea cage. To determine viability of triploid fish in a long-term culture, a large-scaled cold shock method is required to produce a lot of fertilized triploid eggs. The objectives of the present study are (1) to examine the efficacy of large-scaled cold shock induction of triploidy, (2) to determine viability of triploid fish in long-term rearing, and (3) to compare a growth performance of diploid and triploid fish.

## Materials and methods

### Artificial fertilizations and cold shock treatments

Artificial fertilization of yellowtail was performed at two facilities, Kamiura Laboratory and Komame Laboratory of National Research Institute of Aquaculture, in February (trial 1) and April (trial 2) 2015, respectively. Fertilized eggs were treated by cold shock 3 min after fertilization in 15 L containers equipped with nets at the bottom. No temperature difference was observed between the container and surrounding water bath. The conditions of the cold shock treatment were 5 min duration at 0 °C at Kamiura Laboratory, and 20 min duration at 5 °C at Komame Laboratory, and the sea water temperature was regulated by sea water ice and / or a cooling equipment (AZ-280X, Iwaki Co. Ltd., Tokyo).

### Rearing and growth of fish

Treated and untreated fertilized eggs were separately maintained in 1000 L tanks until they hatched. Approximately 5,000 hatched larvae were reared in a 500 L tank with flow-through sea water. Tanks were maintained in a 4000 L water-bath to minimize variance of water temperature among them (water temperature > 20 °C). Fish were fed the L-type rotifer *Brachionus plicatilis* from day 2 to 25, *Artemia* spp. from day 21 to 35 with DHA and EPA enrichment (Hyper-gross, Mrinetech Co. Ltd., Aichi), and dry pellets after day 30. Fish were size segregated and transferred to 1500 L tanks during days 35 to 40. On day 100 or 122 in each trial, body weights of all fish were measured after anesthesia with 2-phenoxyethanol (Wako Co. Ltd., Osaka). Then, the body weights in the mixed rearing were measured on day 151, 185, 212, 245 and 275, whereas those in the separate rearing were measured only on day 150 and 186 because all triploids died in an accident on day 186. In addition, all fish in the mixed rearing were injected with a syringe on day 122 with a PIT tag placed into the ventral part of the body cavity.

### Polyploidy of fish

To determine the polyploidy of treated and

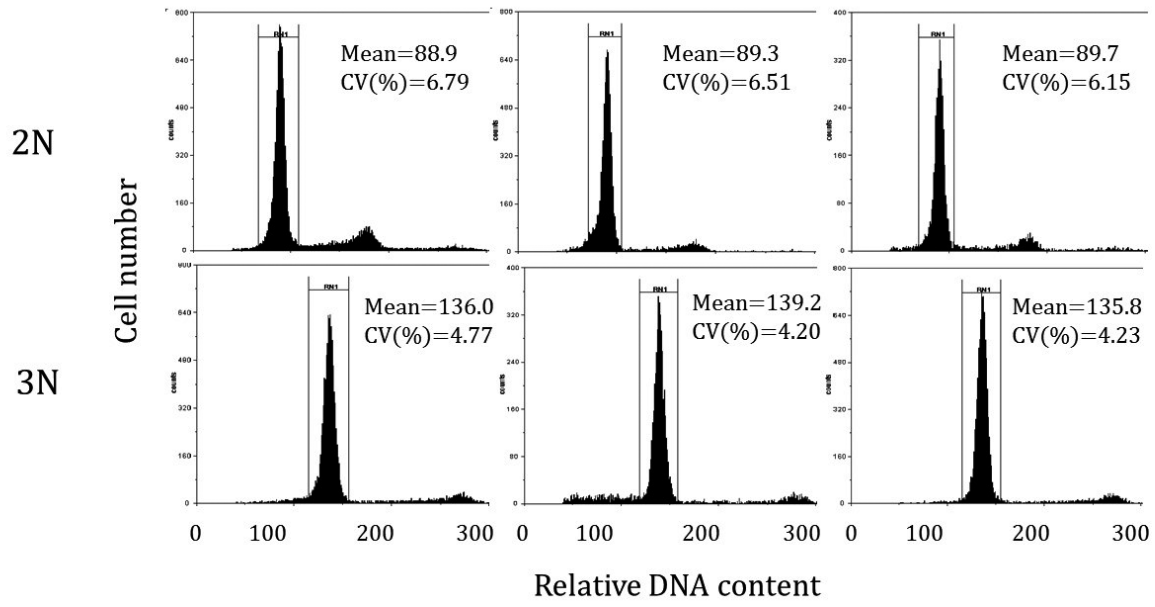


**Fig. 1.** Examples of polyploidy determination for untreated control (upper) and cold shock treated yellowtail (lower). 2 N and 3 N indicate diploid and triploid, respectively. The mean DNA contents of 3N are approximately 1.5 times larger than those for 2N.

untreated larvae, a total of 49 individuals were sampled on day 0 and fixed and preserved in 100% ethanol at 4 °C. These individuals were subjected to flow cytometry (Partec GmbH, Germany) for relative DNA content of the whole body cells in order to determine their ploidy status as shown in Fig. 1.

### Statistics

The data were expressed as the mean ± standard error of the means (SEM), and analyzed by an independent *t*-test.

## Results

### Cold shock treatments and ploidy status

Large-scaled cold shocks were performed on approximately 0.1 million fertilized eggs in trial 1 (separate rearing) and 2 (mixed rearing). Hatching rates in cold shock treatments for 5 min at 0 °C and 20 min at 5 °C, were 18.5% and 36.9%, respectively, whereas those in untreated controls were 78.2 and 22.9%. The triploid rates in cold shock treatments were 100% ($N$ = 8 and 12), whereas those in untreated controls were 0 and 11.8% ($N$ = 15 and 17) on day 0. In addition, we confirmed ploidy status of all individuals using their fin clips at the final measurement. In trial 1, 106 out of 121 treated fish were triploid (87.6%), but all untreated control fish were diploid (100%). In trial 2, treated and untreated control fish were triploid (11 / 11 fish) and diploid (15 / 15 fish), respectively.

### Growth performance of triploid and diploid fish

Growth performances of diploid and triploid fish are shown in Fig. 2. In the separate rearing, triploid and diploid fish were not significantly different in body weight on day 100 ($df$ = 240, $t$ = - 1.636 and $p$ = 0.1032), but at older ages diploid fish showed significantly larger body size than triploid fish (day150; $df$ = 204, $t$ = 4.454 and $p$ < 0.0001, day 186; $df$ = 94, $t$ = 5.659 and $p$ < 0.0001, Fig. 2A). This phenomenon was also observed in the mixed rearing ($df$ = 24, day 122; $t$ = 0.646 and $p$ = 0.5246, day 151; $t$ = 2.057 and $p$ = 0.0507, day 185; $t$ = 2.829 and $p$ = 0.0093, day 212; $t$ = 2.547 and $p$ = 0.0177, day 245; $t$ = 2.210 and $p$ = 0.0369, and day 275; $t$ = 2.285 and $p$ = 0.0315).

## Discussion

The most salient finding of this study was that a large-scaled triploid induction (0.1 million fertilized

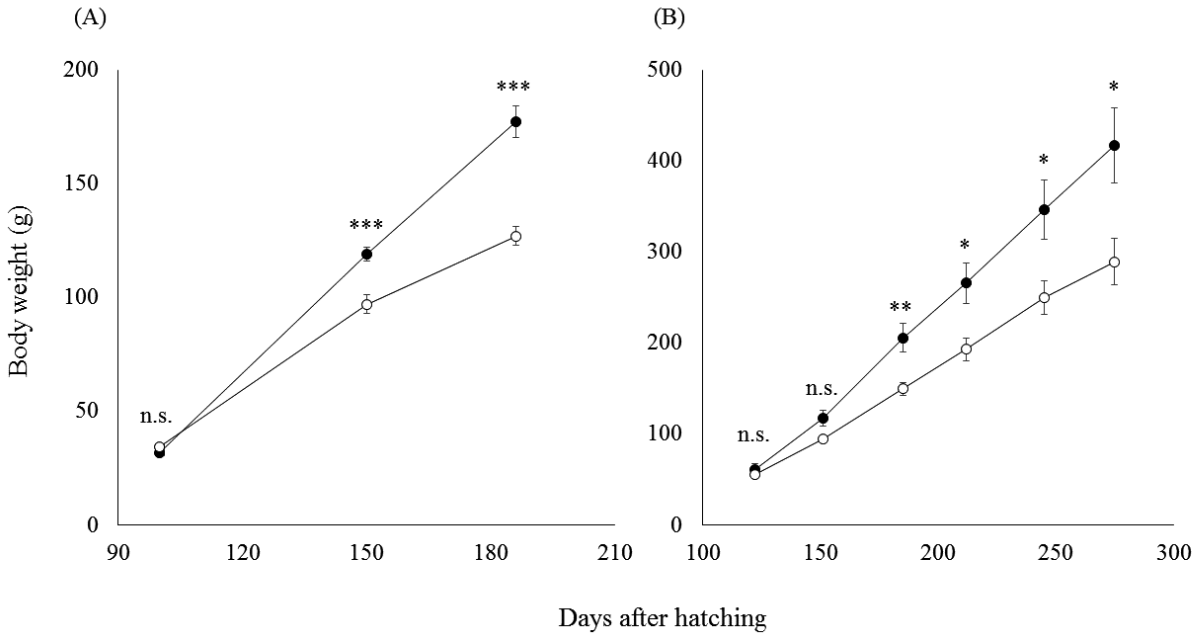


**Fig. 2.** Growth performance of triploid (open circle) and diploid (black circle) under separate rearing (A) and mixed rearing (B) conditions. All statistics were done by independent *t*-test. Symbols of n.s., *, ** and *** mean not significant, $P$ < 0.05, $P$ < 0.01 and $P$ < 0.001, respectively.

eggs) was possible and triploid fish were viable in long-term rearing in yellowtail. However, a growth performance of triploid fish was lower than that of diploid fish in both separate and mixed rearing. In what follows, we will discuss these results as well as some methodological issues in detail.

**A large-scaled cold shock treatment**

In a large-scaled cold shock for fertilized eggs, temperatures in the equipped container and surrounding water bath were the same, suggesting that the equipment used may be applied to larger volumes of fertilized eggs.

**Triploid viability**

In this study, hatching rates of cold shock treated eggs were 18.5 and 36.9% in trial 1 and 2, respectively, whereas those of untreated control were 78.2 and 22.9 %. Usually, survival of triploids is about 70 to 80% of that of the controls (Felip *et al*., 2001). However, the present study showed varying results ranging from 24% to 161% of the hatching rates of untreated controls. Most probably, we still need to improve the maintenance method of fertilized eggs in order to resolve the surprising result.

Triploid fish were observed in untreated controls (trial 2). So far, it is reported in a case study that over ripening of eggs after ovulation increases triploid rates in *Anguilla japonica* (Nomura *et al*., 2013). Therefore, over ripening might be one of the reasons for occurrence of triploids in untreated controls of yellowtail.

**Growth performance of triploid**

The present study demonstrated that triploid yellowtail had a growth delay compared to diploid yellowtail. Similar results have been reported in several other fish species (Felip *et al*., 2001). In contrast, Johnstone *et al*. (1991) reported that non-maturing triploid females showed better growth compared with sexually maturing diploid female fish. In turbot, Cal *et al*. (2006) reported that body weight in diploid and triploid fish started to differentiate at first maturity, and the differences expanded in later age. In our knowledge, we have observed that most triploid female yellowtail showed sexual maturation because the sex determination system of yellowtail is ZZ-ZW type (Fujii *et al*., 2010) and had no abnormal oocytes (Yamaguchi *et al*., unpublished data) as in the case of tilapia (Razak *et al*., 1999). Also, in the aspect of rearing technique Oppedal *et al*. (2003) reported that triploid salmon tended to display enhanced weight gain compared to diploid ones when given continuous lighting conditions. Thus, by improving rearing techniques and/or controlling fish maturation, the growth of triploid yellowtail might be enhanced comparable to the existing production system in diploid yellowtail.

## Conclusion

To summarize, the results of this study demonstrated the feasibility of large-scale production of triploid yellowtail and their use in aquaculture. However, in a comparison between separate and mixed rearing, we found that growth performance of triploid fish was lower than that of diploid fish. Thus, we need to develop appropriate rearing methods for triploid yellowtail.

## Acknowledgment

We thank Mr. Hiroki Ogawa and his colleagues in Aquafarm Inc. for providing parental yellowtail. We thank also Mr. Yasuhiro Shima at National Research Institute of Aquaculture, and Yuichiro Fujinami Ph. D., Mr. Takuro Hotta, Mr. Tsutomu Noda and their colleagues at Seikai National Fisheries Research Institute for providing high quality fertilized eggs for our preliminary study.

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Diploid and triploid in channel catfish *Ictalurus punctatus* were reared in indoor tanks. Triploids were significantly heavier than diploids at 8 months of age and older. Triploid female and male had smaller gonads with altered histology. Triploids converted feed more efficiently, and may provide greater profits in commercial culture than diploids.

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Red blood cell size was measured in brook trout (*Salvelinus fontinalis*) from Phillips Hatchery in Maine to investigate naturally occurring polyploid sterility. In eight brook trout in which gonads were lacking or undeveloped, the red blood cells were large, suggesting polyploidy. The average size of the red blood cells in other sterile fish fit into the normal range but all of the eight fish appeared to have some red blood cells that were polyploid. All polyploids appeared to be mosaics, containing diploid, triploid, or pentaploid cells. The cause of the polyploidy was not determined but may have been caused by the inadvertent exposure of the eggs to low temperatures after fertilization.

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Induced triploidy is widely accepted as the most effective method for producing sterile fish for aquaculture and fisheries management. Artificially produced triploids generally differ from conspecific diploids in three fundamental ways: they are more heterozygous, they have larger but fewer cells in most tissues and organs, and their gonadal development is disrupted to some extent. Despite these basic biological differences, triploids are similar in most respects to diploids when examined at the whole animal level. The only clear differences relate to the effects of impaired gametogenesis on the

reproductive physiology and behavior of triploids, especially in females. Other apparent differences include reduced aggressiveness, occasional specific morphological abnormalities, and inferior performance when reared under suboptimal conditions. The causes of these latter two problems are poorly understood but must be addressed if triploids are to be used more extensively.

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# Application of Germplasm Preservation in Breeding Programs for Molluscan Shellfish Aquaculture and Restoration

Huiping YANG*

**Abstract**: Germplasm are the genetic materials of germ cells, including gametes, embryos, or larvae. Preservation of germplasm is usually achieved through cryopreservation. The technology of cryopreservation has been applied for human artificial reproduction as a clinical treatment for infertility and for livestock as a tool for breeding programs worldwide. For fish and shellfish, cryopreservation has been studied in more than 200 species for preservation of natural resources and conservation of endangered species. For molluscan shellfish aquaculture, this technology can have the following potential applications: ***1) Preservation of specific lines or strains***. Ongoing breeding programs have yielded specific strains and lines, such as disease resistant oysters. Cryopreservation can be used to preserve these valuable strains and provide gametes for assistance of breeding programs. ***2) Preservation of natural wild populations***. The cryopreserved germplasm of natural populations will act as a repository of genetic diversity and allow for the continued adaptive genetic variation for aquaculture populations through infusion of new material from wild populations. In addition, a germplasm repository of wild populations can provide easy access as study materials for researchers. ***3) Creation of self-fertilization inbred lines***. Inbred lines are one of the most valuable resources for breeding programs but difficult to produce and require years of repeated crossing of brothers and sisters or backcrossing. Most bivalves are protandrous, beginning life as males and changing into females as they age. Therefore, with the techniques of non-lethal sperm collection and cryopreservation, self-fertilized lines can be created by using cryopreserved sperm and oocytes from the same individual after sex reversal; ***4) Preservation of sperm from tetraploid oysters***. Triploid-tetraploid technology is probably the most promising in oyster aquaculture because of the superior traits of triploids. Cryopreservation of sperm from tetraploids can extend the commercialization of triploid-tetraploid technology by the sale of frozen sperm and provide cost savings and security for maintaining tetraploids. In addition, cryopreservation of tetraploids produced each year can offer benefit for maintenance of the tetraploid populations. ***5) Assistance for creation of mutant lines***. Mutant breeding is an effective approach for creation of new strains or lines but seldom used in animal breeding programs because of its low efficiency. Recently, a new approach called TILLING (Targeting Induced Local Lesions in Genomes) was developed in plants for creation of mutant lines. To use this new technique for animal mutant breeding, sperm cryopreservation is an absolutely required technique. So far, this approach has been applied to zebrafish and puffer fish. With the genome sequencing accomplished in oysters, establishment of mutant lines by TILLING and sperm cryopreservation will benefit aquaculture and research on oyster functional genetics, and ***6) Assistance of aquaculture hatchery practice for regular and hybrid seed production***. The cryopreserved germplasm materials can function as a reservoir to meet the need for regular and hybrid seed production. For example, the hard clam hybrid offspring of *Mercenaria mercenaria* with *Mercenaria campechiensis* showed fast growth and higher survival.

**Key words**: Germplasm, cryopreservation, shellfish aquaculture

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## Introduction

Germplasm is the living genetic resources which are inheritable and can generate new generations, such as seeds for plants, or germ cells, gametes, embryos, or larvae for animals. Preservation of germplasm can serve as repository (seed banks, gene banks or sperm banks) for animal and plant breeding. Usually, germplasm preservation can be achieved through cold storage (for plant seeds) or cryopreservation (for animal germplasm materials). The technology of cryopreservation has been applied for human artificial reproduction as a clinical treatment for infertility (Bernard and Fuller, 1996; Di Santo *et al*., 2012) and for livestock as a tool for breeding programs worldwide (Curry, 2000). For fish and shellfish, cryopreservation has been studied in more than 200 different species with different applications (Tiersch *et al*., 2007), such as preservation of natural resources to maintain biodiversity and conservation of endangered species.

For molluscan shellfish, the application of germplasm preservation can also serve as germplasm repository for selective breeding (Adams *et al*., 2008), hybrid breeding, commercial seed production, preservation of natural resources, and conservation of endangered species. In addition, due to the characteristic of molluscan shellfish, application of germplasm cryopreservation can be expanded to certain specific areas such as creation of self-fertilization inbred oyster lines (Yang *et al*., 2015), aquaculture of triploid-tetraploids (Dong *et al*., 2005) and possible creation of mutant lines as that for zebrafish (Lawson, 2016).

## Materials and Methods

### Sample collection

In general, collection of germplasm samples for cryopreservation is based on the reproductive biology of the working species. Currently, most aquaculture molluscan species are bivalves (oysters, clams, scallops, and mussels) and a few gastropods, such as abalones. Those species are primarily free spawning with external fertilization, spiral holoblastic embryo development, and a swimming larval period until metamorphosis. Upon release, gametes are usually activated immediately and ready for fertilization within a few hours.

Germplasm collection methods usually can be: 1) *For gametes*: natural spawning, suspension of dissected gonads, or biopsy of gonads. The last two methods are only useful for certain species, such as some oysters and clams, in which gametes from dissected gonad can remain fertile. 2) *For embryos and swimming larvae*: collection is usually performed by filtering through 25 - μm screen and re-suspending in fresh seawater or buffer.

### Viability analysis

For almost all germplasm cryopreservation programs, fertilization and survival have been considered the main criteria for evaluation of cryopreservation success. Additionally, considering the time and resources required for *in vitro* fertilization, other effective and fast viability assays especially for sperm also have been developed and used widely. Viability of fresh or post-thaw sperm can be evaluated by observing motility with a bright-field light microscope and plasm membrane integrity and mitochondria membrane integrity by flow cytometry analysis. For oocytes, viability evaluation can be assisted by observation of the size and morphology besides fertility test. For embryos and larvae, survival and growth can be used for viability evaluation.

### Cryopreservation process of germplasm

Cooling rate is one of the most critical factors for success of germplasm cryopreservation, usually varies with germplasm materials and the working species, and is highly related to the volume, materials, and shape of the packaging container. Accurate cooling rate can be achieved by using liquid nitrogen or dry ice with the following methods: 1) Computer-controlled programmable freezer. The cooling process is controlled by spraying of liquid nitrogen which is pre-set by a programmable computer to achieve accurate cooling rates. 2) Home-made cryokits composed of Styrofoam boxes with liquid nitrogen. The cooling rates need to be pre-determined by measuring the temperature with a digital thermocouple, and cooling rates can be adjusted with the depth of liquid nitrogen, size of the

Styrofoam box and floating boat (for holding the samples to be frozen). 3) Others, such as nitrogen vapor in the storage or shipping dewar or a Styrofoam box filled with dry ice. Upon reaching -80 °C, frozen samples can be plunged into liquid nitrogen for long-term storage in storage dewar.

For sample packaging containers for germplasm cryopreservation, many different types have been used for molluscan germplasm cryopreservation in previous publications, such as French straws (0.25 ml, 0.5 ml, and 5 ml), cryovials, and cryo-plastic bags. The choice of packaging containers need to be facilitated with the accuracy of cooling rates, sample biosecurity, high-throughput processing, freezing and thawing process, and efficiency of storage space.

## Results and discussion

Since the first report of sperm cryopreservation in the Pacific oyster *Crassostrea gigas* in 1971 (Lannan, 1971), a total of 79 publications have been published on germplasm cryopreservation in molluscan shellfish (Table 1). Those studies were majorly on aquaculture species, including bivalves (oysters, mussels, pearl oysters, scallops, and clams) and gastropods (abalones), and most of those were on oyster species from the Family *Ostreidae* (43 publications). A recent publication reviewed the research on germplasm cryopreservation of oysters with detailed summaries (Hassan *et al*., 2015).

Overall, the germplasm materials cryopreserved include sperm, oocytes, embryos, and larvae, covering almost all of the life forms of germplasm (Table 1). However, no research has been found in molluscan shellfish on cryopreservation of primordial germ cells or gonad tissues, which has been developed in fish to generate gonads through transplantation of germ cells in surrogate or sterile mothers (Okutsu *et al*., 2007; Majhi *et al*., 2009). This is probably because of the advantages of mollusks over finfish for egg, embryo or even larval cryopreservation. For fish, egg or embryo cryopreservation still remains as a huge challenge due to the size, yolk contents, and meroblastic embryo cleavage (Yang and Tiersch,

**Table 1.** Summary about publications on molluscan shellfish germplasm cryopreservation

| Family | Species | Number of publication | Materials for Cryopreservation | Year |
|---|---|---|---|---|
| Ostreidae | *Crassostrea gigas* | 30 | Sperm, oocytes, embryos, larvae | 1971-2014 |
| | *Crassostrea virginica* | 9 | Sperm and larvae | 1973-2014 |
| | *Saccostrea cucullata* | | | |
| | *Crassostrea tulipa* | 1 | Sperm | 1991 |
| | *Crassostrea iredalei* | | | |
| | *Crassostrea rhizophorae* | 1 | Sperm and embryos | 2005 |
| | *Saccostrea glomerata* | 1 | Larvae | 2008 |
| | *Ostrea edulis* | 2 | Sperm and trochophores | 2011, 2012 |
| Mytilidae | *Choromytilus chorus* | 1 | Embryos | 1988 |
| | *Mytilus edulis* | 1 | Embryos | 1989 |
| | *Perna canaliculus* | 5 | Sperm, oocytes, trochophores | 2009-2014 |
| | *Mytilus galloprovincialis* | 3 | Sperm, trochophores | 2009-2013 |
| | *Mytilus trossulus* | 1 | Trochophores | 2009 |
| Pteriidae | *Pinctada fucata martensii* | 6 | Sperm and larvae | 2003-2012 |
| | *Pinctada margaritifera* | 4 | Sperm | 2005-2013 |
| Pectinidae | *Argopecten purpuratus* | 1 | Sperm | 2010 |
| | *Pecten maximus* | 1 | Sperm | 2014 |
| Veneridae | *Tapes philippinarum* | 1 | Larvae | 1992 |
| | *Meretrix lusoria* | 1 | Embryos and larvae | 1997 |
| Mactridae | *Spisula sachalinensis* | 1 | Larvae | 2008 |
| Haliotidae | *Haliotis gigantean* | 1 | Sperm | 1983 |
| | *Haliotis discus* | 1 | Sperm | 1983 |
| | *Haliotis diversicolor* | 6 | Sperm, oocytes, and embryos | 1992-2013 |
| | *Haliotis iris* | 1 | Sperm | 2000 |
| | *Haliotis rufescens* | 1 | Sperm | 2005 |
| | *Haliotis midae* | 1 | Embryos | 2008 |
| | *Haliotis laevigata* | 3 | Sperm | 2014 |

2009). For molluscan shellfish, a recent study with the Pacific oyster showed the success of larval cryopreservation, and post-thaw trochophore larvae grew into mature adults and spawned naturally (Suquet *et al*., 2014).

## Outlook for future research

Although many publications have reported research on molluscan shellfish germplasm cryopreservation, most research still has limitations that need to be addressed through future research. The limitations could be generalized as: 1) only very small laboratory-scale trials were reported in almost all of the reports; 2) post-thaw fertilization data was not included in most research to test the effectiveness of cryopreservation protocols; 3) no standardized sample packaging containers were used for possible high-throughput application, and 4) most factors in the freezing process were descriptive without quantification for protocol standardization and repetition, such as sperm concentration, sample volume, and cooling rate. These aspects will be the future research directions in this field.

Since the initiation of sperm cryopreservation in the Pacific oyster in 1971, it has been about 40 years. However, cryopreservation of molluscan shellfish species still remains essentially a research activity with little commercial application. For livestock species, sperm cryopreservation has grown into a billion-dollar global industry in about 50 years since its initiation. Compared to livestock and fishes, most aquaculture molluscan shellfish have the potential advantage for oocyte, embryo and larva cryopreservation. Therefore, germplasm cryopreservation will have great application in research projects, breeding programs, commercial industry, and natural resource conservation. Molluscan shellfish have traditionally been a major component of world aquaculture, its production in 2012 (15.2 million tonnes) accounted for about 22.8% of the total (inland and marine) aquaculture production and 60.3% of the world marine aquaculture production (FAO, 2012). It is predictable that germplasm preservation will be a useful technology to enhance and boost the molluscan shellfish industry.

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(1) Fuller B., Lane N., and Benson E., 2004: Life in the Frozen State, CRC Press, New York, 672pp.

Cryobiology is a research field encompassing disciplines including mathematics, biophysics, cell biology, molecular biology, and metabolism physiology. This book brings together the knowledge about cryobiology from these aspects into one platform for readers to understand this research, and is probably the first major textbook on cryobiology. It includes four themes with a total of 23 chapters, and the four themes are Fundamental Aspects, Life and Death at Low Temperatures, Freezing and Banking of Living Resources, and Medical Applications.

(2) Polge C., Smith A. U., and Parkes A. S., 1949: Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature* **164**, 666.

This is the first paper to report the successful cryopreservation and cold-drying of sperm from fowl by using glycerol as cryoprotectant. The finding in this one-page short report opened the door for human sperm cryopreservation which is now a major clinical treatment for infertility, and bull sperm cryopreservation which is now a huge industry for its breeding programs worldwide. For fish, the first report was published after this report in 1953 in herring (Blaxter J. H. S., 1953: Sperm storage and cross-fertilization of spring and autumn spawning herring. *Nature*, **172**, 1189 – 1190), and this preliminary communication showed that it was possible to cross-fertilize the two spawning 'types' of herring *Clupea harengus* found in the north-east Atlantic by using the cryopreserved sperm.

(3) Tiersch T. R. and Green C. C., 2011: Cryopreservation in Aquatic Species, World Aquaculture Society, Baton Rouge, LA, pp.1034.

This is the 2nd version of the book published electronically by the World Aquaculture Society (www.was.org). The first version is the volume 7 in the series of publication "Advances in World Aquaculture" published in 2000. The revised version is designed as a comprehensive single compendium of information on cryopreservation in aquatic species, and provides a broad overview of the principles, procedure and perspectives which are necessary for development and application of cryopreservation technology. It includes 101 chapters (compared to the 55 in the first edition) organized into 11 sections. This book can assist with teaching, research and program development, and is available for readers to purchase through the World Aquaculture Society with a very affordable price for students and researchers.

(4) Yang H., Hu E., Cuevas-Uribe R., Supan J., Guo X., and Tiersch T. R., 2012: High-throughput sperm cryopreservation of eastern oyster *Crassostrea virginica*. *Aquaculture* **344-349**, 223–230.

This study provided a reliable protocol for sperm cryopreservation in the eastern oyster *Crassostrea virginica* with potential for high-throughput processing. In this study, DMSO yielded the highest post-thaw motility at a cooling rate of 20 °C/min when thawed at 30 or 40 °C among the three tested cryoprotectants. Further evaluation of cooling rates of 10, 15, 20, 25 and 30 °C /min showed that 20 or 25 °C /min yielded the highest post-thaw motility (34 ± 5%) and fertility (77 ± 12%) for French straws and

CBS straws (28 ± 3% and 69 ± 14%). Equilibration times of 10 to 60 min did not cause significant differences in post-thaw motility when freezing with 10% DMSO at a cooling rate of 25 degrees C/min. Also, sperm concentrations ranging from $1\times10^8$ to $1\times10^9$ cells/ml at freezing did not cause significant differences in post-thaw motility. Finally, after thawing, sperm cryopreserved from 16 males with this protocol showed 58 ± 24% fertility (from 18 to 86%) for French straws, and 54 ± 21% fertility for CBS straws (from 18 to 95%). Overall, this research provided an outline template for developing a basic protocol for sperm cryopreservation for any other molluscan shellfish species.

(5) Yang H., Wang Y., Guo X., and Tiersch T. R., 2015: Production of inbred larvae through self-fertilization using oocytes and cryopreserved sperm from the same individuals after sex reversal in eastern oyster *Crassostrea virginica*. *Aquac. Res.* **46**, 2153-2165

The authors in this study reported the production of self-fertilized larvae in eastern oyster *Crassostrea virginica* for the first time, and demonstrated the feasibility of creating self-fertilized inbred lines by use of non-lethal sperm collection and cryopreservation. In this study, small (~1 year old) and large (~2–3 years old) oysters were biopsied for sperm collection. Survival of the biopsied oysters after 1 year was 50% for small oysters and 17% for large oysters, and sex reversal was observed bidirectional (from female to male and also from male to female). Oocytes were collected from sex-reversed females, and self-fertilized with cryopreserved sperm. Of the 24 cryopreserved samples, 14 individuals had ≤ 1% fertility when crossed with oocytes from unrelated females, indicating that the cryopreserved sperm had reduced fertility. The other 10 individuals had a fertility of 39 ± 25% when crossed with oocytes from unrelated females (non-selfing), but showed a significantly lower success of self-fertilization (12 ± 16%) ($P = 0.008$), while aliquots of the same oocytes had a fertilization of 83 ± 11% when crossing with fresh sperm. Larvae were produced in the self-fertilized families (12–94% of the fertilized oocytes), and survived to eyed-larvae stage at days 11–14. Genotyping with 9 microsatellite markers confirmed that the larvae resulted from self-fertilization in four families.

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Bull. Jap. Fish. Res. Edu. Agen. No. 45, 21 − 27, 2017



# Improving Aquaculture Production in *Haliotis* Species through the Development of a Genomic Toolkit

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**Abstract**: Commercial abalone aquaculture has greatly expanded over the past decade, becoming a thriving global industry valued at over $100 million USD. Abalone is one of the few species where culture production dominates the global market as a result of increasing demand and declining natural stocks from overexploitation and disease. U.S. abalone production is also growing due to high market value and demand. Most farms operating in California utilize three native west coast species: red abalone (*Haliotis rufescens*), green abalone (*H. fulgens*), and pink abalone (*H. corrugata*). These species differ in commercially important traits that are key to culture expansion in California and improved production efficiency (e.g., growth rate, disease resistance, thermal tolerance). Next generation sequencing has opened the door for extensive and explorative genetic research on abalone, and several studies were recently published examining transcriptomic data for species including the domestically important red abalone, *H. rufescens*. Recently, researchers working with the Southwest Fisheries Science Center (SWFSC) used restriction site associated DNA sequencing (RAD-Seq) methods to identify genome-wide SNP markers in *H. fulgens* and to examine population structure in wild populations. However, transcriptomic and RAD-mapping analyses were also limited by the paucity of genomic information available for abalone; without knowledge of the genomic structure, it is very difficult to ascertain coverage depth in these studies. We are working to create the first *de novo* abalone genome assembly using sexed *H. rufescens* samples, generate tissue specific transcriptomes for *H. rufescens*, and conduct comparative genomic analyses with other commercially important California abalone. Comparative analyses will include *H. fulgens*, *H. corrugata*, and endangered white (*H. sorenseni*) and black (*H. cracherodii*) abalone. Genomic, transcriptomic, and comparative analyses will improve our understanding of sex-determination, thermal/environmental preference, disease resistance, hybridization outcomes, and local adaptation, especially for commercially important California abalone. This will enable identification of candidate genes of interest and marker development for marker assisted selection to improve aquaculture practices in the U.S. abalone industry and elsewhere. This research will also help guide restoration and wild stock enhancement along the west coast for these species, including for the endangered white and black abalone.

**Key words**: *Haliotis, Haliotis rufescens, Haliotis sorenseni*, genome assembly, RNA-Seq

## Introduction

In the U.S., approximately 200 metric tons of farmed abalone are produced annually (Gordon and Cook, 2013). Most domestic abalone farms operate in California and utilize three native west coast species: red (*Haliotis rufescens*) (Fig. 1), green (*H. fulgens*), and pink abalone (*H. corrugata*) (Allsopp *et al*, 2011) (Fig.2).

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The dominant culture species, red abalone, grows quickly and reaches a large size in culture; popular in the U.S. market, they are also one of the most valuable species in the mollusk industry globally (Robertson, 2012; Aguilar-Espinoza *et al*., 2014; Brokordt *et al*., 2015b). Of the three species, *red abalone* is the most temperate while the green and pink abalone have more southern distributions and are of greater interest for aquaculture production in Southern California and Mexico, where they can be grown at higher water temperatures (McBride and Conte, 1996).

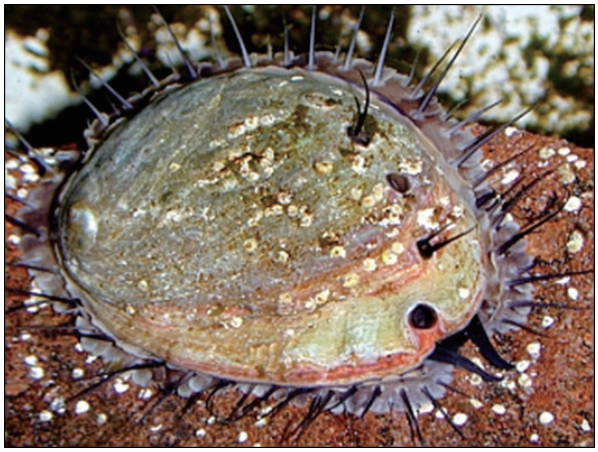

**Fig. 1.** Red abalone (*Haliotis rufescens*)

Although abalone culture is rapidly growing, it has been hindered by several bottlenecks that limit production capacity and efficiency in this industry. Improvements to the abalone stock in disease resistance and the other economically important traits are imperative to reducing production costs and to accelerating growth of the abalone industry, particularly in the United States (Arai and Okamura, 2013). The ability to overcome these bottlenecks will be significantly improved with better genomic resources for abalone. When correctly applied, these techniques may rapidly improve broodstock selection, characterize variation (both beneficial and detrimental), and provide methods to directly improve the value, efficiency, and production in the target species.

Considerable genetic research has been conducted for these species. Genetic markers have been generated to characterize variation in wild and farmed populations, characterize variation in traits of interest, and identify QTLs for important traits (e.g., Kang *et al*., 2010; Rhode *et al*., 2012; Aguilar-Espinoza *et al*., 2014; De Wit and Palumbi, 2013; Gruenthal *et al*., 2014). SWFSC partners have been developing

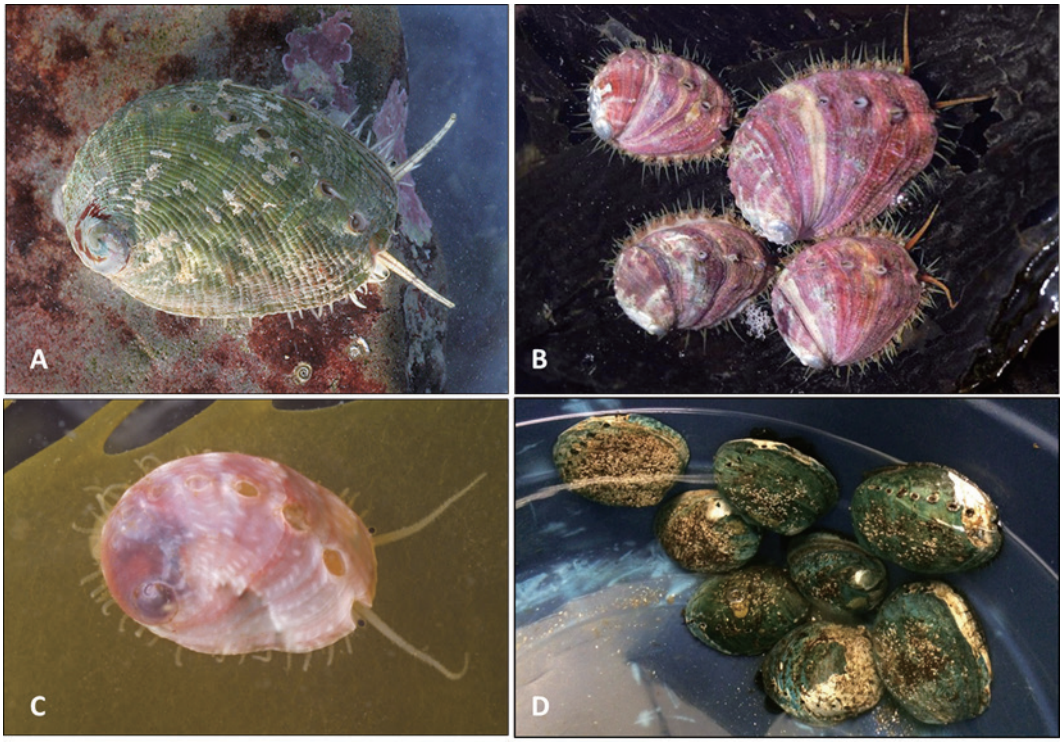


**Fig. 2.** Green abalone (*H. fulgens*) (a), white abalone (*H. sorenseni*) (b), pink abalone (*H. corrugata*) (c), and black abalone (*H. cracherodii*) (d)

suitable parentage and kinship microsatellite panels, genotyping broodstock animals, and applying these markers to monitor survival in outplanted green abalone. Genetic linkage maps have also been constructed for several abalone species using various genetic methods (e.g., amplified fragment length polymorphisms (AFLPs), microsatellites, and single nucleotide polymorphisms (SNPs)) (Liu *et al*., 2006; Sekino and Hara, 2007; Vervalle *et al*., 2013). While linkage-mapping and other genetic studies have started to improve culture practices for abalone, the usefulness of these studies is restricted by the low marker density that limits an understanding of mechanisms controlling traits of interest (Jones *et al*., 2013).

Next generation sequencing has opened the door for extensive and explorative genetic research on abalone, and several studies were recently published examining transcriptomic data for species including the domestically important red abalone (Franchini *et al*., 2011; Bester-Van der Merwe *et al*., 2013; De Wit and Palumbi, 2013). Recently, researchers working with the SWFSC used restriction site associated DNA sequencing (RAD-Seq) methods to identify genome-wide SNP markers in green abalone and examine population structure in wild populations (Gruenthal *et al*., 2014). However, transcriptomic and RAD-mapping analyses were also limited by the paucity of genomic information available for abalone; without knowledge of the genomic structure, it is very difficult to ascertain coverage depth in these studies (Franchini *et al*., 2011; Arai and Okamura, 2013). Additionally, abalone species are poorly represented in public genomic resource databases compared with other aquaculture species (Franchini *et al*., 2011), further limiting usefulness of the available data.

To develop the genomic resources that have benefited other breeding programs, we are working to generate a *de novo* genome assembly for the commercially valuable red abalone, generate tissue specific transcriptomes in red abalone, and conduct comparative genomic analyses through resequencing the pink, green, black (*H. cracherodii*) and white (*H. sorenseni*) abalone. Genomic resource development in red abalone will help identify and characterize economically and/or biologically relevant variation among the abalone species. This information will be an important step toward understanding of the genetic basis of traits that currently limit the growth of domestic abalone culture (i.e., infectious disease, thermal tolerance, slow growth, and the difficulty in determining sex). This research will greatly advance the state of knowledge of abalone culture and improve productivity in the U.S. abalone industry. The work will also provide insight for recovery efforts for the decimated endangered white and black abalone populations.

## Methods

Mature and sexed live red abalone specimens will be sampled for both the genomic and transcriptomic sequencing. Sampling for the transcriptomic work will involve collection of several tissue types (e.g. foot muscle, gonad, digestive gland, epipodium). Sexed (male and female) pink, green, white and black abalone specimens were collected from the abalone culture tanks at the SWFSC and from the California Department of Fish and Wildlife.

Genomic DNA samples were stored in vials containing 95% ethanol; RNA samples submerged in RNAlater (Qiagen) and stored at -20 °C until use. Genomic DNA and RNA were extracted using DNeasy, Genomic Tip, and RNeasy kits (Qiagen), respectively.

Following preparation of the abalone specimens and quality assessment, the DNA and RNA samples were sent to the DNA facility at ISU (Ames, IA) for Illumina and PacBio library preparation and sequencing (Fig. 3). Sequencing will be conducted with the Illumina Hiseq 2500 on two lanes of 150 bp paired-end read data and four lanes of Illumina mate-paired end libraries, with large insert sizes of approximately 12 kb, for at least 100X genomic coverage. Twenty smart cells of PacBio data will be collected for approximately 5x coverage of the genome with expected average read lengths of 6 kb. The resequencing of the other abalone species will involve four lanes of 100bp paired-end Illumina sequencing. For all sequencing data, half of the lanes/smart cells will be male and half will be female.

Raw sequencing reads will be assembled with AllpathsLG (Gnerre *et al*., 2011) and MaSuRCA (Neale *et al*., 2014). The PacBio reads will either be error

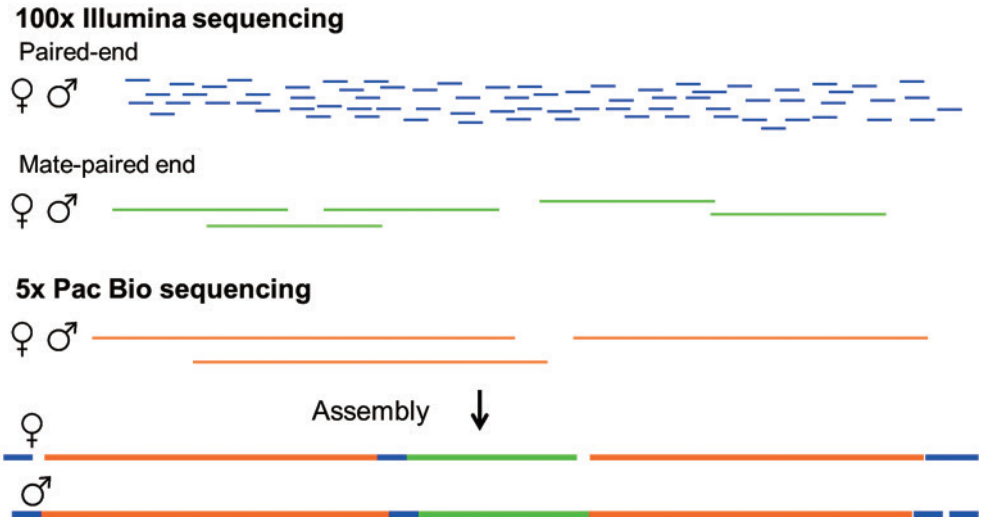


**Fig. 3.** The sequencing strategy using Illumina and PacBio technologies to generate the red abalone (*H. rufescens*) genome

corrected and included in the genome assembly as long "Illumina" reads, or used in super scaffolding after assembly. Transcripts will be assembled *de novo* from the available transcriptome data using Trinity (Haas *et al*., 2013). These transcripts will be aligned to the draft genome produced in the first step. Transcripts that map uniquely to the ends of two scaffolds will be used as evidence to join the two scaffolds. If the transcript alignment spans multiple small scaffolds (one or two exons per scaffold), multiple small scaffolds could be joined. We will use L_RNA_scaffolder (Xue et al. 2013) to perform this step. Gene models will be generated using MAKER (Holt and Yandell, 2011) genome annotation pipeline. The final set of genes will be predicted combining the results from all the predictors, EST evidence, protein alignment and BLAST similarity to other closely related genomes.

Raw reads from the other four species will be aligned to the red abalone genome using GSNAP (Wu and Nacu, 2010). Single Nucleotide Polymorphisms (SNPs) and Insertions/Deletions (InDels) will be called using GATK (McKenna *et al*., 2010). A pseudogenome of each of the four species will be generated using the aligned read file and the SNPs file. The pseudo genome assemblies will be directly comparable to each other and have annotations that correspond to annotations in red abalone, which will provide straightforward cross-referencing among red, green, pink, white, and black abalone gene models.

## Results and discussion

Abalone samples are currently being prepared for sequencing and therefore no sequencing results are available at this time. However, it is expected that the *de novo* assemblies of the resequenced species will be explored for gene loss or gain of candidate genes that are known or suspected to be involved in sex, disease resistance, thermal tolerance, and growth rates (Brokordt *et al*., 2015a; Choi *et al*., 2015; Liang *et al*., 2014; Zippay and Hofmann, 2010; Klingbunga *et al*., 2009; Liu *et al*., 2006). Comparison of synonymous and nonsynonymous nucleotide substitution rates in homologous genes will be performed to measure the evolutionary distance among the five abalone species and detect genomic regions under selective pressure. Genes undergoing selective pressure will be interesting from an aquaculture perspective as they may relate to local adaptation and relevant genetic variation among the species (e.g. thermal tolerance, disease resistance).

Identification of sex determining regions that are specific to one sex will also be explored using the coverage depth of aligned reads to the red abalone genome assembly. Since one male and one female will be sequenced for each species, regions of the genome that show reduced coverage (typically one-half of the coverage for each sex, or one-fourth of the coverage of the combined male/female raw reads)

compared to the rest of the genome are prime candidates for a sex-determining region (Vicoso *et al*., 2013). Markers in these regions will be designed and tested by SWFSC to determine if a sex specific marker can be identified. This will be done for all five species where male and female individuals can be identified for sequencing.

To achieve the greatest impact on sustainable abalone aquaculture, we will create a genomic toolbox for abalone species that facilitates the integration of very large sequencing data sets, molecular markers, QTL data and genetic maps into an easy-to-use web interface. The website will include a Genome Browser utilizing JBrowse (Skinner *et al*., 2009) for visualization of the red abalone genome assembly and tracks that display the raw read and *de novo* assembled alignments of the other species. The genome assembly for red abalone and the pseudo genome assemblies for the other abalone species will be available for download for each species and deposited in the appropriate databases (e.g. NCBI-GenBank).

These tools will enable abalone aquaculture to move past the primary bottlenecks in production by moving toward a marker assisted selection model with breeding and/or hybridization programs that exploit natural diversity in abalone species to improve production capacity and efficiency. By selecting individual abalone or developing hybrids with desirable traits (e.g., disease resistance, thermal tolerance, and growth rate) the value of the aquaculture product will be greatly improved. In support of conservation and restoration efforts, the developed genetic toolkit can be used to monitor the health and viability of wild populations and guide breeding programs for stock replenishment.

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(1) De Wit P. and Palumbi S., 2013: Transcriptome-wide polymorphisms of red abalone (*Haliotis rufescens*) reveal patterns of gene flow and local adaptation. *Mol. Ecol.* 22, 2884-2897.

The authors of this study tested whether signals of environmental selection could be detected in samples of red abalone (*Haliotis rufescens*) collected from three locations in California: Monterey Bay, Sonoma, and north of Cape Mendocino. These particular areas are especially distinct in terms of their temperature, aragonite saturation, exposure to hypoxia stress, and disease pressure; as such, the authors hypothesized that genes related to shell biomineralization, resistance to hypoxia, temperature tolerance, and resistance to pathogens would show the strongest signals of local adaptation. The authors tested this by conducting RNA-Seq analyses on mantle tissue of 39 red abalone individuals from the above locations. A total of 21,579 SNPs were genotyped for each individual, and out of these 691 showed significant differentiation. From this set of 691, 163 loci could be identified through BLAST annotation; many of these genes had functions related to biomineralization, energy metabolism, heat-, and disease- or hypoxia-tolerance. These genes are now candidates for further studies to look for signals of local adaption.

(2) Franchini P., van der Merwe M., and Roodt-Wilding R., 2011: Transcriptome characterization of the South African abalone Haliotis midae using sequencing-by-synthesis. BMC Res. Notes 4, 59.

This study describes the use of applying next generation sequencing technology to develop molecular tools for a South African abalone species, Haliotis midae. They use the Illumina Genome Analyzer II to generate 25 million sequences. Using the transcriptome sequences, they did a de novo assembly that resulted in 27,761 contigs with an average length of 260 bp. Importantly, although abalone have a relatively poor representation in genome databases likely due to their large genome size), a good number of the contigs had BLAST matches to known annotated genes in Genbank; with a stringent e-value set, 16.8% of the contigs had a homologous BLAST match against Genbank. These sequences were assigned to functional categories using GO and KOG databases. The authors were also able to use this data to identify thousands of SNPs, and out of those, they developed 420 primer sets.

(3) Gruenthal K., Witting D., Ford T., Neuman M., Williams J., Pondell, D., Bird A., Caruso N., Hyde J., Seeb L., and Larson W., 2014: Development and application of genomic tools to the restoration of green abalone in southern California. *Conservat. Genet.* 15, 109-121.

The authors describe the use of next generation sequencing technology to develop an extensive set of markers to test population structure and effective population size in green abalone in Southern California. In this study, RADSeq restriction site associated DNA sequencing) was used to generate millions of short sequences, from which, many thousands of SNPs may be identified that span a greater proportion of the genome compared to previous types of marker development. A total of 1209 polymorphic SNPs were developed from this sequencing. While the extensive set of markers did not detect population structure in green abalone in the range that was sampled, they were able to estimate an effective population size Ne) of 1,100-3,600 individuals. Importantly, this work generated valuable genomic resources that can be used to further build the set of tools available to study Haliotis species.

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# Culture Protocols and Production of Triploid Purple-Hinge Rock Scallops

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**Abstract**: The goal of this ongoing research and outreach is to expand the West Coast shellfish industry through creation of triploid seed and demonstration of efficient culture methods for the native purple-hinge rock scallop (*Crassadoma gigantea*). Shellfish aquaculture is a low trophic level means of seafood production that provides many benefits to coastal communities and the environment, while at the same time increasing the supply of locally produced safe and nutritious seafood. There is a strong desire to develop native species for aquaculture development to diversify the shellfish industry and help to avoid concerns often voiced today about the use of non-native species. While new native species of shellfish for aquaculture are highly sought after, there are also genetic concerns associated with rearing native species for aquaculture using hatchery-reared seed that may have undergone significant domestication selection or been produced from distant broodstock populations. This may occur as a normal consequence of rearing in the hatchery environment or through highly directed selection, crossbreeding, or other means to genetically change the production characteristics of the organism. These risks are significant and must be addressed to realize the potential for growth of the U.S. west coast shellfish industry. Issues associated with potential genetic risk to wild rock scallop populations could be resolved through the creation of tetraploid scallop stocks, which could be mated to diploids, producing 100% triploid offspring, or by using chemical means. Scallops were successfully spawned and cultured at the Taylor Shellfish hatchery in Washington State and at the Bodega Marine Laboratory of UC Davis in Bodega Bay, California. Growth and survival of larvae was highly variable among batches and populations, despite broodstock maintained in common conditions and similar larval dietary rations. Causes for this variability and generally low larval survival are being investigated. Initial efforts to culture scallop larvae relied mainly on C-Isochrysis sp. These efforts produced weak larvae with low survival and nutrition was identified early as a limiting factor. Subsequent efforts relied on a mixed diet of C-Isochrysis sp., Nannochloropsis sp., Pavlova sp., Chaetoceros sp., and Thallasiosira sp. The optimal timing for production of 3N scallops by inhibition of second polar body extrusion using 6-DMAP has been determined to be a 20 min treatment 55 - 60 min post fertilization at 17 °C. The optimal dosage of 6-DMAP for production of 3N scallops by inhibition of second polar body extrusion has been determined to be 425 uM.

**Key words**: Purple-hinged rock scallop, *Crassadoma gigantea*, triploid, 6-DMAP

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A bacteriological study was carried out in a scallop hatchery in western Norway. The hatchery had suffered severe mortalities during the larval stages, and prophylactic use of antibacterial agents was necessary to produce larvae. A number of bacterial strains were isolated from the hatchery. A challenge test was performed with the isolates. Six of the strains caused mortalities not statistically different from *Vibrio pectenicida*, a known scallop pathogen. From 16s rDNA analysis on these strains, the phylogenetic tree indicated two groups of apparent pathogens; one strain that resembles the Alteromonas/Pseudoalteromonas group and a cluster of strains that resemble the species *Vibrio splendidus*. Since the antibacterial agent used in the hatchery was chloramphenicol, which is now banned in Norway and Europe, application of alternative antibacterial agents were investigated. In this study the minimum inhibitory concentration (MIC) values of chloramphenicol, florfenicol, fluemequine, the combination trimethoprim/sulfadiazine, oxytetracycline, oxolinic acid and Pyceze on bacterial strains isolated from scallop larvae were investigated. Based on these MIC values, procedures for the treatment of scallop larvae with antibacterial agents were evaluated. Since the therapeutic procedures will be used in a marine environment, the antagonizing effect of seawater on some of the antibacterial agents was measured. For fluemequine and trimethoprim/sulfadiazine the average MIC values increased significantly when using seawater with salinity of 25 ppt compared to 2% NaCl.

(4) Caines J. and Crocker K. 1999: Hatchery production of sea scallop spat (*Placopecten magellanicus*) in Newfoundland, Canada.

A commercial hatchery for production of sea scallop spat was commissioned in 1995 at Belleoram, Newfoundland, by the provincial Department of Fisheries and Aquaculture. Four annual production seasonal cycles have been completed to date, where spat have been transferred to grow-out at shellfish farms. The annual yield from the hatchery has been 2 – 25% of the planned target of 20 million spat per year. This presentation will report our experiences in 1998 regarding algal culture, broodstock conditioning, larval growth, spat settlement and growth, and bacteriological monitoring of water quality. Algae (flagellates and diatoms) were grown in a continuous culture system purchased from Seasalter Shellfisheries in England. The intent was to provide scallop larvae and spat with a diet of mixed species of varied lipid and carbohydrate content. The following flagellates and diatoms were cultured in 500-L polyethylene bags under continuous light at 22 degree C with the addition of CO sub(2): *Isochrysis galbana, T.Iso, Pavlova lutheri, Tetraselmis suecica, Chaetoceros calcitrans, C. ceratosporum, C. muelleri, Thalassiosira pseudonana, T. weissflogii, and Chroomonas salina*. The growth rate was approximately 0.30 and 0.35 divisions/day for the flagellates and diatoms respectively.

(5) Meng Q., Bao Z., Wang Z., Wang S., and Hu J., 2012: Growth and reproductive performance of triploid Yesso scallops (*Patinopecten yessoensis*) induced by hypotonic shock. *J. Shell. Res.* **31**, 1113-1122.

The successful induction of triploid embryos or larvae has been performed in *Patinopecten yessoensis* during the past two decades. However, no research has been reported about the performance of triploid *P. yessoensis* cultured in the field. This study induced triploidy in *P. yessoensis* by hypotonic shock and compared the growth and reproductive performance of triploids and diploids reared under commercial conditions for up to 24 months. The main results of this study are as follows: Triploid scallops were smaller in size and weight compared with diploids and had a retarded absolute growth rate (AGR). After 24 months of cultivation, the mean shell height, shell length, shell width, and body weight of triploids were 9%, 10%, 9%,

and 25% less than diploids, respectively ($P < 0.01$). Although normal in sex ratio, the reproductive potential of triploids was significantly reduced. Only 87% of the triploids exhibited sex-discernible gonads during the breeding season. None of the male triploids spawned, and the percentage of female spawners among the triploid population was only 27% of that for the diploid population. The relative fecundity of triploid females was only 4% of diploid females. Triploid eggs produced mostly aneuploid larvae and had an extremely small chance of generating viable offspring when fertilized by sperm from diploid males. Most aneuploid larvae died before the D-shaped stage, and no survival exceeded seven days. The potential to yield viable offspring from the triploid population was estimated to be only 4% of that of the diploid population. Despite the growth disadvantage of triploids, this study may support, in part, the energy reallocation hypothesis because triploid AGR was similar to diploid AGR (2% variance) during the sexual maturation season. Our results also indicate that there would be no growth advantage, but instead a disadvantage, for triploid *P. yessoensis* growing at the experiment site. In addition, this research provides the first evidence that viable triploid molluscs can be induced by hypotonic shock, of which the practical and evolutionary implications are also discussed.

(6) Cogswell A. T., Roach S. E., and MacDonald B. W., 2006: Triploid bay scallops (*Argopecten irradians*): induction methodology, early gonadic development and growth. Canadian Technical Report of Fisheries and Aquatic Sciences, 2635pp.

Triploid (3N) Pacific oysters (*Crassostrea gigas*) account for more than 50% of total oyster production in the US. The success of this species has created an interest in producing triploids of other commercial shellfish species, including scallops, clams and mussels. Here, we report on 3N induction trials with the bay scallop, *Argopecten irradians*. The most commonly employed 3N induction technique involves exposing early embryos to chemicals (e.g., Cytochalasin B (CB)). CB inhibits the release of the second polar body immediately following fertilization, causing retention of both pairs of female chromosomes in addition to the male chromosome set. It does this by disrupting actin polymerization. Four concentrations of CB were evaluated for ability to produce 3N larvae. The efficacy of current and proposed 3N induction techniques (i.e., 4N x 2N crosses) and the commercial potential of 3N bay scallops are discussed.

(7) Ruiz-Verdugo C. A., Allen S. K., and Ibarra A. M., 2001: Family differences in success of triploid induction and effects of triploidy on fecundity of catarina scallop (*Argopecten ventricosus*). *Aquaculture* **201**, 19-33.

Mass induction of triploidy in the catarina scallop (*Argopecten ventricosus*) results in low success in the percentage of triploids produced. To understand whether this is a treatment effect affecting all eggs equally, families were individually induced to triploidy with cytochalasin-B (CB), comparing their survival from egg to D-larvae and spat and the percent of triploidy within families. Differences in percent triploidy success were evident between families, obtaining some with no triploids and some with high triploidy. Among the possible causes for these differences are quality of eggs, different developmental rates, and differences in susceptibility to the treatment (CB or DMSO) itself. Regardless of those differences, overall triploidy production was increased by inducing individually eggs of each scallop rather than in mixed egg batches. In the first experiment, it was improved by 17%, and in the second experiment by 42%, as indicated by the weighted mean of triploids among the families and when compared with previous results with this same species, where triploidy success was 58%. In a second experiment with three different families, the growth and fecundity of triploid and diploid catarina scallop were evaluated. The growth superiority of triploids was confirmed. The results indicated that triploid catarina scallop had a significantly reduced fecundity when compared with diploid scallop. The reduced fecundity appears to be mostly of a random nature, possibly associated with a reduced capability to produce balanced gametes. Whereas the successful production of tetraploid catarina scallop from fertile triploid scallop is in principle possible, the low number of eggs shed by triploid catarina scallop could diminish that success rate, even more if single triploid females are required to optimize tetraploid induction.

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# Big Data in Agriculture and the USDA/ARS Initiative

Jeffrey SILVERSTEIN*

**Abstract**: In recent years, technological achievements have permitted the relatively inexpensive and rapid production of vast amounts of data. The large and often complex datasets produced in the scientific sphere demand new approaches to gain value and to turn data into information. Management of the growth in the volume, variety, and velocity of data is often referred to as the 'Big Data problem'. The Agricultural Research Service of the U.S. Department of Agriculture (USDA/ARS) has long been a strong, science-based, problem solving agency. In the past, our computational infrastructure has primarily been based on meeting administrative needs and security requirements, whereas computational, analytical, and sharing of scientific data were regarded as secondary priorities. The Big Data problem has required a reassessment of scientific computing needs. In February 2013, we held a workshop led by ARS scientists to assess scientific data needs, and this resulted in a $25 million initiative to develop the USDA/ARS capacity to collect, share, and analyze Big Data. Our Big Data initiative contains three elements. First, we have developed a dedicated scientific research network (SCInet) which will leverage Internet2 to facilitate large scale transfer of research data at high-speeds with low latency. Second, we have constructed a high-performance computing (HPC) system with high memory, high processing capacity and the potential to burst computational workloads to commercial resources when necessary. SCInet and the HPC have been largely constructed and connected in the first 18 months of the initiative. Finally, we are developing a virtual research support core of individuals who will provide scientific computational and informatics support. These individuals will work with agency scientists to facilitate specific projects and will also develop standardized solutions and training for common challenges. Key steps of this initiative, 1) determining the needs of the agency, 2) developing a plan to connect more than 90 locations in the agency, 3) building the technical capacity in our labs, and 4) controlling costs due to restrictive budgets are challenges that will be shared and discussed.

**Key words**: Big Data, Internet2, informatics

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# Genomic Selection in Aquaculture

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**Abstract**: Recent advances in genotyping and sequencing technology facilitate handling thousands of SNPs from hundreds of samples. This enables us genomic prediction of breeding values for economically important traits not only for livestock and plants but also for aquaculture species. In this article, we review six empirical studies on genomic prediction in aquaculture. Implementation of genomic selection for aquaculture is steadily progressing with no doubt, and we will see the tangible and practical outcomes of genomic selection within several years.

**Key words**: Genomic selection, NGS, selective breeding, SNP

## Introduction

Genomic selection (GS) introduced by Meuwissen *et al.* (2001) is a form of marker-assisted selection where the genomic estimated breeding value (GEBV) is predicted as the sum of additive genetic effects of genome-wide dense genetic markers. All loci affecting the trait (e.g. quantitative trait loci; QTL) are supposed to be in linkage disequilibrium with at least one marker. Therefore, to predict marker effects for highly polygenic traits, such as human height and disease (Yang *et al*., 2015; Abraham *et al*., 2015), hundreds of thousands of genetic markers are required. When the idea was proposed for the first time, it was highly challenging to genotype thousands of markers from hundreds of specimens. However, recent advances in genotyping technology put it into practice.

Succinctly, GS consists of two steps. The first step is estimation of marker effects in a test (training) group, and the second step is prediction of GEBV of selection candidates (validation group), sibs and/or relatives of individuals of the training group. There are two major methods for marker effect estimation: GBLUP and BayesB. To use these methods, a prior distribution of marker effects is required, and one of the major differences between the two methods is the assumption of the distribution and variances of marker effects. Normal distribution with constant variance is assumed for GBLUP, and therefore the model is equivalent to a conventional BLUP (PBLUP) animal model; a numerator relationship matrix estimated from pedigree information is substituted by a realized relationship matrix estimated from genome-wide SNP information in GBLUP. On the other hand, non-normal distribution is assumed for BayesB, where only a subset of markers has effects and these effects follow a reflected exponential distribution. Simulation data revealed that the prediction accuracy of these two methods superior to that of traditional BLUP (Meuwissen *et al*., 2001). This is mainly because genomic prediction takes the Mendelian sampling term into account (Daetwyler *et al*., 2007). Because of its high prediction accuracy,

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**Table 1.** Summary of species, traits, genotyping platform and prediction method researches about genomic selection for aquaculture

| Species | Trait | Genotyping platform | Prediction method | Citation |
|---|---|---|---|---|
| Atlantic salmon (*Salmo salar*) | parasite resistance | SNP array | GBLUP | Ødegård *et al.* (2014) |
| | fille color | | | |
| | body size | SNP array | GBLUP | Tsai *et al.* (2015) |
| | parasite resistance | SNP array | GBLUP | Tsai *et al.* (2016) |
| Rainbow trout (*Onchorhynchus mykiss*) | disease resistance | SNP array, RAD | ssGBLUP, wssGBLUP, BayesB, BayesC | Vallejo *et al.* (2016) |
| Yellow croaker (*Larimichthys crocea*) | body size | Genotyping-by-Sequencing (GBS) | GBLUP, emBayesB | Dong *et al.* (2106) |
| | fatty acid composition | | | |
| Yesso scallop (*Patinopecten yessoensis*) | body size | 2d RAD-seq | GBLUP, LASSO, Bayesian LASSO, BayesA, BayesB, rrBLUP | Dou *et al.* (2016) |
| Coho salmon (*Onchorhynchus kisutch*) | body size | ddRAD-seq | GBLUP | Hosoya *et al.* (submitted) |

* adjusted prediction accuracy: the correlation between the GEBV of the test population and the actual phenotypes divided by the square toot of the heritability

genomic selection is now widely used in livestock and plants, but there has been limited realization in aquaculture.

With the aids of new genotyping technologies, such as SNP array and restriction enzyme associated DNA sequencing (RAD-seq), genomic selection becomes applicable not only for the major livestock species and plants but also for aquatic species. So far, at least six studies have reported the possibility of genomic selection in aquatic species (Table 1). In this report, we review these studies. Five are about finfish, and the other is about scallop. Three of the studies were done by researchers from North Europe, one was from North America, and the other from Mainland China. All these studies have been published within the last couple of years, and more attempts of GS for a wide range of aquatic species will be published from all over the world.

### Atlantic salmon

As mentioned above, there are three articles on Atlantic salmon, and one of them (Ødegård *et al.*, 2014) was the first report attempting to incorporate GS in aquaculture. Atlantic salmon is one of the most important aquaculture species as its estimated global economic values was $ 7.8 billion in 2010 (FAO, 2010). It is also known as a genomic model species for aquaculture as its chromosomal-level genome assembly (GCA_000233375.4) is available (Lien *et al.*, 2016). Additionally, dense SNP chips (Axiom® Salmon Genotyping Array, Affimetrix) are commercially available. All those three studies used SNP chips for genotyping. Analyzed traits for this species are salmon lice (*Lepeophtheirus salmonis*) resistance (LR: estimated heritabilities = 0.14) and fillet color (estimated heritabilities = 0.43) (Ødegård *et al.*, 2014); weight (estimated heritabilities = 0.5) and length (estimated heritabilities = 0.6) (Tsai *et al.*, 2015); LR (estimated heritabilities = 0.2 - 0.3) (Tsai *et al.*, 2016).

GEBV for these traits were predicted by GBLUP. Relatively high prediction accuracy was generated for traits with higher heritability. Interestingly, while over 50k SNPs are required to gain better prediction accuracy for livestock species, 5k SNPs are sufficient for Atlantic salmon within-family prediction. However, for prediction across populations or year groups, where genetic relationships are more distant between training and validation groups, accuracy was substantially low even when 30k SNPs were used for the prediction (Tsai et. al., 2016). In such cases, using larger population sample sizes and higher-density SNP genotypes will improve prediction accuracy. Thus, phenotyping training populations consisting of animals closely-related to the selection candidates and genotyping relatively lower-density of SNPs (5k) will gain better cost performance for commercial salmon breeding schemes. This strategy is supported by simulation

studies (e.g. Sonesson and Meuwissen, 2009) and generally works for populations from most aquaculture breeding programs since relatively limited numbers of broodstock are used in most of the aquaculture breeding programs.

**Rainbow trout**

Vallejo *et al*., (2016) reported an attempt to implement genomic selection for bacterial cold water disease (BCWD) resistance to the National Center for Cool and Cold Water Aquaculture (NCCCWA) BCWD resistance breeding line. Several major resistance QTL (including a QTL of PVE = 58%) have been detected for the trait using the same breeding line (Palti *et al*., 2015). However, because of complex genetic architecture and high genetic variation, the authors postulated that GS gives better performance for genetic improvement in BCWD resistance than the marker-assisted selection based on QTL analyses.

Genomic predictions for survival days and survival status were done using single step GBLUP (ssGBLUP), weighted ssGBLUP (wssGBLUP), BayesB and BayesC. ssGBLUP is an integrated version of GBLUP and PBLUP. While GBLUP and PBLUP use either genomic or pedigree information to construct a relationship matrix (i.e. G matrix and A matrix), ssGBLUP uses both types of information (H matrix) (Aguilar *et al*., 2010). wssGBLUP is an extended version of ssGBLUP, where QTL effects are weighted. Overall predictive abilities were similar among the GS models and PBLUP. ssGBLUP showed slightly better performance compared to the Bayesian methods. This will be partly because a large number of individuals was used for the training population in ssGBLUP. Construction of H matrix for ssGBLUP seems somewhat complicated since it includes "tuning" steps. However, this step can increase the performance of genomic prediction. Moreover, because individuals with phenotype, but without genotype, data can be included in the training population under ssGBLUP model, it is possible to increase the sample size of training population without increasing genotyping cost. Therefore, ssGBLUP will be one of the most powerful solutions for genomic prediction of GEBV. However, pedigree records are not typically maintained in aquaculture because of the difficulty in the tagging of individual larvae and the maintenance of separate families. Since the number of selection candidates is large and the value of the selection candidate is low for aquaculture species, the cost of pedigree recording may not be negligible, and this will be a major obstacle to implement ssGBLUP into selective breeding programs in aquaculture.

**Large yellow croaker**

Genomic selection is also attempted on non-salmonid fish. Dong *et al*. (2016) reported the feasibility of genomic selection in the traits of growth rate and meat quality (i.e. the percentage of n-3 highly unsaturated fatty acids (n-3HUFA) in muscle) of large yellow croaker (*Larimichthys crocea*). They first estimated heritability and then compared predictive ability between GBLUP and emBayesB. emBayesB is an alteration of BayesB. The Markov Chain Monte Carlo (MCMC) technique (i.e. Gibbs sampling) used in BayesB for the model fitting requires large computational time with dense SNP data. On the other hand, MCMC is replaced by the Expectation-Maximization (EM) algorithm in the emBayesB approach. This enables us fast but accurate GEBV prediction (Shepherd *et al*., 2010).

Estimated heritability for body weight, body length and n-3HUFA were 0.604, 0.586 and 0.438, respectively. GBLUP was superior to emBayesB in the predictive abilities for body weight and body length (GBLUP: BW = 0.41 and BL= 0.4; emBayesB: BW = 0.37 and BL= 0.37), but not for the n-3HUFA trait with relatively lower heritability (GBLUP: 0.30; emBayesB: 0.32). The differences are probably due to the number of QTLs affecting the traits; the number of QTLs is expected to be smaller for the n-3HUFA trait than that for body weight and body length. The results suggested the importance of testing algorithms on specific traits to gain the best prediction performance. The authors also estimated that at least 1000 individuals in the training population are required to get prediction accuracy of 0.8 by fitting the curve of prediction accuracy. The number will be affordable when GBS is used for genotyping.

**Scallop**

A species other than finfish tested for the

possibility of implementation of GS is Yesso scallop (*Patinopecten yessoensis*) (Dou *et al*. 2016). In the study, the performance of 2b-RAD sequencing methods (a type of RAD-seq, or genotyped-by-sequencing GBS), where the uniform fragments produced by type IIB restriction endonucleases are sequenced: Wang *et al*., 2012) was evaluated for shell length, shell width and shell height. The prediction accuracies calculated under models of GBLUP, LASSO, Bayesian LASSO, BayesA, BayesB and rrBLUP were compared.

The real dataset involved 349 individuals consisting of two full-sib families and three bi-parental families. A high-quality 2,364 putative SNPs with an average calling rate of 84% was obtained by 2b-RAD (minor allele frequency > 5%; SNP calling frequency > 70%). The estimated heritability of the three traits using the entire population were 0.36 - 0.48. Those values varied among families (0.28 - 0.61 for SH, 0.26 - 0.60 for SL, and 0.15 - 0.48 for SW). This implies large differences in genetic diversity among families. The (adjusted) prediction accuracies varied from 0.30 to 0.60 across the three traits, showing 2b-RAD to be a powerful and cost-effective genotyping method for GS for Yesso scallop breeding programs. The prediction accuracy of GBLUP, BayesA and BayesB outperformed the other methods across the three traits. This is partly because these three models can effectively capture the polygenic resemblance and genetic relationships (Neves *et al*., 2012; Resende *et al*., 2012; Moser *et al*., 2009). However, the performance is largely depending on the number of samples, population structure and heritability of the traits, and the three methods may not always give better performance than the others in different populations and traits.

## Conclusion

It seems a relatively small number of SNPs (~ 5k) is required for genomic prediction of aquaculture species compared to that of livestock (>50k) to obtain practical levels of prediction accuracy. Our result on coho salmon (Hosoya *et al*., submitted) is also supportive of this idea. The reason is partly because the generation is still young and LD size is large in many aquaculture breeding populations compared to the livestock populations. Although GBLUP and Bayesian methods showed good performance in prediction accuracy, it will be better to compare the performance among between prediction models before deciding the model to use because the performance is largely depending on the number of samples, population structure and heritability of the traits.

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# Genetic Selection on Animals using Pedigree, Phenotypic, and Genomic Information

Shogo TSURUTA*

**Abstract**: Genetic selection of quantitative traits in animals has been performed for a century in the United States. Especially, in the last two decades, field data analysis of dairy cattle, beef cattle, swine, and chicken has contributed to livestock industries. We have analyzed genetic components in animal production, reproduction, and disease traits using our own computer programs (so-called BLUPF90 family). Those programs have been used by breed associations and breeding companies in routine genetic evaluations. Recently, we have developed a method called single-step genomic BLUP (ssGBLUP) to predict genomic breeding values by combining pedigree, phenotypes, and genotypes (SNP markers).

With ssGBLUP, we found that the accuracy of genomic breeding values can be increased in dairy and beef cattle, pigs, and chickens by 10-30% compared with traditional breeding values. This methodology has been expanded to use a large number of genotyped animals (> 1 million). The application programs are available on our website at http://nce.ads.uga.edu/projects/programs/~. In this paper, we explain general BLUP and ssGBLUP algorithms applicable to livestock and fish breeding. Also, we show evidence of genetic progress by genetic selection and results from genomic analyses in US Holsteins.

Genomic selection has a greater advantage when more genotyped animals are available because predictions are more accurate and more complex models can be applied. When animals are genotyped in the earlier stages of life, genetic progress can be accelerated and genetic gains can be magnified. The ssGBLUP methodologies are applicable to other organisms, such as farmed fish, shellfish, anadromous fish (salmon), honey bees, and even plants.

**Key words**: Genetic selection, BLUP, genomic information

## Introduction

Genetic selection of quantitative traits in US Holsteins using genetic evaluation has been performed for 80 years in the United States (USDA-ARS Animal Improvement Program 2013). In the beginning, artificial selection was conducted using only phenotypes. Later, pedigree information was added from parent-offspring relationships, siblings, and families to all available information among related animals. Around the same time, advanced statistical methods were developed (e.g., regression analysis, least square analysis, and best linear unbiased prediction (BLUP) with an inverse of the relationship matrix) to estimate genetic parameters and to predict breeding values for economically important traits in animals. As a result, animal productions have dramatically increased in the last half-century.

Genetic progress can be equated to a function of accuracy of the selection, genetic variation (standard deviation), selection intensity, and generation interval. To accelerate genetic progress for quantitative traits, we need to increase the first three parameters and

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decrease the last one, ignoring all possible interactions. Accuracy can be increased by adding genomic information into traditional breeding values estimated with phenotypes and pedigrees. We conducted several studies with genomic information using a single step genomic BLUP (ssGBLUP) to increase accuracy in genomic breeding values (Aguilar *et al*., 2010; Tsuruta *et al*., 2011; Lourenco *et al*., 2015; Fragomeni *et al*., 2015). Genetic variation can be increased by outbreeding and mutation. Selection intensity can be increased by increasing the population size. Generation interval can be reduced by evaluating an animal's performance at the early stages of its life, using genomic information.

Unfortunately, in the US and in Japan, genetic selection in fish and shellfish has not garnered much popularity compared to other livestock. The main reasons may include 1) recording phenotypes and identifying individuals were expensive, 2) farming was much more expensive than fishing, and 3) as a result, genetic analyses were not well conducted. However, these conditions have been changing over the years. As seafood gains popularity, overfishing becomes a problem for popular species, and the balanced ocean ecosystem could collapse. Fortunately, a complete breeding cycle technology in aquaculture for some species has been successfully developed. In addition, low-cost single nucleotide polymorphism (SNP) marker information is now available for genomic selection.

The objective of this paper was to show how genetic selection has been efficiently conducted in livestock production in the US and to explain its potential for improving marine species as well.

## Materials and Methods

For 34,506 US Holstein bulls, 42,503 SNP markers were available to predict genomic breeding values and SNP marker effects for 305-day (d) milk yield and cow mortality, using ssGBLUP. Details are described in Tsuruta *et al*. (2015).

In general, there are two options in genetic evaluation processes: 1) estimating genetic parameters (heritability and genetic correlation) and breeding values simultaneously or 2) estimating genetic parameters first and then breeding values (empirical BLUP), assuming that those variance components are known. Using matrix notation, a general mixed model equation (MME) in animal breeding and genetics can be written as

$$\begin{bmatrix} \mathbf{X}'\mathbf{R}^{-1}\mathbf{X} & \mathbf{X}'\mathbf{R}^{-1}\mathbf{Z} \\ \mathbf{Z}'\mathbf{R}^{-1}\mathbf{X} & \mathbf{Z}'\mathbf{R}^{-1}\mathbf{Z} + \mathbf{A}^{-1}\dfrac{1}{\sigma_u^2} \end{bmatrix} \begin{bmatrix} \mathbf{b} \\ \mathbf{a} \end{bmatrix} = \begin{bmatrix} \mathbf{X}'\mathbf{R}^{-1}\mathbf{y} \\ \mathbf{Z}'\mathbf{R}^{-1}\mathbf{y} \end{bmatrix}$$

where **b** and **a** are unknown vectors and **X** and **Z** are design matrices for fixed and random effects, respectively. The additive genetic variance for a (breeding values) and the residual variance for e are described as

$$\mathrm{Var}\begin{bmatrix} \mathbf{u} \\ \mathbf{e} \end{bmatrix} = \begin{bmatrix} \mathbf{G} & 0 \\ 0 & \mathbf{R} \end{bmatrix} = \begin{bmatrix} \mathbf{A}\sigma_u^2 & 0 \\ 0 & \mathbf{I}\sigma_e^2 \end{bmatrix}$$

where A is a pedigree or numerator relationship matrix; **I** is an identity matrix; $\sigma_u^2$ and $\sigma_e^2$ are variance components for additive genetic and residual effects, respectively. Therefore, heritability can be calculated as $\sigma_a^2 / (\sigma_e^2 + \sigma_a^2)$. To obtain solutions for **b** (BLUE) and **a** (BLUP) from MME, the preconditioning conjugate gradient (PCG) algorithm is widely used owing to its stable convergence property (Strandén and Lidauer, 1999; Tsuruta *et al*., 2001).

For ssGBLUP (Legarra *et al*., 2009; Aguilar *et al*., 2010), simply replace $\mathbf{A}^{-1}$ by $\mathbf{H}^{-1}$ as described below:

$$\begin{bmatrix} \mathbf{X}'\mathbf{R}^{-1}\mathbf{X} & \mathbf{X}'\mathbf{R}^{-1}\mathbf{Z} \\ \mathbf{Z}'\mathbf{R}^{-1}\mathbf{X} & \mathbf{Z}'\mathbf{R}^{-1}\mathbf{Z} + \mathbf{H}^{-1} \end{bmatrix} \begin{bmatrix} \mathbf{b} \\ \mathbf{a} \end{bmatrix} = \begin{bmatrix} \mathbf{X}'\mathbf{R}^{-1}\mathbf{y} \\ \mathbf{Z}'\mathbf{R}^{-1}\mathbf{y} \end{bmatrix}$$

$$\mathbf{H}^{-1} = \mathbf{A}^{-1} + \begin{bmatrix} 0 & 0 \\ 0 & \tau\mathbf{G}^{-1} - \omega\mathbf{A}_{22}^{-1} \end{bmatrix}$$

where **G** is a genomic relationship matrix defined as follows (VanRaden, 2008):

$$\mathbf{G} = \frac{\mathbf{W}\mathbf{D}\mathbf{W}'}{2\sum_{i=1}^{n} p_i(1-p_i)}$$

where $p_i$ = allele frequency at locus $i$; **D** = weight for each locus (**I** if assuming the same variance); **W** = a design matrix as follows:

$$w_{ii} = \begin{cases} 0 - 2p_j & homozygous \\ 1 - 2p_j & heterozygous \\ 2 - 2p_j & homozygous \end{cases}$$

For a genome-wide association study (GWAS), SNP marker effects and SNP variances can be estimated by solving **a**=**Wu** where **u** is a vector of SNP marker effects (Strandén and Garrick, 2009). The BLUPF90 family programs (Misztal *et al*., 2002) for predicting genomic breeding values and estimating variance components are available at http://nce.ads.uga.edu/~.

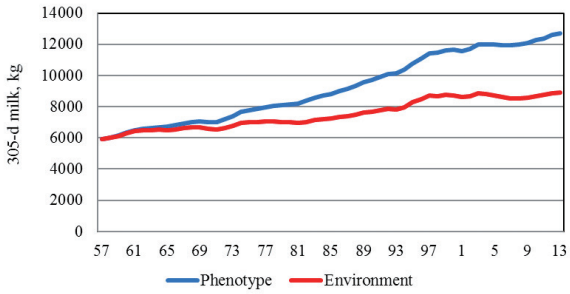


**Fig. 1.** Phenotypic and environmental trend for 305-d milk yield in US Holsteins

## Results and discussion

### Selection index

A selection index is widely used when selecting animals on multiple traits. Table 1 shows various selection indexes used in US Holsteins (VanRaden and Cole, 2014). A selection index can be weighted differently for each trait, depending on the selection goal. Each dairy farm has a unique strategy to improve their animals in that environment (e.g., focusing on milk production or cheese production).

### Genetic trend

Phenotypic and environmental trends of 305-d milk yield in US Holsteins (Fig. 1) were calculated based on cow's EBV published in 2015 from Council on Dairy Cattle Breeding (CDCB 2015). The difference between phenotypic and environmental trends, which started in 1957 assuming no genetic trend at first, can be distinguished as genetic progress or gain.

### Genomic selection

Since the USDA-ARS started genomic evaluation using SNP markers as genotypes in 2008, genomic evaluation is gaining popularity in other breeds and species. As previously mentioned in $\mathbf{H}^{-1}$, MME contains three different relationship matrices: **A**, **G**, and $\mathbf{A}_{22}$. Due to different definitions and bases on **A** (or $\mathbf{A}_{22}$) and **G**, distributions of the matrix elements look inconsistent (Fig. 2) when 42,503 SNP markers for 34,506 US Holstein bulls were analyzed (Tsuruta *et al*., 2015). The inverse matrices (A22 and G-1) can be consistent, but the base adjustment could still be required for a large population with many generations.

In Fig. 3, according to the genetic progress formula

**Table 1.** Relative weights (%) on four different selection indexes for US Holsteins

| Trait | Relative weight (%) | | | |
|---|---|---|---|---|
| | Net Merit $ | Cheese Merit $ | Fluid Merit $ | Grazing Merit $ |
| Protein | 20 | 24 | 0 | 18 |
| Fat | 22 | 19 | 23 | 20 |
| Milk | −1 | −9 | 23 | −1 |
| Productive life | 19 | 16 | 20 | 10 |
| Somatic cell score | −7 | −7 | −3 | −6 |
| Udder | 8 | 6 | 8 | 8 |
| Feet/legs | 3 | 2 | 3 | 3 |
| Body size | −5 | −4 | −5 | −4 |
| Daughter pregnancy rate | 7 | 6 | 7 | 19 |
| Heifer conception rate | 2 | 1 | 2 | 3 |
| Cow conception rate | 1 | 1 | 2 | 5 |
| Caving ability ($) | 5 | 4 | 5 | 5 |

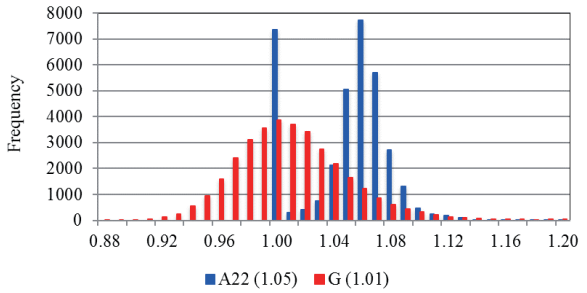


**Fig. 2.** Distribution of diagonals of genomic relationship (G) and pedigree relationship ($A_{22}$) matrices (mean values > 1.0 in parentheses indicate inbreeding) in US Holsteins

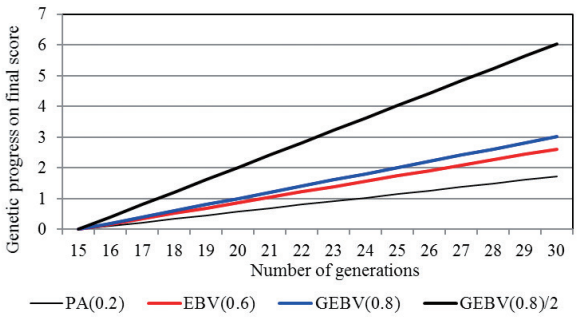


**Fig. 3.** Future prediction of genetic trend for final score in US Holsteins

described before, future genetic trends for final score with heritability (0.31) in US Holsteins were calculated with several conditions that assume that reliability in GEBV is 0.2 higher than that in EBV and reliability in EBV is 0.4 higher than that in parent average (PA). The solid thick line is the highest genetic trend when making the generation interval a half, indicating that reducing generation interval will increase genetic gains significantly. This becomes possible by genotyping and evaluating animals in the early stages of their life before sexual maturity.

## GWAS

Using the ssGBLUP algorithm, SNP marker effects and variances were estimated for 305-d milk yield and cow mortality in US Holsteins (Tsuruta *et al.*, 2015). Figs. 4(a-d) show genetic variance (%) explained by SNP markers on each chromosome for 305-d milk yield in the first lactation and cow mortality in the first three lactations for US Holsteins. The Manhattan plots indicated that the SNP variance on chromosome

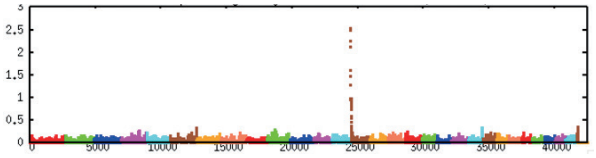

**Fig. 4a.** Genetic variance (%) explained by SNP markers for 305-d milk yield in first lactation

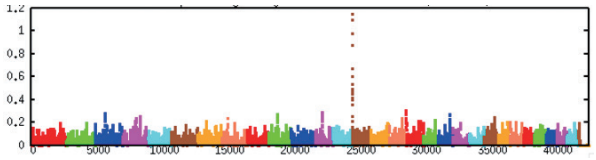

**Fig. 4b.** Genetic variance (%) explained by SNP markers for cow mortality in first lactation

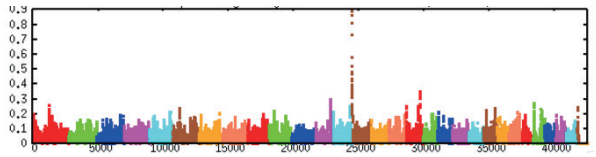

**Fig. 4c.** Genetic variance (%) explained by SNP markers for cow mortality in second lactation

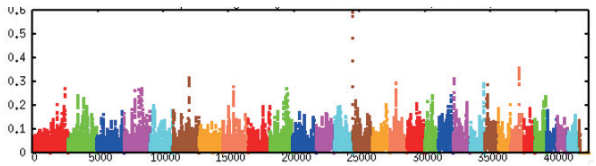

**Fig. 4d.** Genetic variance (%) explained by SNP markers for cow mortality in third lactation.

14 was significantly large (2.5%) for milk yield and the variance for cow mortality decreased as the number of lactations increased, suggesting that younger cows died for a reason more genetically related to milk production than older cows.

## Genetic selection in aquaculture

Norway has been successfully conducting genetic selection in Atlantic salmon for decades. Table 2 shows four different breeding programs that select animals for four to 11 traits.

The FAO published results for "genetic improvement" in fish (FAO Fisheries and Aquaculture Department 2015), showing some genetic progress in fish and shellfish. Selective breeding improved growth rate 50% after 10

**Table 2.** Traits presently selected in the four breeding programs for Atlantic salmon in Norway (Thomassen *et al.* 2007)

| Trait | Breeding program | | | |
|---|---|---|---|---|
| | A | B | C | D |
| Growth in freshwater | √ | √ | √ | √ |
| Body weight at slaughter | √ | √ | √ | √ |
| Early sexual maturity | √ | √ | | |
| Furunculosis | √ | √ | | |
| Infectious salmon anaemia | √ | √ | | |
| Infectious pancreatic necrosis | √ | √ | √ | |
| Dressing percentage | √ | √ | | |
| Fillet yield | √ | √ | | |
| Fillet color | √ | √ | √ | √ |
| Fillet fat | √ | √ | | |
| Deformity | √ | √ | | |
| Body shape | √ | | | |

generations in coho salmon, 20% per generation in gilthead sea bream, 10 – 13% live weight in oyster, and 60% in tilapia. Maturity and time of spawning, physiological tolerance (stress), and disease and pollutant resistances were also improved.

If genetic selection can successfully be applied, fish farming may become the most profitable option in the fish industry. If sufficient facilities and resources (ocean, river, or lake) are available, strong selection pressure can be put on breeding lines because fish and shellfish produce a large number of eggs in a single spawning (that could be many full sibs and half sibs). A superior individual within full sibs can be identified by using genotypes. If genetic selection is systematically conducted in a complete breeding cycle by recording pedigrees (at least selection lines), phenotypes, and genotypes, the genetic progress in fish farming will be maximized. Genetic selection on economically important traits for farmed fish, shellfish, and anadromous fish is environmentally friendly and sustainable because no gene or gamete manipulation is involved (i.e., only gene frequencies are changed). It also has the capacity to multiply seafood production while maintaining product quality.

## Conclusions

Genomic selection has a greater advantage over genetic selection when young animals are genotyped because predicted breeding values are more accurate than the traditional breeding values and the generation interval can be reduced. Genetic and genomic selection can be applied to any quantitative trait for marine animals, such as farmed fish, shellfish, and anadromous fish, if accurate pedigree information, sufficient phenotypes, and/or genotypes are available. Aquaculture has great potential in food supply all over the world, and fish production can be significantly increased by sustainable genetic selection.

## Acknowledgements

This paper was made possible by numerous studies in our animal breeding and genetics group at the University of Georgia, including current faculties (Ignacy Misztal and Daniela Lourenco), postdocs (Yutaka Masuda and Breno Fragomeni), a former postdoc (Andres Legarra), and a former graduate student (Ignacio Aguilar). We thank the livestock industry and USDA for their continued support.

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(5) Fragomeni B. O., Lourenco D. A. L., Tsuruta S., Masuda Y., Aguilar I., Legarra A., Lawlor T. J., and Misztal I., 2015: Use of genomic recursions in single-step genomic BLUP with a large number of genotypes. *J. Dairy Sci.* **98**, 4090-4094.

The authors have been working on estimation of genetic parameters and prediction of breeding values for economically important traits in animals. They have recently developed a method called single-step genomic BLUP (ssGBLUP) to predict genomic breeding values by combining pedigree, phenotypic, and genomic information and have implemented the method to their existing computer (BLUPF90) programs (e.g., BLUP, REML, and Gibbs Sampler) to use SNP marker information. Recently, they published several studies of ssGBLUP to maximize accuracy and minimize bias in genomic breeding values, applying this methodology to dairy cattle, beef cattle, pigs, and chickens. Currently, they have been working on how to include a large number of genotyped animals (> 1 million) in genomic evaluation. Breed associations and breeding companies that have been using their software are now planning to introduce ssGBLUP to routine evaluations. The same or similar methodology can be applied to fish breeding. The BLUPF90 family of programs and the manual are available at http://nce.ads.uga.edu/~.

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# Exploring Transcriptomic Patterns in Slow- and Fast-growing Seriola dorsalis Larvae

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**Abstract**: *Seriola* species have traditionally been a major component of global commercial and recreational fisheries, and in recent years, aquaculture value of these fish has grown into a ~$1.3 billion industry. The California yellowtail (*Seriola dorsalis*) is a strong candidate for development of offshore commercial aquaculture in southern California and neighboring Baja California. Although production from broodstock populations has been successful, it has not yet reached a sustainable level where it can satisfy the aquaculture demand, largely due to difficulties from highly variable survival and growth rates through the larval stages. Given the extremely fast growth and the major physical changes that occur during the earliest life stages, one way to examine variability in survival and growth is at the gene and molecular levels across those early-developmental periods. To improve our understanding of the molecular processes underlying development, we examined RNA-Seq profiles for several early life stages of yellowtail, categorized as either slow- or fast-growing. Gene expression was measured in three replicates of pooled larval samples at 2, 7, and 17 days post hatch for these two growth categories. Using the Illumina platform, an average of sixty million reads were obtained per replicate; genes of related function were sorted into clusters, and those found at high frequency in the differential gene expression set were identified. Differences in molecular pathways, biological processes, and gene regulating patterns between the two fitness groups were examined. There were many differentially expressed genes across developmental stages and between the fitness groups. For example, genes involved in oxidative phosphorylation pathways revealed interesting patterns both across developmental stages and between slow- and fast-growing larvae.

**Key words**: Aquaculture, Seriola dorsalis, RNA-Seq, growth-heterogeneity, larvae

## Introduction

In the United States, the native California yellowtail (*S. dorsalis*) is considered a prime candidate for aquaculture development in Southern California and is the target for an aquaculture industry poised for rapid growth in North America, with offshore net pens in place off northern Baja California, and proposals to develop similar pens off the coast of Southern California. However, the feasibility of commercial-scale culture for this and other *Seriola* species hinges on reliable juvenile production from broodstock populations. In Japan, *Seriola* culture has traditionally relied on harvesting and growout of wild juveniles, but more recently, focus has shifted to closed life-cycle production to help alleviate pressure on natural populations and to generate a more predictable supply of juveniles (Ohara *et al*., 2005;

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Ozaki *et al*., 2013; Aoki *et al*., 2014). Similar to aquaculture production of other marine fish, hatchery production of *Seriola* species in the U.S. and elsewhere has been hindered by high larval mortality (Woolley *et al*., 2014) and a propensity for growth heterogeneity and deformities developed during larval and early juvenile stages that limit the production capacity and efficiency. Growth heterogeneity may be especially problematic for *Seriola*, as it has been estimated in *S. lalandi*, for example, that small fish constitute up to 42% of an offspring population, with many of those individuals exhibiting developmental issues leading to their demise (Moran, 2007).

Although other aspects of research (e.g., disease, nutrition, environmental factors, tank conditions) have supported the development of the yellowtail aquaculture industry, genetic improvement programs for *Seriola* culture are in the beginning stages (Ozaki *et al*., 2013). To date, genetic research aimed at improving *Seriola* aquaculture has included: construction of linkage maps at varying levels of coverage (including a map utilizing a hybrid cross between *S. quinqueradiata* and *S. lalandi*, Ohara *et al*., 2005), development of genetic markers (microsatellites and single nucleotide polymorphisms (SNPs)), identification of markers associated with candidate genes of interest, mapping of genes, estimation of heritability for commercially-relevant traits, and identification of quantitative trait loci (QTLs) associated with disease resistance (Ozaki *et al*., 2013; Whatmore *et al*., 2013; Aoki *et al*., 2014; Fuji *et al*., 2014). However, up until this point, bioinformatics data resulting from next generation sequencing has not been applied toward improving aquaculture for *Seriola*. The development of large-scale genomic resources has become increasingly accessible and affordable for non-model organisms. These types of genetic resources have been developed and used extensively in agriculture and livestock breeding for decades to improve product quality and quantity. Only more recently have these genomic approaches been applied to select aquaculture species (e.g. rainbow trout, Atlantic salmon, tilapia, catfish, flounder, Atlantic cod) (Terova *et al*., 2013; Dunham *et al*., 2014). Applying these genomic approaches to *Seriola* would greatly aid in the selection for economically important traits (Aoki *et al*., 2014) and improve the understanding of the biological, biochemical, and molecular networks involved in larval development, which could then be used to improve culturing techniques (Benzekri *et al*., 2014; Mazurais *et al*., 2011).

In this study, we describe the generation of whole larval transcriptomes for the California yellowtail (*S. dorsalis*) at three developmental stages: 2, 7, and 17 days post hatching (dph) using RNA-Seq on the Illumina HiSeq 2500 sequencing platform. Transcriptional profiles and differential gene expression were also investigated for slow- and fast-growing larvae over these same stages. We will present the findings of the transcriptomic investigations. Through this study, we hope to apply the gene expression results to generate a better understanding of the mechanisms and timing of larval development in *S. dorsalis*, and of the genes and processes involved in the observed growth heterogeneity that has limited *Seriola* aquaculture production.

## Methods

Larval yellowtail were collected from spawning events using the wild-caught broodstock population held at Hubbs-SeaWorld Research Institute (HSWRI) (San Diego, CA). Yellowtail larvae were sampled opportunistically at 2, 7, and 17 dph with three replicates for each time point, and the replicates within each time point were sampled from separate spawning events. Depending on developmental stage, one to several larvae were placed on a microscope slide and euthanized with a lethal dose of MS-222. The larvae were quickly photographed, measured, and sorted by size into vials containing RNAlater® (Ambion). The vials of RNAlater® were kept on ice until being placed in -20 °C storage at the NMFS laboratory.

As a proxy for physical larval fitness (i.e., overall larval growth and robustness), the smallest and largest larvae, hereafter referred to as slow-growing (SG) and fast-growing (FG) larvae, were sampled within each spawning event at the three time points used for this experiment, with three replicates at each time point. For each replicate, 10 whole larvae

were pooled for the tissue homogenization. Pooling was used to: 1) minimize differences between individual larvae and focus on general differences in larval age and fitness groups and 2) provide adequate amounts of RNA for the analyses, which was problematic in single specimens at the earlier time points (i.e., 2 dph). Whole larvae were utilized at the three time points to best characterize organism-wide developmental changes, particularly as many organ systems were not developed at 2 dph.

RNA-Seq was conducted on the Illumina Hiseq 2500, and a total of 600 million reads were generated for the 18 samples (30 million reads/replicate). Raw read data were assembled using Trinity (Haas *et al*., 2013), and DESeq (Anders & Huber, 2010) was used to calculate the differential expression (DE) of the genes for every combination of timepoint and group (SG and FG fish). For the exploration of gene ontology (GO) enrichment, only transcripts with a fragments per kilobase of exon per million fragments mapped (FPKM) value of greater than four in at least one of the 18 samples was retained, resulting in a total of 54,858 high confidence transcripts. GO enrichment was performed on each subset of DE genes identified by DEseq for each comparison with a false discovery rate (FDR) corrected q-value cutoff of 0.05. GO enrichment was also performed on additional subsets of transcripts that had opposite transcriptional trends spanning the three time points between the SG and FG samples. The transcripts were required to have at least 15 normalized read counts across all 18 samples, and there had to be at least a twofold change between the averaged counts of biological replicates for days two and 17, which represented the start and end points of the sampling. GO terms were further analyzed for broad GO term categories using CateGOrizer and MGI_GO_slim2 categories (Hu *et al*., 2008), and pathway analysis was conducted using PathVisio 3 (Kutmon *et al*., 2015).

Lemon Tree analysis (Bonnet *et al*., 2015; Joshi *et al*., 2009) was performed to identify gene co-expression modules and assign regulators to those modules. Counts (FPKM) were normalized for library size, using RSEM (Li and Dewey, 2011). All 18 samples were used for clustering. Transcripts were filtered for having at least one sample with FPKM greater than four, resulting in 47,609 transcripts. Modules with significant interaction terms (FDR = 0.1) were selected for further analysis. Modules were sorted by magnitude of difference between groups in mean expression change over time. Modules with large expression increases over time in FG fish and large decrease over time in SG (and vice versa) were of particular interest in order to identify developmental gene differences or regulatory differences between these two groups. Module genes were pooled by pattern as described above. GO enrichment analysis (FDR = 0.05) was then performed within Cytoscape (v 3.2.1) (Smoot *et al*., 2001; Shannon *et al*., 2003) using the BiNGO (Maere *et al*. 2005) plugin, and zebrafish gene ontologies (http://zeogs.molgen.mpg.de/). Non-regulatory transcripts with ZFIN (Zebrafish Information Network) (Sprague *et al*., 2005) gene IDs were used as the reference gene set for enrichment analysis.

## Results and discussion

For slow-growing (SG) fish between 2 and 7 dph and between 7 and 17 dph, 343 and 765 significantly enriched GO categories were identified, respectively. In fast-growing (FG) fish, 604 and 904 GO categories were identified as significantly enriched between 2 and 7 dph and between 7 and 17 dph. For SG and FG fish at both time period comparisons (2-7 dph and 7-17 dph), the GO categories of metabolism and catalytic activity contained the largest proportion of enriched terms (Fig. 1).

A large proportion of enriched terms fell under the transport category for all but the 2-7 dph SG fish, where it was significantly lower than other comparisons. Interestingly, the proportion of enriched terms in the cellular metabolism category was significantly higher in SG fish (both time periods) than in FG fish. Within the cellular metabolism category, nucleic acid metabolism was higher for 2-7 dph and 7-17 dph SG fish (5.1% and 4.6%, respectively) than for FG fish (1.8% and 1.9%). However, enriched GO terms under primary metabolism were higher between 7-17 dph in the FG fish but not in SG fish and not different between SG and FG fish in the 2-7 dph comparisons (Fig.1).

The normalized count data for the three biological replicates at each time point were averaged. These

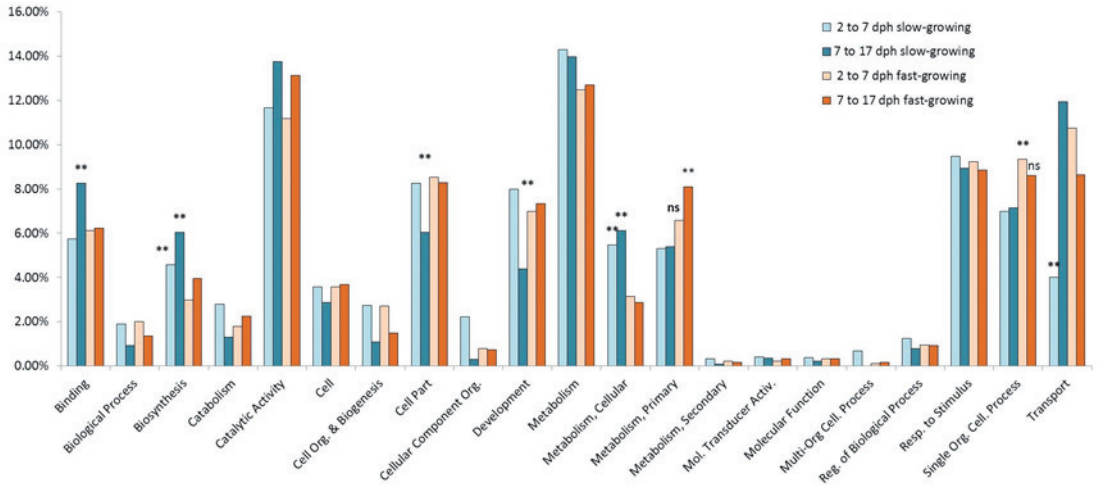


**Fig. 1.** Proportions of enriched DE GO terms assigned to GO categories between two time periods, 2 to 7 days post hatch (dph) and 7 to 17 dph, for SG and FG fish. Significant differences in GO categories between growth category or time period are represented by * ($p < 0.05$) and ** ($p < 0.01$), and ns (not significant) is used to clarify significance relationships when necessary.

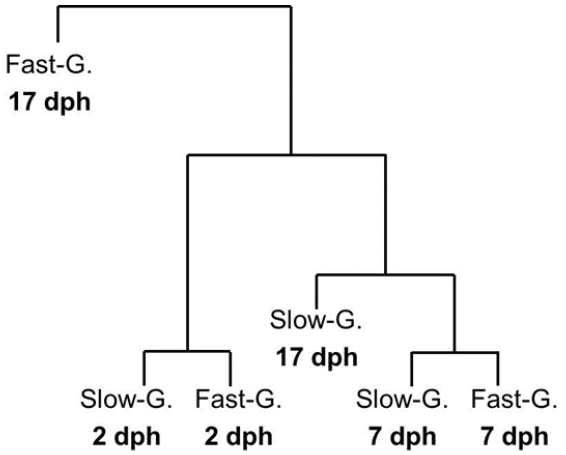


**Fig. 2.** A hierarchical clustering dendogram for all gene transcripts to visualize the overall similarity between time points of slow-growing (SG) and fast-growing (FG) fish.

averaged values were then used to generate a hierarchical clustering dendrogram using hclust (method = "average") (Murtagh and Legendre, 2014; Murtagh, 1985) to visualize the overall similarity between time points of SG and FG fish. Similar to the phenotypic characterizations we used to classify the SG and FG fish, we note the transcriptional profiles of 2 dph and 7 dph cluster together, whereas by 17 dph, the FG fish show a significantly different transcriptional profile than the SG fish. In fact, the SG fish at 17 dph are transcriptionally more similar to 7 dph and 2 dph FG and SG fish than to FG fish at 17 dph (Fig. 2).

In the module analysis, initial clustering produced 878 modules, and 63 of these had significant interaction terms (FDR = 0.1). Genes from these modules were further grouped into expression categories: 1) up-regulation in FG and down-regulation in SG fish (31 modules) and 2) up-regulation in SG and down-regulation in FG fish (12 modules). For modules showing preferential increases in FG fish, top GO terms (and associated top hierarchical enriched GO terms within the GO network) for module genes were regulation of metabolic process, regulation of biological process, biosynthetic process, ribosome biogenesis, nucleobase-nucleoside-nucleotide and nucleic acid metabolism, response to hypoxia, mesenchyme cell migration, cellular component assembly, small molecule metabolism and generation of precursor metabolites and energy, and transport. The percentage terms under the GO categories of translation, generation of precursor metabolism and energy, cellular nitrogen-compound metabolism, generation precursor metabolites and energy, biosynthetic process, and small molecule metabolism were significantly higher in FG fish than in SG fish (Fig. 3).

For the 12 modules exhibiting differential increases

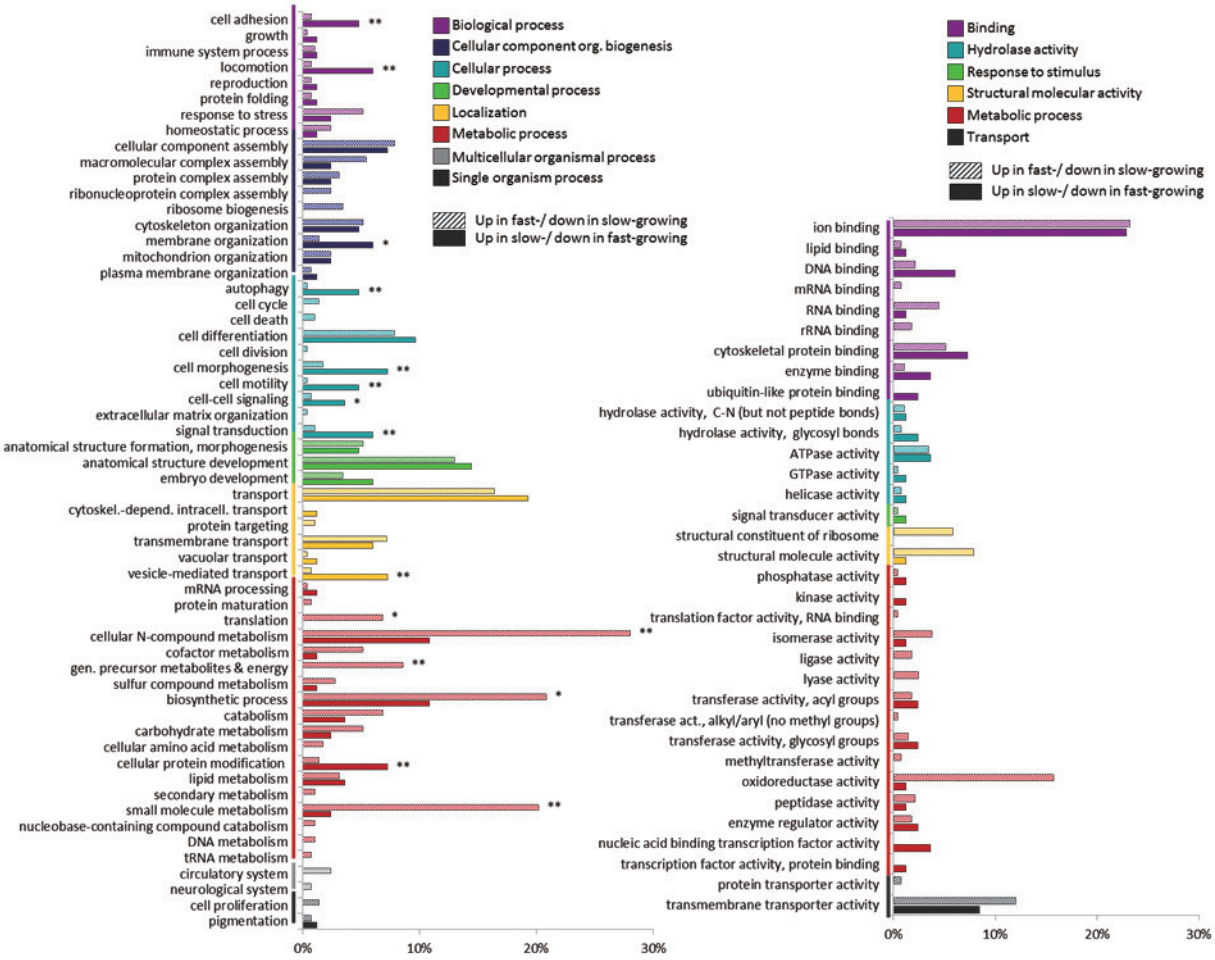


**Fig. 3.** From the significant modules, the proportions of terms identified in GO categories for genes in biological processes (A) and molecular function (B). Bar color reflects inclusion in the broader parent GO categories listed in the key provided for each graph. GO categories significantly higher for either the FG or SG larvae are denoted by *($P < 0.05$) and ** ($P < 0.01$).

in SG fish, enriched GO terms associated with the non-regulatory genes included response to nutrient levels (starvation), cellular response to stimulus, autophagy and Schwann cell development. Terms under GO categories associated with cell adhesion, locomotion, membrane organization, autophagy, cell motility, cell morphogenesis, vesicle-mediated transport, cell-protein modification, signal transduction, and cell-cell signaling were significantly higher in SG fish module genes (Fig. 3).

Pathway analysis of SG and FG larvae at the time points revealed two pathways with obvious trends in developmental progression: oxidative phosphorylation (Fig.4) and the electron transport chain. The overall trend for these pathways included higher gene expression for most genes in these pathways in SG fish at 2 dph and a complete reversal by 17 dph, with primarily higher gene expression in FG fish. Pathway analysis within growth groups indicate that SG fish show decreasing gene expression in these pathways as development continues, while FG show increasing gene expression over the same 15 day period.

It is not immediately obvious why 2 dph SG larvae exhibit up-regulation in oxidative phosphorylation and electron-transport chain pathways compared to FG larvae at the same time point. These larvae appear to be generating or attempting to generate higher levels of energy that do not translate into growth. Quickly the SG larvae lag behind the FG larvae, in size and in developmental stages; instead they up-regulate processes related to starvation responses and autophagy. One hypothesis is that one

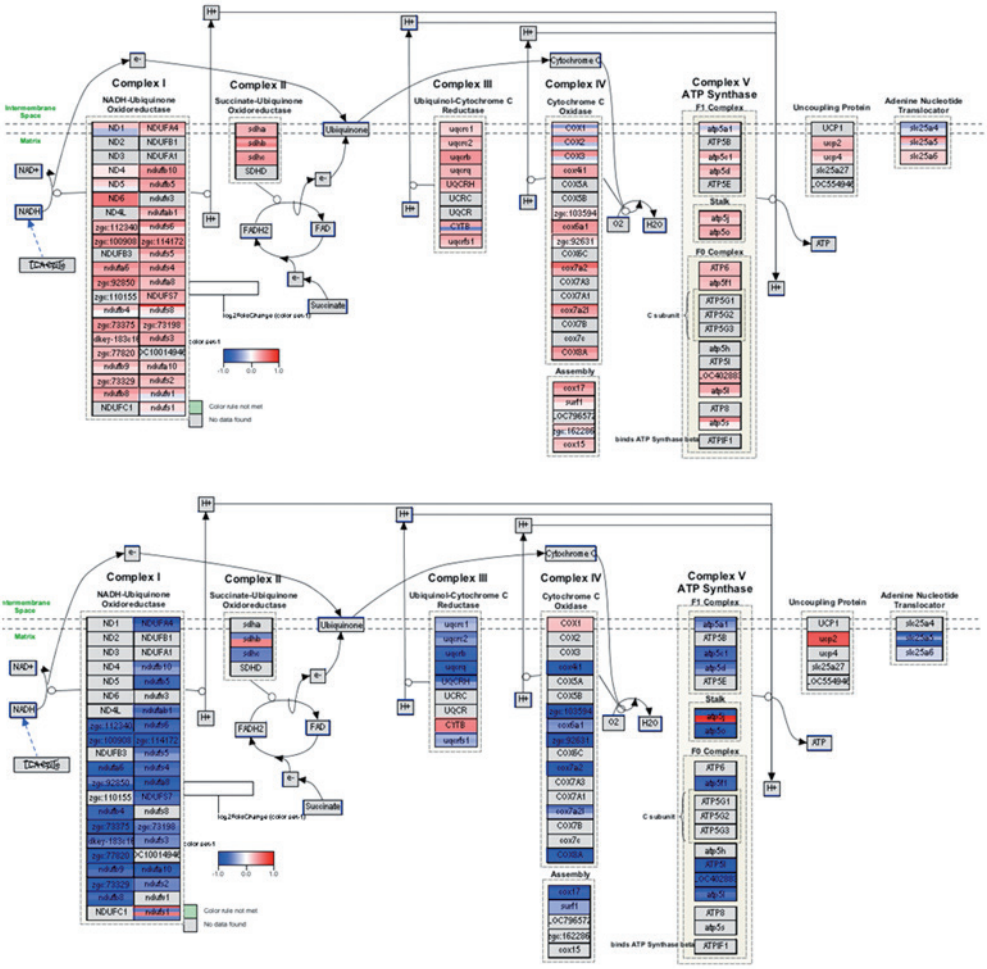


**Fig. 4.** Gene expression comparison (insert expression info) in the electron transport chain pathway between 2 dph (A) and 17 dph (B) FG and SG larvae. Gene boxes in pink/red indicate higher gene expression in SG larvae, and gene boxes in blue indicate higher gene expression in FG larvae.

or more of the ATP-generating pathways contain a mutation or some alteration that makes the pathway(s) less efficient, and instead of generating energy, these processes end up taking a higher metabolic toll on the SG larvae (Meyer and Manahan, 2010; Kocmarek *et al*., 2014). Alternatively, it has been documented that starvation can result in different metabolic adaptations in fish larvae (Salem *et al*., 2007), and it may be possible that the up-regulated gene expression patterns may result from inabilities to translate yolk-reserves into energy in the egg or yolk-sac larval stages. Next steps in this research will include exploring significantly associated regulatory genes, investigating the stages earlier than 2 dph, and examining mitochondrial function in the slow-growing larvae.

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(1) Kocmarek A., Ferguson M., and Danzmann R., 2014: Differential gene expression in small and large rainbow trout derived from two seasonal spawning groups. *BMC Genomics* **15**, 57.

In this study, the authors wanted to identify genes that showed similar expression patterns between large and small rainbow trout from different spawning seasons in two types of tissue: liver and white muscle. The goal in identifying these genes was to be able to develop growth-related markers for use in breeding programs. They hypothesized that 1) genes related to carbohydrate and lipid metabolism, energy production, insulin, and growth factors would be down-regulated in both tissue types in the small fish, 2) that genes involved in cytoskeletal structuring would be down-regulated in small fish, while myostatin will be up-regulated, and 3) that liver-specific lipid binding, cytoplasmic components, signaling, and transcription would be up-regulated in small fish. They observed that genes related to immune function were up regulated in large fish; suggesting that enhanced growth is associated with enhanced immune function. They also found that genes related to transcription, translation, and protein production were up regulated in small fish (from Sept.) in white muscle, which supports patterns previously detected in liver tissue. This indicates that protein production in small fish may not be translating effectively into finished proteins. This study was able to identify patterns of differential gene expression in small and large rainbow trout; this will enable future studies to delve deeper into the genes related to differences in growth.

(2) Meyer E. and Manahan D., 2010: Gene expression profiling of genetically determined growth variation in bivalve larvae (*Crassostrea gigas*). *J. Exp. Biol.* **213**, 749-758.

The authors compared gene expression patterns in larvae of the Pacific oyster (*Crassostrea gigas*) that exhibited slow- and fast-growth (these larvae were produced from experimental crosses). Based on a previous transcriptome-wide analysis, a set of 181 candidate genes for growth heterogeneity were analyzed with the goal of understanding the biological processes underlying the differential growth rates. Of the genes identified by GenBank, ribosomal proteins were the most abundant, comprising 50% of the total genes with 17 different ribosomal protein genes. The genes included nine components of the large ribosomal subunit, and eight components of the small ribosomal subunit. Some of these genes were up-regulated in the fast-growing larvae (n = 6), while others were up-regulated in the slow-growing larvae (n = 11). Since ribosome biogenesis is a significant metabolic cost in cell proliferation, any changes in this pathway would likely have a large effect on the

overall energy metabolism. The authors hypothesize that in the slow-growing larvae there may be a high metabolic cost to synthesizing and degrading excess ribosomal protein copies resulting from the higher expression of those genes.

(3) Moran D., 2007: Size heterogeneity, growth potential and aggression in juvenile yellowtail kingfish (*Seriola lalandi Valenciennes*). *Aquac. Res.* **38**, 1254-1264.

In this study, the authors are describing the occurrence of size heterogeneity and aggressive behaviors in cultures of *Seriola lalandi*; this was done to examine the effectiveness of size-grading in reducing aggression and mortality, and increasing growth rates. To do this, graded and ungraded juveniles were compared for various measures of aggression and growth, and a RNA/DNA ratio was used as a measure of growth rate. The authors found that size heterogeneity became more pronounced at 12 days post hatch (dph) when *Artemia* are offered as a food source. While the large and aggressive juveniles only accounted for 8% of the population, the small grade juveniles that received the aggression accounted for 42% of the population. In the ungraded treatment, this aggression was associated with mortality for most small fish. However, even without aggression, the small-grade juveniles did not gain weight or increase their RNA/DNA ratio after 12 days. The authors believe that these small fish appear to be on a degenerative developmental strategy without any influence of the larger aggressive fish.

(3) Salem M., Silverstein J., Rexroad III C., and Yao J., 2007: Effect of starvation on global gene expression and proteolysis in rainbow trout (*Oncorhynchus mykiss*). *BMC Genomics* **8**, 328.

The authors of this study used microarrays to identify genes and pathways involved in the starvation response and protein turnover in rainbow trout, and to identify metabolic adaptations that occur in the liver during these starvation periods. This study was of interest because, as the authors point out, examining changes in metabolism during starvation is an effective way to identify relationships between metabolic pathways and body processes. The experiments showed down-regulated expression of genes involved in protein biosynthesis in the starved fish, but no significant changes in protein catabolic pathways, and a slight increase in 20S proteasome activity. Responses in the liver to starvation included an overall decline in gene expression associated with decreasing tissue metabolism, a reduction in protein synthetic capacity, an impairment of ATP-biosynthesis, and lower expression in hepatic lipid and fatty acid transport.

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# Modeling the Variable Effects of Using Wild and Cultured Broodstock on the Fitness Risk Due to Escaped Farmed Fish

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**Abstract**: Various negative ecological and genetic impacts may occur when farmed fish escape and mix with wild conspecifics. Genetic impacts result due to interbreeding between wild fish and escapees and may result in reduced genetic diversity within and among populations and a loss of fitness. Loss of genetic diversity and fitness compromise the adaptive potential of a mixed (wild plus cultured) population, making it potentially less able to respond to changes in environmental conditions (e.g. climate change).

Simplified, risk to wild population fitness due to interbreeding with escapees is a function of the number of escapees relative to wild census size, the genetic difference between escapees and wild fish, and the fitness of escapees in the wild relative to wild fish. The Offshore Mariculture Escapes Genetics Assessment (OMEGA) model simulates the magnitude of this risk. To illustrate OMEGA's application, we present results from evaluations of a planned pilot project for Almaco jack (*Seriola rivoliana*) in Hawaii and contrast them with those obtained from evaluation of a theoretical sablefish (*Anoplopoma fimbria*) aquaculture program originally used to verify the model. Escape scenarios for both species varied from low to high base leak rates, cage failure probabilities, and catastrophic cage failure probabilities. Whereas the high escapes scenario for sablefish resulted in a significant impact to wild population fitness, even a total loss of almaco jack had a negligible effect. Key differences in the simulated fitness risk associated with each program included, but are not limited to, broodstock source (domesticated or wild), encounter rate between wild fish and escapees, and the scale (size and longevity) of the operation.

**Key words**: Almaco jack, fitness, genetic risk, offshore aquaculture, sablefish

## Introduction

The rapid development of offshore marine finfish aquaculture worldwide has raised concerns due, in part, to the potential negative genetic and fitness impacts escaped farmed fish may have on natural populations (Hindar *et al*., 1991; Tufto, 2010; Waples *et al*., 2012). Fitness is measured in terms of an individual's relative reproductive success. The fitness of escapees in the wild is often inversely correlated with the level of domestication those fish experienced in culture (Baskett *et al*., 2012). Genetic and fitness-related impacts to the wild population then occur when escaped individuals from these cultured populations interbreed with wild conspecifics (Baskett *et al*., 2013). Changes in gene expression due to adaptation to the hatchery environment and fitness declines in a mixed population due to domestication can occur after only one or two generations (Araki *et al*., 2008; Christie *et al*., 2016).

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These impacts can threaten the adaptive potential of the wild population, which may make it less able to respond to and survive environmental changes (Tringali and Bert, 1998).

There is little scientific data, however, that reliably assigns the risk to fitness due to escapees, particularly for mariculture. Thus, existing regulatory standards addressing escapes are largely preventative, theoretical, or qualitative rather than quantitative. To address this knowledge gap, the NOAA Fisheries Office of Aquaculture (OAQ) developed a research initiative designed to explore the genetic interactions between wild and cultured fish. As part of this initiative, the OAQ solicited ICF International, Inc., (ICF) to develop the Offshore Mariculture Escapes Genetics Assessment (OMEGA) model. OMEGA is a Microsoft Excel-based program designed to simulate the risk posed to wild population fitness by escapes from marine aquaculture programs.

Simplified, risk to wild population fitness due to interbreeding with escapees is a function of the number of escapees relative to wild census size, the genetic difference between escapees and wild fish, and the fitness of escapees in the wild relative to wild fish. OMEGA is organized around three interacting components that incorporate these factors, including the aquaculture operation itself, wild population characteristics, and the potential for interaction between escapes and wild conspecifics. Under these components, OMEGA employs a modular format to describe assumptions used to model the potential interactions and impacts of escapees in the wild. There are nine modules requiring user input, ranging from basic settings and preferences for saving the workspace and running simulations to entering wild and cultured population data. Currently, there are 103 possible user-defined variables (Index of User Inputs available upon request), some of which are optional and a few that represent alternatives for which the user chooses one of two or more options.

Once the model has been parameterized with data describing the three interacting components, OMEGA employs a single trait phenotypic fitness model developed by Ford (2002) to simulate risk. This underlying model is a quantitative two population analysis of differential selection regimes and the effect of gene flow between the populations on the mean trait value. Initially, each of the two populations is exposed to a separate environment (hatchery or wild) and, therefore, selective regime (domestication or natural). The mean trait value of each population is equal to its environmental (and fitness) optimum, and any shift away from either environmental optimum trait value leads to a reduction in fitness (Lande, 2007; Tufto, 2010).

OMEGA is intended to provide insight into the variables affecting risk, help identify information gaps and research priorities, explore options for operational design or modification, and inform policy and management decisions (Volk *et al*., 2015). To illustrate OMEGA's application, we present results from evaluations of a theoretical sablefish (*Anoplopoma fimbria*) program and a real world pilot project for Almaco jack (*Seriola rivoliana*).

## Methods

Escape scenarios for both species varied from low to high base leak rates, cage failure probabilities, and catastrophic cage failure probabilities.

### Sablefish

Sablefish are a highly-prized groundfish found along the North Pacific Rim. The wild population includes a single stock along the west coast of the U.S. Fish live up to 80 years but old fish are rare. The female spawning biomass is 79 - 82 metric tons (mt), with maturity reached by six years. Models for population dynamics and harvest are well-established in this species.

Commercial culture of sablefish currently occurs in Washington State, USA, and British Columbia, Canada. The OMEGA scenarios were originally developed for model verification and explored a low versus a high incidence of escapes, with a high encounter rate between wild and farmed fish (Volk *et al*., 2015). A hypothetical sablefish hatchery program was sited in the U.S. Pacific Northwest and included using a domesticated broodstock to produce up to 10,000 mt of fish stocked among 50 offshore cage sites. Culture methods were recently developed, with fish reaching harvest size in one year.

OMEGA was run multiple times. For the low escapes scenario, the base leak rate and cage failure

probability was set at a static 0.1%, regardless of fish size. The annual probability of a catastrophic event was set at 5% through the first 20 years of operation, decreasing to 1% by year 26 and thereafter, with a static assumed program loss of 20% per event across all years. For the high escapes scenario, the base leak rate, cage failure probability, and probability and magnitude of losses due to catastrophic events were increased. Base leak rate was dependent on fish size, with 3% of the smallest fish and 0.5% of the larger fish escaping. Cage failure probability was 0.5% for smaller fish and 1% for larger fish. Meanwhile, the probability of catastrophic events was set at 10%, with a 60% loss of fish per event, in year 1, which decreased to 5%, with a 40% loss, by year 16. Thereafter, probability was 5%, with a 20% program loss.

**Almaco jack**

Almaco jack (also known as kahala or kampachi in Hawaii) have a circumglobal distribution in tropical and temperate waters. Fish tend to school near reef slopes, offshore banks, and other objects, and they are often found in mixed schools with the greater amberjack (*S. dumerili*). The wild population in Hawaii is poorly characterized, and data used for populating OMEGA was supplemented with information from congenerics (e.g. greater amberjack size at maturity of 2.5 kg and fecundity at 130 thousand eggs per kg body weight).

In terms of U.S. commercial culture, Kampachi Farms has recently engaged in iterative open-ocean pilot projects directed at expanding offshore farming of almaco jack to a commercial scale near Kailua-Kona, HI, USA, and elsewhere. The OMEGA scenarios for Kampachi are based on well-developed operational plans. The project will use wild broodstock as required for permitting in Hawaii. Culture methods are established, and fish reach harvest size of 1.8 kg in one year. The F1 juveniles will be stocked in a tethered cage located approximately 11 km offshore of Kona, with production of about 14 mt per year for each of two years.

OMEGA was run multiple times. The assumed number of escapes was set at 50-100% per year. The natural spawning and total biomass was set equal to catches reported for all carangids in the Western Pacific Region reef fish trend report (2010). The encounter rate between wild and farmed almaco jack is likely to be low due to behavior characteristics (i.e. schooling near objects). Escapee survival and reproductive capabilities are known to be poor from previous pilot projects.

## Results

Over the default 100-year timespan of the simulations, the low escapes scenario for sablefish resulted in escapee percentage peaking at ≤ 5% of the total population and the spawning biomass soon after each escape event, with larger and/or successive events resulting in higher percentages, but gradually shrinking to < 1% of the population. High chronic escapes coupled with several acute escape events, in contrast, had a longer lasting impact on population make-up. The high escape scenario resulted in escapees comprising a significantly higher proportion of the population, averaging 5 -7%. Correspondingly, fitness in the wild was reduced (shifted toward the hatchery optimum; Fig. 1) more under the high escape scenario, whereas the low escape scenario remained near the wild optimum.

Whereas the high escapes scenario for sablefish resulted in a significant impact to wild population fitness, even a total loss of almaco jack had a

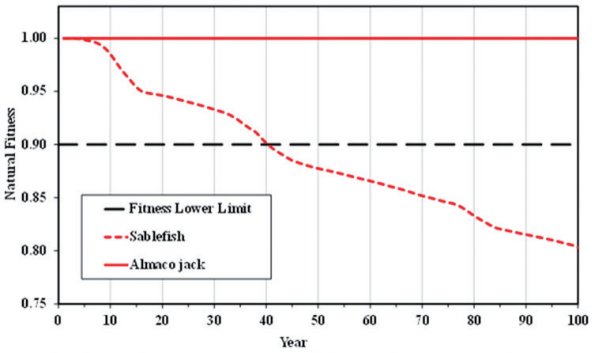


**Fig. 1.** Relative fitness effects for a sablefish (broken red line) and an almaco jack (solid red line) escape scenario, both with a high number and frequency of escapes. Almaco jack scenario results similar to no aquaculture. Dotted black line represents a hypothetical regulatory maximum 10% decrease in mixed population fitness.

negligible effect on the mean trait value (Fig. 1). The proportion of the wild almaco jack population and spawning biomass made up of escapees peaked at 0.45% and 0.87%, respectively, of the over 100-year span of the simulation. No significant decrease in fitness (< 0.02%) was predicted even when all 30,000 fish escaped and survived. Fitness impacts were realized, but still not significant, in attempts to force the model to show a fitness effect (i.e. to "break the model"), with a small estimated natural biomass, significantly more than two years of production, 100% annual escape and encounter rates, and 100% survival and spawning of escapees.

## Discussion

Results were in line with expectations according to Ford (2002), as well as results from related research (e.g. Baskett *et al*., 2013). Key differences in the simulated fitness risk associated with each program included, but were not limited to, broodstock source (domesticated or wild), encounter rate, and the scale (size and longevity) of the operation. The sablefish culture program was hypothetical and used originally to verify that the OMEGA was operating as expected. Were an offshore program to be developed for the species, results from these hypothetical scenarios could be used as a starting point in terms of designing a low risk program. Topics that may be important to consider when designing a program based in part on results from OMEGA include, but are not limited to, whether any scenarios are economically viable or ecologically sustainable and whether engineering or biological technology to minimize risk is available and economically feasible. In contrast, the almaco jack pilot program produced negligible effects. These results were included in an environment assessment submitted as part of the permitting process for the proposed pilot project for Kampachi Farms. It is recommended that reassessment of risk be performed, however, if Kampachi Farms wishes to modify protocols or scale production up to commercial levels.

OMEGA is still a work in progress, however, and requires further testing. The next steps toward finalizing the OMEGA model package for broader use include fostering external collaborations to develop model scenarios, evaluate model parameters, and validate the model with data from current and planned aquaculture operations worldwide; developing targeted scenarios, such as exploring the magnitude of risk associated with 1) using wild or selected broodstock; 2) widening the fitness gap between farmed and wild fish or keeping fish "wild-like;" focused research like induced sterility (e.g. triploidy; Hindar *et al*., 1991), and setting and enforcing spatiotemporal escape limits. Downstream steps include performing a sensitivity analysis of case studies, determining acceptable default parameters, and developing an economic analysis module. Work will also be directed at making OMEGA fully operational and friendly to a variety of user groups (e.g. managers, industry professionals, researchers), paving the way for the model to play a key role in genetic risk assessments for offshore culture in the U.S. and elsewhere.

Aquaculture must simultaneously support commercial interests; increase the availability of safe and nutritious fish, shellfish, and other products for consumers; and protect wild populations (IWG-A 2014). Toward that end, more specific questions that OMEGA may help user groups answer include how significant the impacts of escapes surviving to reproduce are and what the effects of our decisions about aquaculture operations, such a broodstock management (e.g. wild or selected), may be. More broadly, the intent is to use OMEGA to help define an acceptable risk to marine resources (e.g. 5% or 10% fitness decrease), as well as use it as a decision-making tool to assist in the assessment and management of marine aquaculture operations such that they may remain both commercially viable and environmentally responsible.

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OMEGA v1.0 and its user guide are freely available for download on the OAQ website at www.nmfs.noaa.

gov/aquaculture/science/omega_model_homepage.html.

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(1) Baskett M. L., Burgess S. C., and Waples R. S., 2013: Assessing strategies to minimize unintended fitness consequences of aquaculture on wild populations. *Evol. Appl.* **6**, 1090-1108.

Baskett *et al.* (2013) address various factors associated with the management of cultured populations and model their potential fitness impacts should farmed fish escape into the wild. Factors assessed include the origin and level of domestication in the cultured stock (i.e. degree of maladaptation), induced sterility in the cultured stock, and the magnitude and frequency of escapes (e.g. continuous low level leakage versus rare catastrophic events). Results indicate that, up to a point, the magnitude of the fitness impact rises as the maladaptation of escaped cultured fish increases; an extremely maladapted, non-local origin cultured population may actually have effects similar to a weakly diverged stock. Second, sterilization reduces unintended fitness consequences rapidly. Finally, it is more effective to reduce low-level leakage than guard against sporadic large-scale escape events.

(2) Ford M. J., 2002: Selection in captivity during supportive breeding may reduce fitness in the wild. *Conserv. Biol.* **16**, 815-825.

Escaped cultured fish may cause a potential loss of fitness in the wild, if breeding occurs between escapees and wild conspecifics. This drop in fitness is associated with a difference in the optimum trait values for hatchery and natural environments. The single-trait phenotypic fitness model in Ford (2002) describes how mean phenotype values of the mixed population (captive plus wild fish) may shift relative to the optimum values for each environment, based on the presence/absence and amount of gene flow (interbreeding) between the cultured escaped and wild fish. The overall fitness effect depends on the magnitude of the difference in optimum trait value, trait heritability, and selection pressure against domestication in the wild, as well as habitat capacity,

magnitude and frequency of escape events, wild and captive population demographics, and the potential for interaction between wild fish and escapees. Ford (2202) was used most notably in Pacific Northwest salmonids, where the All-H Analyzer, or AHA, model helped the Hatchery Scientific Review Group explore the potential fitness consequences of supplementing wild populations and of cultured fish straying into wild populations.

(3) Hindar K., Ryman N., and Utter, F., 1991: Genetic effects of cultured fish on natural fish population. *Can. J. Fish. Aquat. Sci.* **48**, 945-957.

Hindar *et al.* (1991) represents one of the original manuscripts reviewing the genetic impact escaped farmed fish may have on wild populations and is one of the most often cited. The authors recommend several strategies for protecting the genetic integrity of wild populations, many of which remain the focus of aquaculture programs today. These strategies include improved containment technology and recovery, sterilization of the culture stock, and better breeding practices, coupled with monitoring the genetic contribution of escaped fish to the mixed population.

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

Waples *et al.* (2012) is a comprehensive overview of the potential genetic impacts to wild populations associated with marine aquaculture. As such, the authors "provide managers with a better understanding of the genetic effects of marine aquaculture on natural populations," with the intent of informing policy and management decision-making. The document synthesizes relevant information and provides key references, identifies research priorities, provides a risk assessment framework, and gives recommendations for monitoring and evaluation toward sustainable marine aquaculture development in the US. Waples *et al.* (2012) focuses on commercial aquaculture of marine finfish but presents it in light of decades of research on salmon hatcheries and marine stock enhancement.

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# Did Farmed Coho Salmon *Oncorhynchus kisutch* that Escaped during the Earthquake and Tsunami Disaster in 2011 Interbreed with Native Masu Salmon *Oncorhynchus masou*?

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**Abstract**: During the Great East Japan Earthquake on March 11, 2011, several million individuals of farmed coho salmon (*Oncorhynchus kisutch*) escaped from aquaculture facilities in the Northeast Pacific Ocean, Tohoku, Japan. In fall of the same year, sexually mature coho salmon migrated up rivers in this area. Farmed coho salmon that migrate up rivers to breed may affect the genetic material of native salmon species and result in weakened populations. Especially, there is a strong concern that coho salmon may cross with the native masu salmon (*Oncorhynchus masou*); it is known that hybrids of these species have survivability. In this study, we surveyed masu salmon landed at a local fish market, using genetic and morphological methods in order to determine whether there are hybrids. As a result, hybrids were not found in this survey. Therefore, at this moment the impact on the genetic resources of masu salmon is considered to be low.

**Key words**: *Farmed coho salmon (Oncorhynchus kisutch), escaped, masu salmon (Oncorhynchus masou), hybrids, DNA fragment analysis*

## Introduction

Coho salmon (*Oncorhynchus kisutch*) has been transplanted from North America to Hokkaido since the 1970s (Ishida *et al*., 1975, 1976; Nara *et al*., 1979; Umeda *et al*., 1981), but has not become naturalized in Japan. They migrate to waters off Hokkaido for feeding, and sometimes stray and go up rivers in Hokkaido (Kikuchi *et al*., 1998); however, they do not regularly spawn in Japan. Sea farming of coho salmon has been conducted around the Tohoku Pacific coast in Japan since 1975. In recent years, production has remained at over 10,000 tons per year.

Due to the effects of the Great East Japan Earthquake and tsunami on March 11, 2011, all farmed coho salmon (more than 5 million fishes) escaped into the Northeast Pacific Ocean off Tohoku, northeastern Japan. In fall of the same year, sexually mature coho salmon migrated up rivers in this area. These coho salmon may have affected the genetic material of native salmon species and resulted in weakened populations. There is a strong concern that coho salmon may cross with the native masu salmon (*Oncorhynchus masou*) because it is known that hybrids of these species have survivability (Ito *et al*., 2006). On the other hand, the ability to survive is low for crosses with chum salmon (*Oncorhynchus keta*) (Foerster, 1935). The upriver season is different between masu salmon and coho salmon; however, the spawning season overlaps for these species. The spawning season of masu salmon (Honshu pacific region) is from September to November (Kiso, 1995), while that of coho salmon is from November to January (Koseki, 2013). It is unclear how many years

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hybrids will need to mature. However, based on the maturation age of both species being 3 years old, the possibility that hybrids returned in spring of 2014 was considered to be high. In this study, we surveyed masu salmon landed at a local fish market, using genetic and morphological methods in order to determine the presence of hybrids.

## Materials and methods

We conducted visual checks of masu and coho salmon landed at a Miyako fish market from May to September, 2014 (approx. 2,000 individuals) (Fig. 1). In general, it is possible to distinguish both species by checking the gill raker and the radial silver stripes on the caudal fin (Fig. 2). We surveyed presence of individuals with mixed characteristics. The reasons we choose this market were as follows: (1) large number of escaped coho salmon were landed at this market during summer to autumn season in 2011 and (2) upriver coho salmon were detected in several rivers near the market. We selected 39 masu, and 5

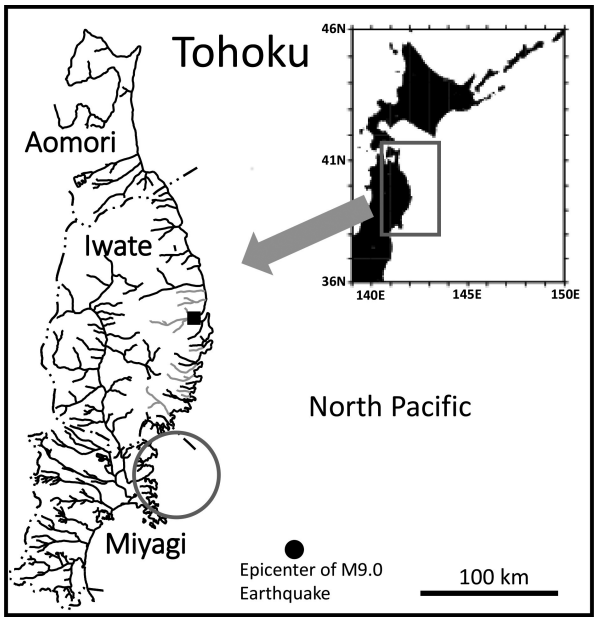


**Fig. 1.** Map of the Tohoku Pacific Coast. Rivers marked in gray indicate that adult coho salmon were detected in 2011. Open circle indicates the main farming area. "■" indicate study site.

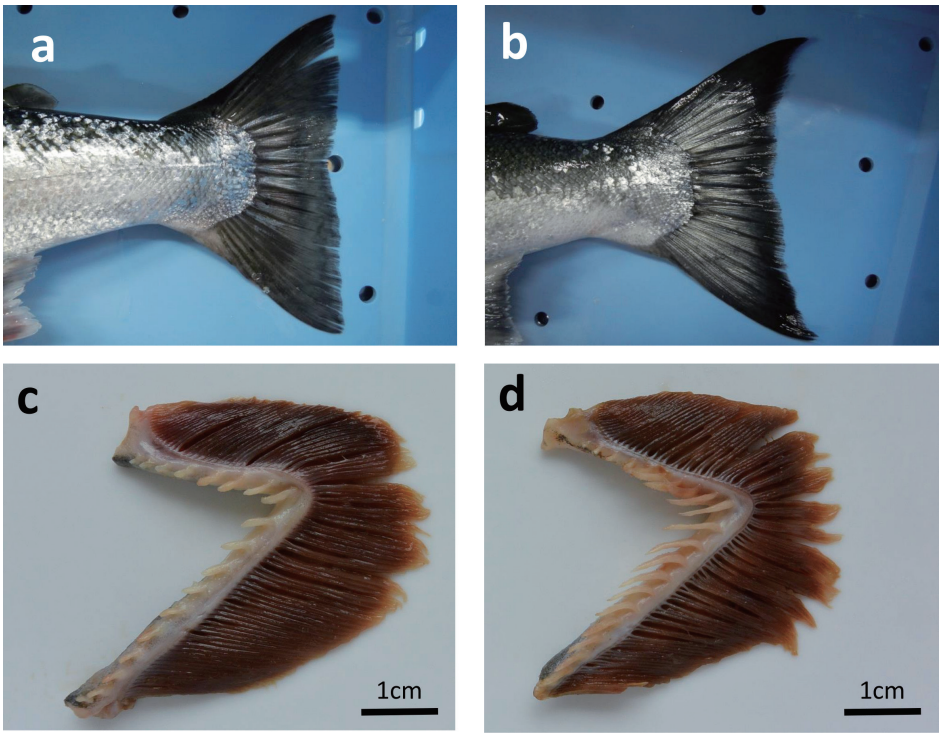


**Fig. 2.** Comparison of the tail and the first gill of adult coho and masu salmon. Upper panels shows tail of masu (a: fork length 57.0cm) and coho (b: fork length 60.6cm), lower panels shows the first gill of masu (**c**) and coho (**d**). Radial silver stripes on the caudal fin are obvious in coho, and the gill rakers longer and slender in coho.

coho salmon (all individuals were sampled during the survey) for more detailed analysis. For the morphological comparison, we compared the number of rays of each fin, and the number and length of the gill rakers. We conducted sequence analysis of the intron C of the growth hormone 1 (GH-1) using a primer set with 17 bp adapter sequence for the fluorescent label. PCR amplified product of masu salmon (256bp) was 34 bp less than that of coho salmon (290 bp) (Fig. 3), thus making it possible to

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                                For primer(OSH3) ->
AF541855  345:CTAAGGCACTACATCTCAGTGCCAGAGGTGTCACTGCARACCCTGGTTTGATTCCAGACTGGTATTTCAA 414
AF005925  701:CTAAGGCACTACATCTCAGTGCCAGAGGTGTCACTGCAGACCCTGGTTCGATTCCAGACT-GTATTTCAA 769
              ************************************ ********* *********** *********

AF541855  415:ACGGCTGTGATTGTGAGTCCCATAGGGCGGCACACAATTCTCCCAG------------------------ 460
AF005925  770:ACAGCTGTGATTGTGAGTCCCATAGGGCGGCACACAATTCTCCCAGCGTCGTTAGGGTTTGGCCGGGGTT 839
              ** ****************************************

AF541855  461:---------CAGTGTCTTCAACTAAGGTAGATAAAACAACCACATATCACAATCATTGCAAGTAAAACCA 521
AF005925  840:GCAATACCTCAGTGTCTTCAAATAAGGTAGATAAAACAACCACAT------ATCATTGCAAGTAAAACCA 903
                       ************ ***********************      ******************

AF541855  522:TCACTGTCTAATTGGTGGTTTCTCTACATCTCCA-------TTTTGTGCTTTTCTGTACAGGAATCCCGC 584
AF005925  904:TCACTGTCTAATCGGTGGTTTCTCTATGTCTACATTCTCTGTTTTGTGCTTTTCTGTACAGGAAACCCGC 973
              ************ *************  *** **       ********************** *****

            <- Rev primer+17bp Tag(OSH4)
AF541855  585:CCCAAAAGTATTTCACTCAATCATGTAAATATGGCATCTGAAGCTGTACAATACAACCCAACTTCATTTT 654
AF005925  974:CCCAAAAGTATTTCACTCAATCATGTAAATAGGGCATCTCAAGCTGTACAATACAACTCAACTTCATTTT 1043
              ******************************** ******* ***************** ************

AF541855  655:CCAATTATCTGTGGTTTCTCTACATCTTCACACACAG                                691
AF005925 1044:CCAATAATCTGTGGTTTCTCTACATCTTCACACACAG                                1080
              ***** ******************************
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**Fig. 3.** Alignment of the intron C of the growth hormone 1 (GH-1) between masu salmon (GenBank: AF541855) and coho salmon (AF005925). PCR product amplified using primer set OSH3 and OSH4 of masu salmon is 34 bp less than that of coho salmon.

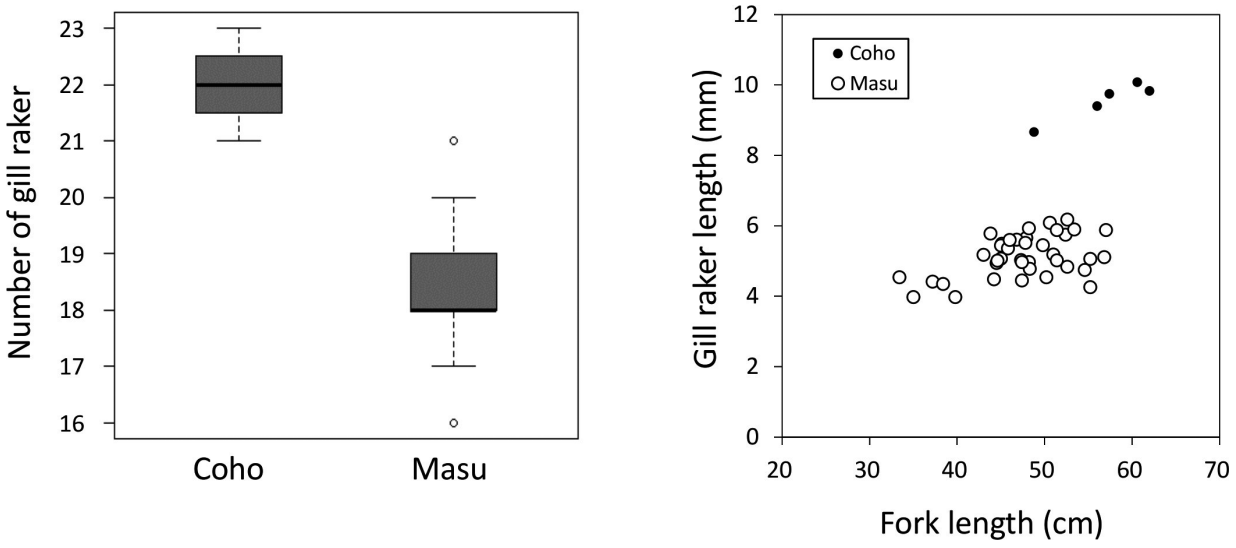


**Fig. 4.** Box plot of the number of gill rakers (left panel), and relationship between the longest gill raker length in the first gill and the fork length (right panel).

distinguish the two species. The amplicon was examined in both to see if there was a hybrid.

## Results and Discussion

As a result of the morphological comparison, the range of number of fin rays overlapped, and there was no significant difference in the two groups (Table 1). The number and length of gill rakers were larger and longer in coho salmon (Fig. 4). Individuals with mixed characteristics were not found.

As a result of DNA fragment analysis, hybrids were not confirmed. Identification of species from DNA analysis of all the individuals agreed with the species identification judged from the morphological comparison (Fig. 5). Therefore, based on the current survey, the impact of escaped coho salmon on the genetic resources of masu salmon is considered to be low. However, three of five of coho salmon were of the 2011 brood, and their gonads were developed. These individuals may have derived from the escaped coho salmon during the 2011 earthquake. Therefore, it is necessary to carefully monitor the occurrence of hybrids in the near future.

**Table 1.** The number of fin rays in masu salmon and coho salmon

| | Masu salmon *O. masou* | Coho salmon *O. kisutch* |
|---|---|---|
| n | 39 | 5 |
| Dorsal fin rays | 12-16 | 11-12 |
| Caudal fin rays | 19-25 | 19-26 |
| Anal fin rays | 12-15 | 13-15 |
| Pelvic fin rays | 9-11 | 10-11 |
| Pectoral fin rays | 12-15 | 14-16 |

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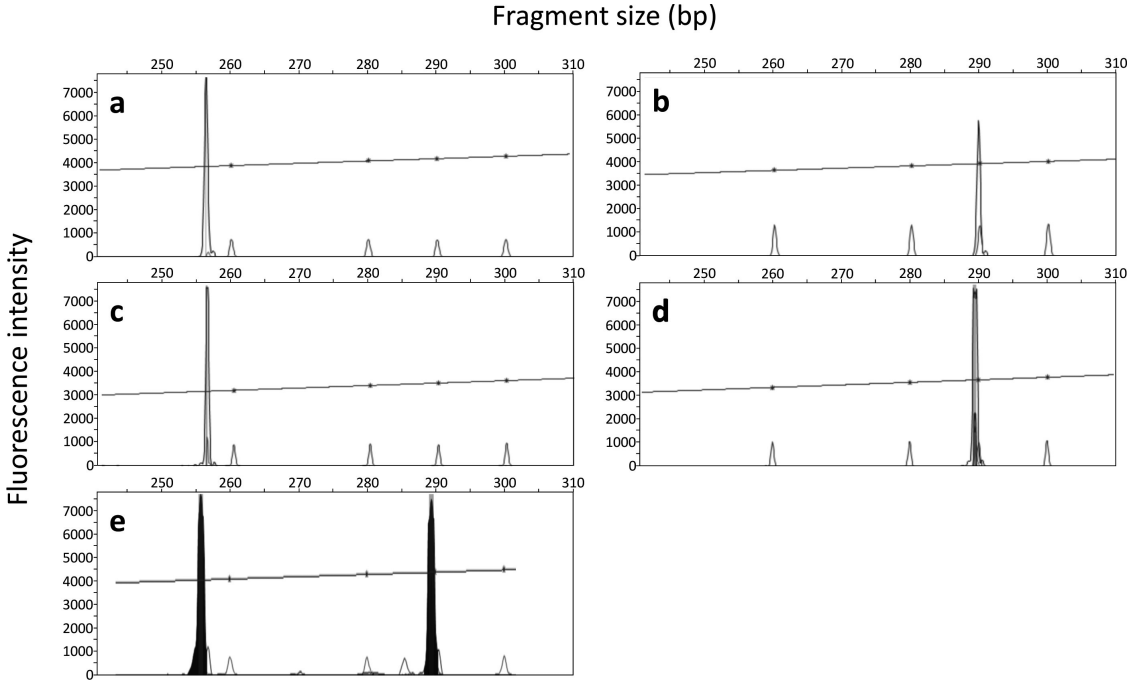


**Fig. 5.** Fragment size of the PCR amplified products of masu and coho salmon. Upper panel shows control of masu **(a)** and coho **(b)** salmon. Middle panel shows present specimens of masu **(c)** and coho **(d)**. Lower panel shows artificial cross of masu (male) and coho (female) **(e)**, the amplicon is seen in both 256 bp and 290 bp.

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# Evaluation of the Tsunami Impact on the Genetic Diversity of the Marbled Flounder *Pseudopleuronectes yokohamae* in Sendai Bay, Miyagi, Japan

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**Abstract**: Evaluating genetic diversity is a fundamental step for stock management. However, it can be influenced by environmental and demographic changes, and thus it should be carefully investigated, especially when drastic disturbances have occurred. Marbled flounder *Pseudopleuronectes yokohamae* is a commercially valued fish in Japan, and possibly consists of multiple management units due to its benthic lifestyle including low dispersal ability. In Sendai Bay, the population is thought to form a single management unit, and its spawning ground has been protected for stock conservation. Since this population could have been strongly affected by the huge tsunami associated with the 2011 off the Pacific coast of Tohoku Earthquake, the tsunami impact on the genetic diversity of the population of the marbled flounder in Sendai Bay needs to be evaluated.

We firstly developed genomic resources for this species using next generation sequencing technology and *de novo* assembly, which generated a total of 525,502 contigs and 10,732,070 unassembled reads. We then designed a total of 331,368 microsatellite primer pairs from the genomic data, and characterized sixteen primer pairs as novel microsatellite loci. Using these microsatellite markers, significant genetic divergence was found between the east and west sides of Boso Peninsula (pairwise $F_{ST}$ = 0.02305 – 0.19784). At the east side, genetic homogeneity was observed over the wide area from Onagawa Bay to Choshi including Sendai Bay, except for Mutsu Bay. The genetic variability of the Sendai Bay population did not show any considerable changes after the tsunami from April 2012 to February 2014 ($H_E$ = 0.5061 – 0.5463) and was relatively higher compared to those of the western populations of Boso Peninsula ($H_E$ = 0.3817 – 0.4557). These results suggest that the tsunami impact on the Sendai Bay population of the marbled flounder is considered to be minor but long-term monitoring may be needed.

**Key words**: Marbled flounder, *Pseudopleuronectes yokohamae*, genetic diversity, management units, Sendai Bay

## Introduction

Evaluating genetic diversity of species and / or populations of interest is a fundamental step for appropriate stock management. The information on genetic diversity such as population structure, migration rate between populations and population size helps identify management units and translocation ranges, and monitoring those alterations provides the insights into the population vulnerability

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and viability (Waples *et al*., 2008). On the other hand, genetic diversity is influenced by environmental and demographic changes like climate change, natural disaster and human activities. Direct effects involve population bottleneck and varied migration between populations, whereas indirect ones cause changes in habitat structure and community composition (Banks *et al*., 2013). Genetic diversity should be, therefore, carefully investigated, especially in the situation where drastic disturbances have occurred.

The 2011 off the Pacific coast of Tohoku Earthquake occurred on March 11, 2011, with a magnitude 9.0 generated a large tsunami that struck the Pacific coastline of eastern Japan (Mori *et al*., 2012). In addition to social, economic and physical impacts, ecological ones have been recently studied and reported in this area. Urabe *et al*. (2015) examined the macrobenthic animal communities at nine intertidal flat in Sendai Bay and the Sanriku Coast and found that 30 – 80% of taxa disappeared after the tsunami. Hata *et al*. (2016) investigated the ecological traits of ayu *Plecoglossus altivelis* with a one-year amphidromous life history and concluded that the tsunami drastically changed the ecological traits of this fish on the Sanriku Coast, such as hatching date and oceanic growth period. It can be, therefore, expected that this environmental event also affects genetic diversity of aquatic organisms in this area.

Marbled flounder *Pseudopleuronectes yokohamae* is a commercially valued fish across Japan. Due to the benthic lifestyle including low dispersal ability of the marbled flounder, this species possibly consists of multiple management units over its geographic distribution. In Sendai Bay, the population is considered to form a single management unit, and its spawning ground has been protected for stock conservation. This population, however, could have been strongly influenced by the great tsunami that occurred along the Pacific coast in Japan in 2011, and consequently the genetic diversity could be changed. Accordingly, the temporal genetic variation of the Sendai Bay population needs to be investigated and the geographic range of the management unit should be determined based on the genetic population structure of this species inferred together with other Pacific Ocean populations.

In this study, we firstly introduce our previous studies to develop the genomic resources and novel microsatellite markers for the marbled flounder, then demonstrate the results of population genetic analyses of this species using newly developed markers, and finally discussed the impact of that tsunami on the genetic diversity of this fish.

## Materials and Methods

### Development of the genomic resource and novel microsatellite markers

A 200-bp library was prepared using the genomic DNA extracted from a single adult specimen of the marbled flounder collected in Sendai Bay in 2012. IonTorrent PGM sequencing was performed and a single round of *de novo* assembly was conducted. To test if the assembled contigs can function as reference in genomic analyses, IonTorrent RAD-seq was performed using six individuals of this fish and SNPs were discovered by mapping RAD-tag reads to those contigs. The detailed procedure was provided in Genomic Resources Development Consortium *et al*. (2015). Using the software QDD version 3.0 (Meglécz *et al*., 2010), microsatellite sequences were searched for in the obtained genome sequence data of *P. yokohamae* and primers were designed for those microsatellites with a default configuration. The assembled contig sequences and unassembled reads from *de novo* assembly were separately used as input data in the software. Based on PCR amplification, visual confirmation of polymorphisms on an agarose gel and accurate genotyping, the primer pairs were chosen for subsequent population genetic analyses (Minegishi *et al*., 2015).

### Population genetic analyses

Temporal change in genetic diversity of the marbled flounder after the tsunami impact was investigated using thirteen populations collected in Sendai Bay from April 2012 to February 2014 ($N$ = 807). Nine populations from the Pacific Ocean coasts (Mutsu Bay, Onagawa Bay, Sendai Bay, Ibaraki, Choshi, Tokyo Bay [Funabashi, Hagiu, Takeoka, and Uraga], Sagami Bay, Hamana Lake, and Mikawa Bay; $N$ = 596) were also analyzed for comparisons (Fig. 1). Genomic DNA was extracted from fin clips and genotyping was performed at the newly developed

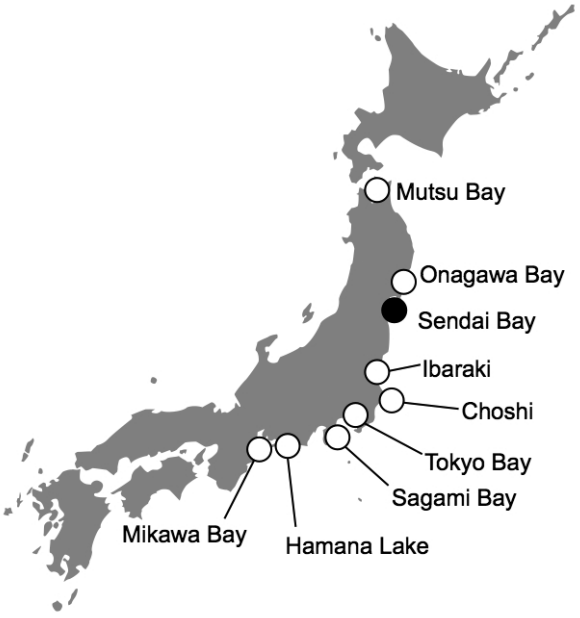


**Fig. 1.** Distribution of the marbled flounder (bold lines) and sampling sites in the present study. The black circle shows Sendai Bay. Boso Peninsula locates between Choshi and Tokyo Bay.

sixteen microsatellite loci. As the presence of null alleles was suggested at locus Psyo075, this locus was excluded from further analyses. Population genetic analyses were conducted using Arlequin ver. 3.5 (Excoffier *et al*., 2005). The detailed experimental conditions for genotyping were described in Minegishi *et al*. (2015).

## Results and Discussion

### Development of the genomic resource and novel microsatellite markers

IonTorrent PGM sequencing and *de novo* assembly generated a total of 525,502 contigs (N50 = 1994) and 10,732,070 unassembled reads. The summed length of the contigs was 547.8 megabases, corresponding to 81.8% of the genome of this species (670 megabases; Ojima and Yamamoto, 1990). This suggests that this genome sequences data contains most information of the genome of this species. RAD-seq reads (91.92 – 95.34% of the reads) were successfully mapped to these contig sequences, which subsequently yielded 75,472 SNPs at maximum, indicating the utility of this genome data as a reference sequence in the genome-scale analyses (Genomic Resources Development Consortium *et al*., 2015). Microsatellites were found in 781,773 sequences in the unassembled reads, and a total of 331,368 primer sets were successfully designed. In contrast, no microsatellites were detected in the contigs. Screening based on PCR amplification, visual confirmation of polymorphisms on an agarose gel and accurate genotyping resulted in sixteen primer pairs that are polymorphic and applicable for population genetic analyses of this species (Minegishi *et al*., 2015). These results indicate that the obtained genome sequence data can be used as a genomic resource of this species and contributes to finding novel DNA markers such as SNPs and other microsatellites and the genes associated with various useful phenotypes including faster growth and high resistance to virus infection.

### Evaluation of the genetic diversity of the Sendai Bay population of the marbled flounder

Population genetic analyses using genotyping data from the microsatellites developed above found significant genetic divergence between the east and west sides of Boso Peninsula (pairwise $F_{ST}$ = 0.02305 – 0.19784). On the east side of Boso Peninsula, the Mutsu Bay population was differentiated ($F_{ST}$ = 0.03829, $P < 0.001$) from other populations and gene flow was observed over the wide area from Onagawa Bay to Choshi including Sendai Bay. The genetic

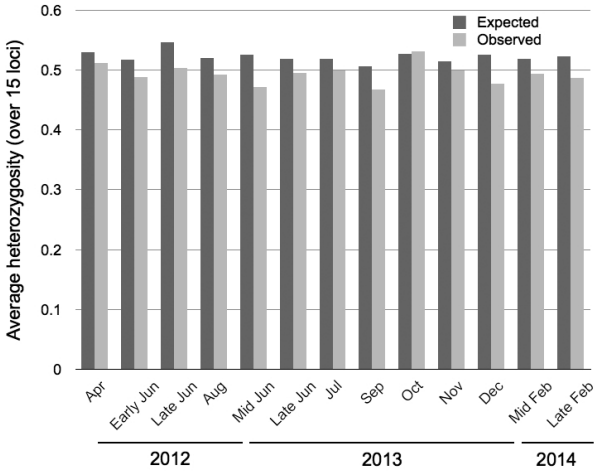


**Fig. 2.** Temporal change of the average heterozygosity over 15 microsatellite loci of the Sendai Bay population sampled from April 2012 to late February 2014. Dark and light grey indicates expected and observed values, respectively.

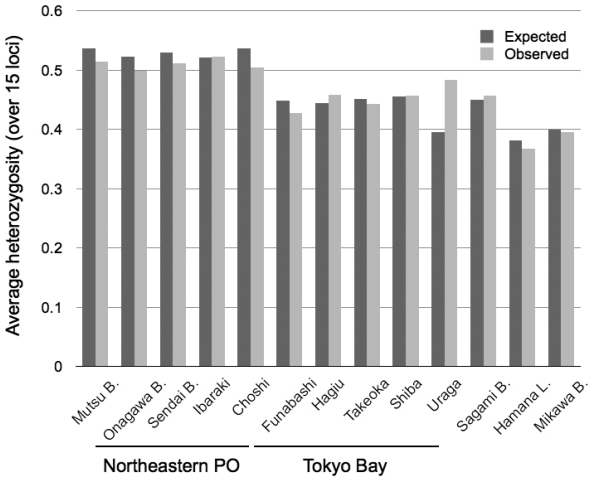


**Fig. 3.** Comparison of the average heterozygosity over 15 microsatellite loci of nine populations from Mutsu Bay to Mikawa Bay. Dark and light grey indicates expected and observed values, respectively.

variability of the Sendai Bay population did not show any drastic changes during the sampling period from April 2012 to February 2014 ($H_E$ = 0.5061 – 0.5463) (Fig. 2) and no signal of a genetic bottleneck was observed. Moreover, the genetic diversity of the Sendai Bay population was relatively higher compared to those of the western populations of Boso Peninsula such as Tokyo Bay and Mikawa Bay ($H_E$ = 0.3817 – 0.4557) (Fig. 3) . These results indicate that the Sendai Bay population should be treated as a single management unit together with other populations in the area ranging from Onagawa Bay to Choshi for stock management. In addition, unlike macrobenthic animals and ayu mentioned earlier, the tsunami impact on the Sendai Bay population of the marbled flounder is considered to be minor, but further monitoring may be needed since a long-term effect of the tsunami impact on marine ecosystems could emerge in the future.

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Environmental disturbance underpins the dynamics and diversity of many of the ecosystems of the world, yet its influence on the patterns and distribution of genetic diversity is poorly appreciated. We argue here that disturbance history may be the major driver that shapes patterns of genetic diversity in many natural populations. We outline how disturbance influences genetic diversity through changes in both selective processes and demographically driven, selectively neutral processes. Our review highlights the opportunities and challenges presented by genetic approaches, such as landscape genomics, for better understanding and predicting the demographic and evolutionary responses of natural populations to disturbance. Developing this understanding is now critical because disturbance regimes are changing rapidly in a human-modified world.

(2) Waples R. S., Punt A. E., and Cope J. M., 2008: Integrating genetic data into management of marine resources: how can we do it better? *Fish Fish*. **9**, 423–449.

Molecular genetic data have found widespread application in the identification of population and conservation units for aquatic species. However, integration of genetic information into actual management has been slow, and explicit and quantitative inclusion of genetic data into fisheries models is rare. In part, this reflects the inherent difficulty in using genetic markers to draw inferences about demographic independence, which is generally the information of the greatest short-term interest to fishery managers. However, practical management constraints, institutional structures and communication issues have also contributed to the lack of integration. This paper identifies some of the organizational, conceptual and technical barriers that have hampered full use of genetics data in stock assessment and hence fishery management and outlines how such use could be enhanced.

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# Experimental Hybridization and Competition between Atlantic Salmon (*Salmo salar*) and Native Salmonid Species in Japan

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**Abstract**: Atlantic salmon (*Salmo salar*) is a leading aquaculture species that has been extensively bred in Norway using selective DNA marker technologies. The commercial application of a high-growth transgenic Atlantic salmon (namely AquAdvantage® Salmon) was approved by the United State Food and Drug Administration in November 2015. AquAdvantage salmon is a strain of Atlantic salmon that has been genetically modified by the integration of a growth-hormone regulating gene from a Chinook salmon and the antifreeze protein gene promoter from the ocean pout. The GM strain grows at least twice as fast as regular farmed Atlantic salmon. Should it become a candidate for commercial production in Japan, there is not enough available information on the biology and ecology of Atlantic salmon in Japanese waters for comparison. To remedy this in part, we evaluated the maturation of experimentally reared Atlantic salmon in Japan, and tested the potential of the species for hybridization and competition with four native salmonid species. Seasonal water temperature was a primary determinant of maturation of the Atlantic salmon. Crossed with Amago salmon, Masu salmon, and Biwa trout, most of the hybrid embryos ceased to develop between mesoderm induction and axis formation. However, a relatively low number of the Atlantic salmon and native white potted char (*Salvelinus leucomaenis*) hybrid, as confirmed through restriction fragment length polymerase analysis, survived for 2 years. In addition, we reared different life stages of Atlantic salmon and three native salmonid species in tanks or ponds to determine the extent of interspecific and intraspecific competition for food and space, as well as related changes in body mass. We found that competition was dependent on the combination of salmonid species and life stage of the different species.

**Key words**: Atlantic salmon (*Salmo salar*), competition, interspecific crosses, maturation, native salmonids in Japan

## Introduction

The distribution of Atlantic salmon (*Salmo salar*) depends on water temperature and is native to the North Atlantic, where it is generally considered to comprise three populations: North American, European and Baltic. As with other salmonids, the anadromous life cycle of this species is complex (Mills, 1989, 1991; Hendry and Cragg-Hine, 1997; Urke *et al*., 2010). Young Atlantic salmon spend one to four years in their natal river. When they develop a highly evolved chemotactic system, they migrate

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toward the Atlantic Ocean and follow on sea surface currents and feed on plankton or juveniles of other fish species such as herring. After a year to up to five years of good growth, they move to the sea surface currents that transport them back to their natal river. It is a major misconception that salmon swim thousands of kilometers in the sea (Hendry and Cragg-Hine, 2000; Jonsson and Jonsson, 2007; Øystein *et al*., 2010).

Since the mid-1960s, Atlantic salmon aquaculture has grown well beyond the native range of the species, with Norway, Chile, Scotland and Canada currently the largest producers (Marine Harvest, 2015). Atlantic salmon can now be farmed through their entire life cycle, with brood stock selected for a number of traits, including rapid growth, hardiness and good edible yield, and the adults are often cultured in marine net pens or sea cages.

Nearly one third of the total world production of Atlantic salmon occurs in regions where this species is exotic. There is an evidence of successful spawning in three streams in British Columbia, Canada, but whether escaped Atlantic salmon have established breeding populations along the North American West Coast remains uncertain (Volpe *et al*., 2001a). Spawning of escaped farmed Atlantic salmon has not been documented in Chile or Tasmania. Outside its native range the Atlantic salmon is a poor colonizer. Because Atlantic salmon are attacked by other salmonid fishes, such as native brown trout (Heggenes *et al*., 2002), the probability of escaped Atlantic salmon establishing populations in exotic environments seems low, although the possibility of this occurring cannot be ruled out. It is difficult to predict if or how Atlantic salmon adapt to a new environment, partly because research on the potential impacts in such a habitat is limited (McGinnity *et al*., 2003).

Released or escaped cultured salmon have the potential to compete with the wild stocks for food, space, and breeding partners (Van Zwol *et al*., 2012). As a result of the morphological, physiological, ecological, and behavioral changes that may occur among artificially reared salmon, their competitive ability is likely to differ from that of the wild fish. These changes are partly phenotypic and partly genotypic (Perrier *et al*., 2013). For instance, genetic change among hatchery-reared fish is a form of selection, with differential mortality among the genotypes, with brood stock chosen based on commercial production traits such as high adult body mass and fast growth rate (Perrier *et al*., 2013). Escaped male salmon are often larger than the wild fish, making them more attractive to females and more successful in spawning, even though they may be less fit genetically (Hendry and Cragg-Hine, 2000).

Numerous researchers have described interspecific and intraspecific competition between Atlantic salmon and other salmonid species for all life stages (Fausch, 1998; Bremset and Heggenes, 2001; Volpe *et al*., 2001b; Heggenes *et al*., 2002; Van Zwol *et al*., 2012). Most studies to date have suggested that the performance of wild steelhead, rainbow and brown trout is superior to that of Atlantic salmon, even with significant behavioral differences observed among these species. In experimental tank environments, the behavior of Atlantic salmon has been described to be influenced by that of other salmonid species in a manner similar to that occurs in natural river environments (Stradmeyer *et al*., 2008; Berg *et al*., 2014).

Fletcher *et al*. (2004) reported genetically modified (GM) Atlantic salmon to grow faster than non-GM farm-raised Atlantic salmon. The GM salmon contain an rDNA construct comprising a growth hormone gene from Chinook salmon, under the control of a gene promoter derived from the ocean pout (*Macrozoarces americanus*). The GM salmon have proven to grow faster before the pre-smolt stage and can reach market weight one year faster than the regular farmed Atlantic salmon (Du *et al*., 1992; Cook *et al*., 2000; Fletcher *et al*., 2004; Deitch *et al*., 2006).

AquaBounty Technologies Inc. first applied to sell a GM strain of Atlantic salmon (AquAdvantage® Salmon) in North America. Based on the proposed physical and biological containment measures, the United States Food and Drug Administration deemed that the likelihood of the GM salmon escaping from land-based facilities (in Canada and Panama), thereafter surviving in the ocean or freshwater and interbreeding with wild-type salmon, is remote. Accordingly, they issued a 'Finding of No Significant Impact' (FONSI) on November 19, 2015, thereby approving the company's application to produce the

salmon commercially.

The only report of farmed Atlantic salmon in Japan comes from Aomori Prefecture for 1983–1988 (Yoshida and Matsuzaka 1991). Thus, there is scant information describing the biology and ecology of Atlantic salmon in Japanese conditions for comparison with AquAdvantage salmon, which would help in determining whether AquaBounty Technologies can apply for commercial production of the GM strain in Japan. Therefore, to partly remedy the lack of comparative data, we evaluated the maturation of Atlantic salmon cultured at two locations in Japan. Next, we tested the potential of the species for hybridization with four native salmonid species, and meanwhile sought to evaluate the levels of intraspecific competition and interspecific competition, with three native salmonid species, under contained conditions.

## Materials and methods

### Experimental salmonids

To investigate maturation of Atlantic salmon (*Salmo salar*) reared in Japan, we obtained hatched fry from the Hokkaido University Nanae Freshwater Station (Hakodate) and transferred them to the National Research Institute of Aquaculture's Tamaki (Mie Prefecture) and to Nikko inland station (Tochigi Prefecture) (the latter being in the coldest region on Honshu Island during winter). To investigate competition and the potential for crossbreeding with native salmonids, Biwa trout (*Oncorhynchus masou rhodurus*) were sourced from Lake Biwa Museum (Shiga Prefecture) and transferred to Tamaki station; Masu salmon (*O. masou masou*) and white-spotted char (*S. leucomaenis*) were sourced from Nikko station and transferred to Tamaki station; and the Amago salmon (*O. masou ishikawae*) were bred at Tamaki station.

### Crossbreeding the Atlantic salmon with four native salmonid species

We monitored the water temperature for the immature Atlantic salmon cultured in outdoor tanks (15 m × 2.5 m × 1 m) at Tamaki station from January to September. The immature salmon were then transferred to an indoor 2 tons tank for rearing between October and the following January to decrease the water temperature to approximately 10°C. A separate batch of immature Atlantic salmon were cultured indoors throughout the year at Nikko station in spring water maintained at approximately 10°C. Between late November and December, once the salmon female had matured, we squeezed eggs from females and sperm from males, and then artificially fertilized the salmon to ensure the quality of the egg and sperm. Male Atlantic salmon were maturated from the end of October at Tamaki station, and we exposed the sperm of mature Atlantic salmon to the eggs of mature Amago salmon, Masu salmon, Biwa trout and white-spotted char to induce hybridization.

### Competition between juvenile Atlantic salmon, and with Amago salmon and Biwa trout

To estimate intraspecific and interspecific competition between juvenile fish, ten individuals each of Atlantic salmon, Amago salmon and Biwa trout of average weight of 30 g were reared for 6 months in separate 2 m × 50 cm × 25 cm tanks with transparent fronts. In addition, we reared five Atlantic salmon with five Amago salmons, and five Atlantic salmon with five Biwa trout for 3 months. During this period, we used time-lapse video to record the position of each fish at 5 - minute intervals for 1 hour each day, and we weighed the juvenile fish approximately every 2 weeks.

### Competition between the adult Atlantic salmon and three native salmonid species

To quantify interspecific and intraspecific competition between adult fish, 6 individuals each of Atlantic salmon, Amago salmon and Biwa trout of average weight of 250 g, were reared in separate 5 m × 1.5 m × 1 m ponds. Additionally, we reared 3 individuals each of Atlantic, Amago and Masu salmon and Biwa trout together for approximately 2 months. For 1 hour per day over a 2-week period, the position of each fish was recorded using two underwater video cameras. And, each day for 2 weeks, we counted the number of fish consuming the food pellets offered, using the camera footage for 30 minutes each feeding. Lastly, the fish were weighed every 2 weeks during the approximately 2 - month

period.

## Results and Discussion

### Maturation of Atlantic salmon reared on Honshu Island, Japan

Seasonal water temperature was an important determinant of the maturation of the experimentally reared Atlantic salmon. At the more northern Nikko inland station, the salmon could be bred year-round in relatively cold water, maintained at approximately 10 °C (Fig. 1A). However, the survival of the fry grown from artificially fertilized eggs was < 0.1% over a five-year period (2011–2015). Only at the more southern Tamaki station, in 2012, did survival of similarly derived fry reach 4%. In 2012, the water temperature between January and September ranged from 13 to 15 °C, after which it decreased to approximately 10 °C from late October to November (Fig. 1B). In order to mature Atlantic salmon at water temperature similar to that in 2015, Atlantic salmon were reared outdoor from January to September at water temperatures from 12 to 15 °C, after which they were transferred to an indoor 2 tons black breeding tank, from October to December, where the temperature was maintained at approximately 10 °C (Fig. 1C). These conditions produced survival to the fry stage.

The natural breeding grounds of Atlantic salmon are rivers in Europe and the east coast of North America (Øystein *et al*., 2010). The bulk of individuals of the ocean-run form live in freshwater for the first 2 years of life, after which they smolt and migrate to the Atlantic Ocean to feed, grow and mature for 1 or more years. The adults utilize the ocean surface

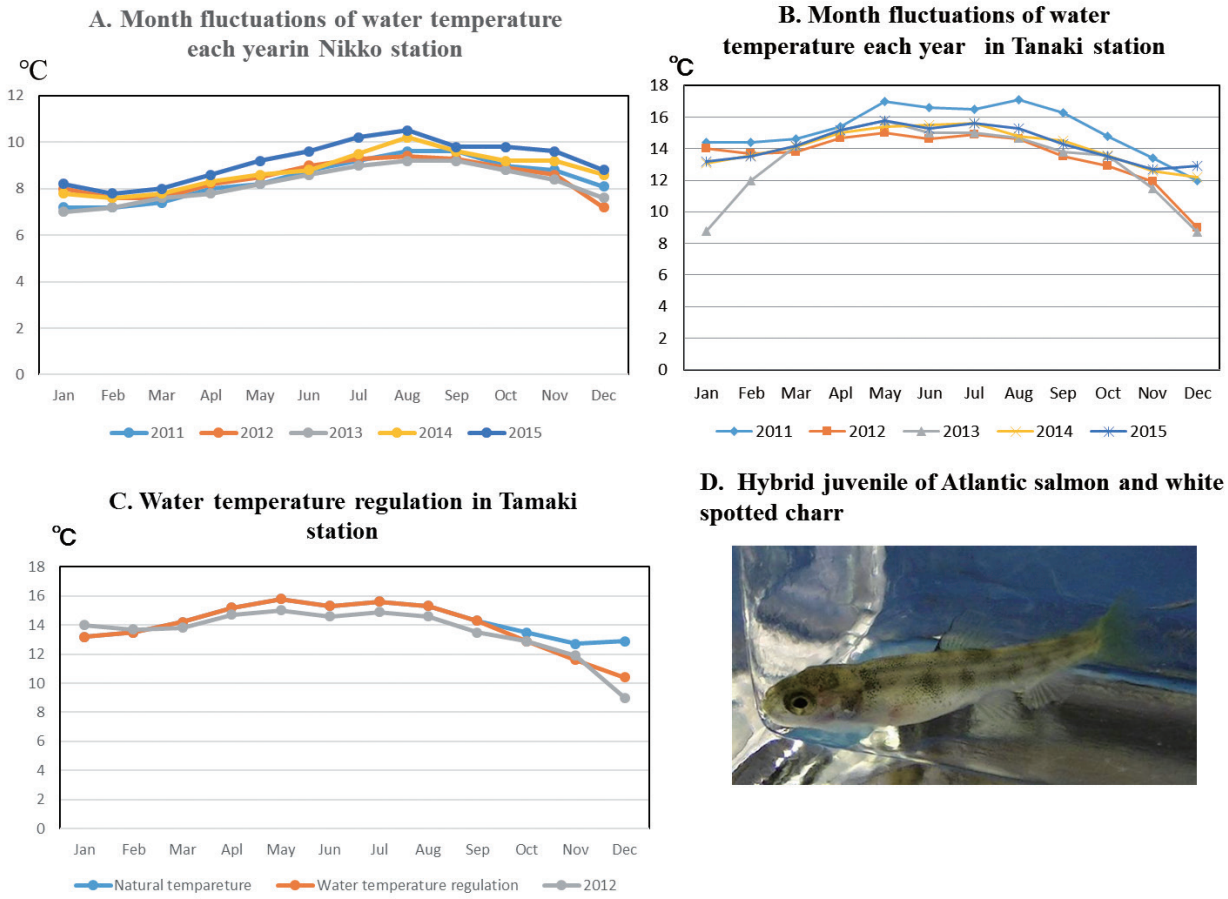


**Fig. 1.** Water temperature at Tamaki and Nikko stations, and an Atlantic salmon × white-spotted char hybrid fry. A: Water temperatures at Nikko station. B: Water temperatures at Tamaki station. C: Regulated water temperature in the indoor 2-t water black breeding tank at Tamaki station. D: Atlantic salmon and white-spotted char hybrid fry.

currents (Hendry and Cragg-Hine, 2000) to return to their natal river to spawn, generally from April to September (with peak spawning in October and November in Canadian waters). Atlantic salmon complete their maturation in the sea, and return to their natal river before or during summer with higher water temperatures (Bromage and Roberts, 1994; Jonsson and Jonsson, 2007). Rapid reductions in water temperature during October or November induce spawning (Taranger *et al*., 2010; Good, 2015). In 2012, the year that we successfully managed to artificially fertilize the salmon and hatch out embryos to the fry stage, the culture water temperatures were similar what to this species would encounter in the wild in its native range. Natural populations of Atlantic salmon live at high latitudes, with most aquaculture facilities also traditionally located at high latitudes (e.g., Norway, Chile, Canada, United Kingdom, and Alaska in the US). Both experimental stations in our study, however, are situated at somewhat lower latitudes. The maturation of Atlantic salmon is understood to be a complex process variously affected by seasonal water temperature, photoperiod, growth rate while at sea, and age (Taranger *et al*., 2010). Future research on these aspects will be required to determine the total appropriate conditions without water temperature for the artificial breeding of Atlantic salmon in Japan.

**Hybridization between the Atlantic salmon and four salmon species native to Japan**

We attempted to crossbreed Atlantic salmon with four native salmonid species, at both Tamaki and Nikko station. Most cross-fertilized embryos of Atlantic salmon with Amago salmon (*Oncorhynchus masou ishikawae*), Masu salmon (*Oncorhynchus masou masou*), Biwa trout (*Oncorhynchus masou rhodurus*), and white-spotted char (*Salvelinus leucomaenis*) died between mesoderm induction and axis formation because mesoderm was induced and cell cycle rate closed to that of somatic cells at the stage. However, some embryos of female white-spotted char crossed with male Atlantic salmon did hatch. These hybrid fry have been previously identified using restriction fragment length polymorphism (RFLP) analysis of a PCR-amplified fragment of the 16S rRNA gene and the second intron of the aromatase gene (Masaoka *et al*., 2015). In our experiments, the surviving fry achieved a relatively short body length and had a slightly irregular body form (Fig. 1D); however, we did not determine to what life stage the hybrids might have survived or whether they could have ever reached maturity.

Ban *et al*. (2013) crossed Atlantic salmon with several salmonid species nativ to Japan with a low level of success, and almost all the hybrid embryos were gynogenetic. Likewise, those authors reported an even lower level of success with hybrid embryos of male Atlantic salmon crossed with female Japanese char. We similarly observed poor survival of the Atlantic salmon and white-spotted char hybrid fry. Thus far, the potential for natural hybridization between these species in Japan appears to be unlikely, indicating that escaped farmed Atlantic salmon have little potential to impact the genetic fitness of the wild salmonid stocks in Japan.

On the other hand, interspecific hybridization between Atlantic salmon and brown trout was possibly the first hybrid fish ever described; natural hybridization of these species was mentioned by Willughbeii in 1686 (Makhrov, 2008), and artificial hybridization of these species has been known since the early 19th century (Leaniz and Verspoor, 1989; Jansson and Ost, 1997). Today, the hybridization of Atlantic salmon and brown trout provides a good model for studying the factors and consequences of interspecific hybridization among salmonids (Garcia-Vazquez *et al*., 2004; Quilodrán *et al*., 2003; Ban *et al*., 2013).

**Interspecific competition among juveniles**

Prior to our experiments, no published information was available on competition between Atlantic salmon and salmonid species native to Japan. To evaluate competition for food and space, and the resulting differences in body mass, we reared two different life stages (juvenile and before maturation) of Atlantic salmon together with three native salmonid species.

Most of the Atlantic salmon juveniles (average weight 20 g) reared in a 2 m × 50 cm × 25 cm aquarium gained weight during the same seasonal period but with different individual growth rates.

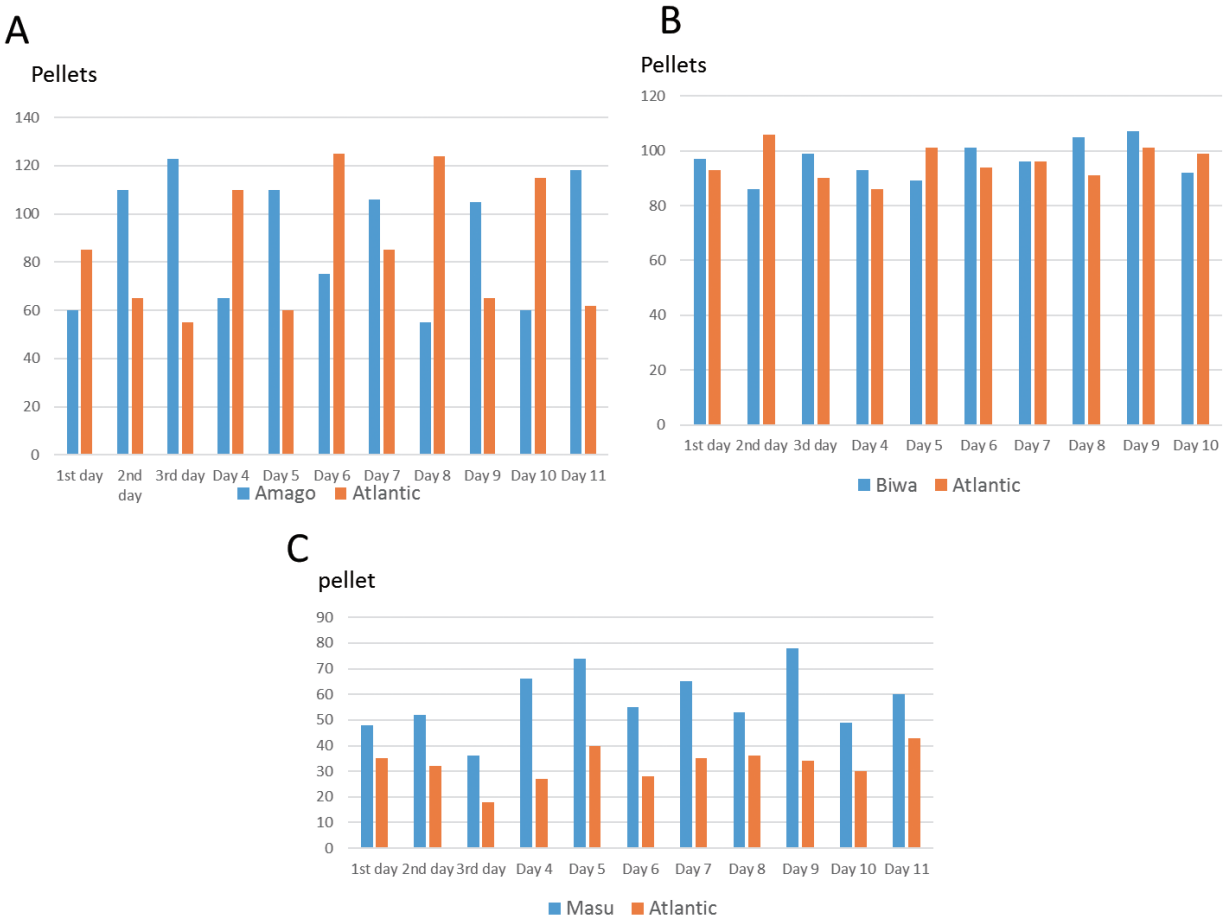


**Fig. 2.** Food competition between Atlantic salmon, Amago salmon, Biwa trout and Masu salmon. A: Competition for food between Atlantic salmon and Amago salmon. B: Competition for food between Atlantic salmon and Biwa trout. C: Competition for food between Atlantic salmon and Masu salmon. X-axis: time (days) from commencement of experiment; Y-axis: total number of food pellets consumed by 3 individual salmon.

This also occurred among ten juvenile Amago salmons (average weight 20 g) reared in a single-species tank, however their individual growth rates were greater than that of the juvenile Atlantic salmon. Interspecific competition for food between juvenile Atlantic salmon and Amago salmon appeared weak; intraspecific competition for food among juvenile Amago salmon appeared stronger than what was observed among Atlantic salmon. Among ten Biwa trout juveniles (average weight 30 g) reared in one aquarium, the weight gain of one was considerably large, and that of another much less so pronounced. Finally, intraspecific competition for food appeared stronger among the juvenile Biwa salmon than among either Amago salmon or Atlantic salmon.

Kept in sympatry, most of the five individuals each of Atlantic salmon and Amago salmon gained weight during the same period, but with overall differences in growth likely caused by intraspecific competition among one or the other of the species. Thus, interspecific competition between juvenile Atlantic salmon and Amago salmon appeared weak. When we reared five individual Atlantic salmon together with five Biwa trout, the weight of one Biwa trout individual increased substantially, whereas the weights of the other juvenile Biwa trout and the Atlantic salmon increased relatively slowly. The largest Biwa trout was aggressive toward the Atlantic salmon to the point of damaging the fins of the latter. Moreover, the Atlantic salmon appeared to avoid the Biwa trout by aggregating in a corner of

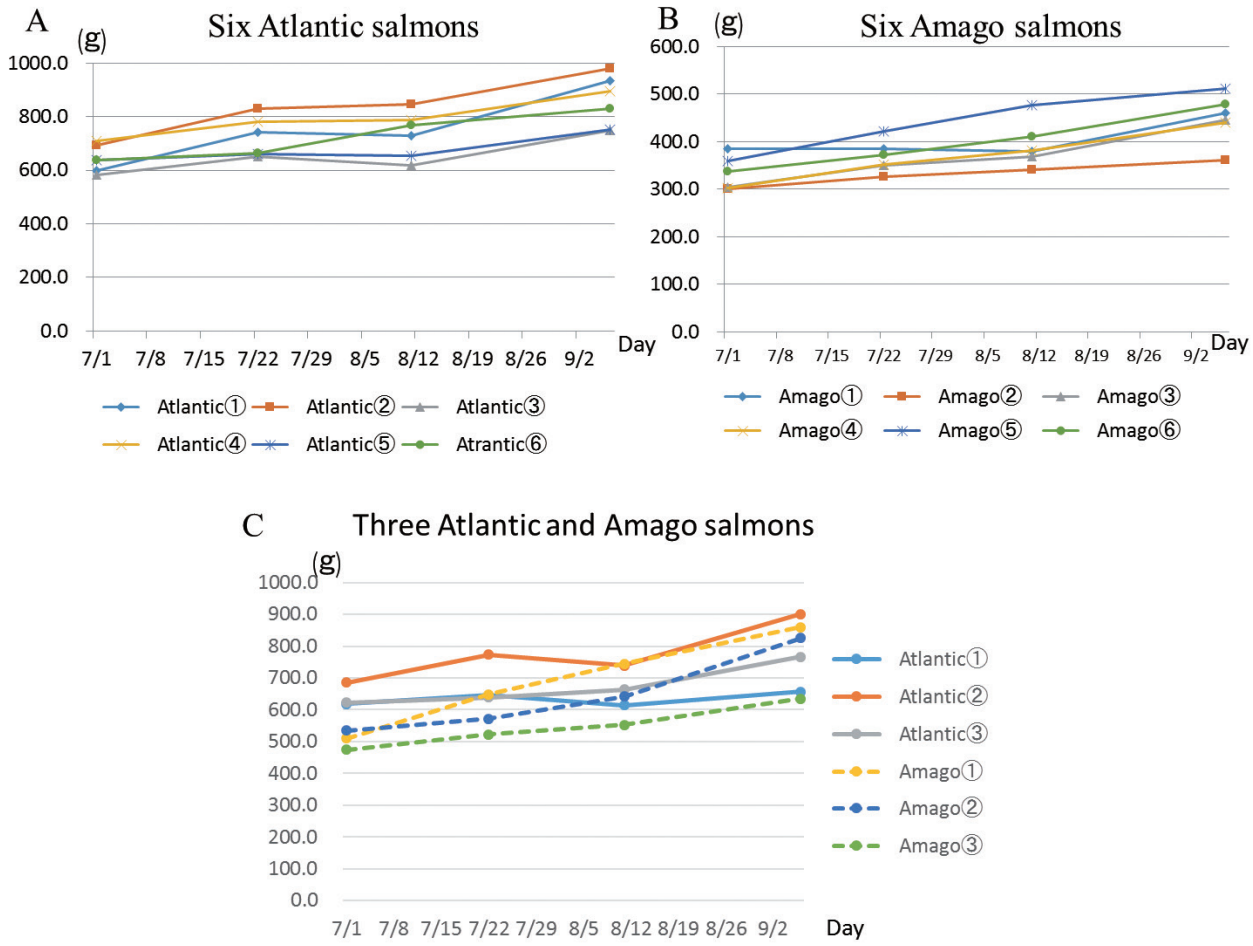


**Fig. 3.** Body weight change in the competition test between Atlantic salmon and Amago salmon. A: The body weight change of 6 individual Atlantic salmon reared in a single-species pool. X-axis: time (day); Y-axis: body weight (g). B: The body weight change of 6 individual Amago salmon reared in a single-species pool. C: The body weight change of 3 Atlantic salmon and 3 Amago salmon reared and fed together in one pool.

the aquarium. Thus, we surmise that interspecific competition between juvenile Biwa trout and Atlantic salmon was strong in the containment conditions.

**Intra and Interspecific competition among subadults**

When we investigated outdoor inter- and intraspecific competition between Atlantic salmon and Amago salmon, or Biwa trout or Masu salmon before maturation, Atlantic salmon raised in single-species ponds increased weight at the same period with little difference in growth rate (Fig.3A, 4A, 5A). Subadult intraspecific competition was deemed weak. We also deemed intraspecific competition between subadult Amago salmon was weak because Amago individuals increased their weight with little difference in growth rate (Fig 3B). Biwa trout raised in a single-species pond gained weight slowly (Fig 4B). For the six Masu salmon raised in a single-species pond, the weight increased considerably in three individuals and decreased slowly in one individual (Fig 5B). Our results suggest stronger intraspecific competition in Masu salmon than Amago salmon and Biwa trout.

We investigated interspecific competition by observing three individual Atlantic salmon reared together with three Amago salmon, three Biwa trout, or three Masu salmon, before maturation, in 5 m × 1.5 m × 1 m outdoor ponds. When we cultured Atlantic salmon and these native species of salmon in one pond, the amount of food that Atlantic salmon could eat changed depending on the salmon species

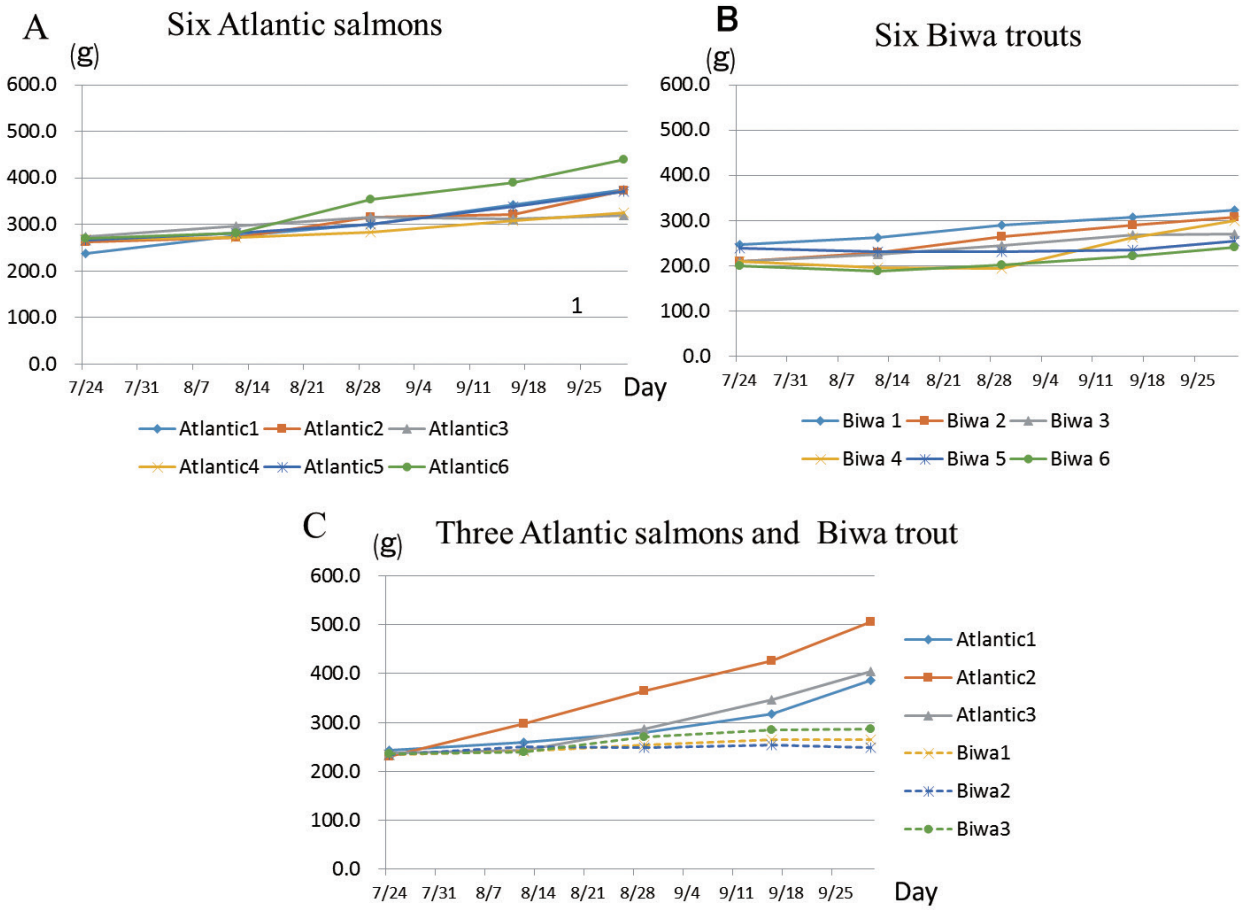


**Fig. 4.** Body weight change in the competition test between Atlantic salmon and Biwa trout. A: The body weight change of 6 individual Atlantic salmon reared in a single-species pool. X-axis: time (day); Y-axis: body weight (g). B: The body weight change of 6 individual Biwa trout reared in a single-species pool. C: The body weight change of 3 Atlantic salmon and 3 Biwa trout reared and fed together in one pool.

combined with Atlantic salmon (Fig.2). We found that the overall growth of the Amago salmon exceeded that of the Atlantic salmon regardless of the fish being kept in separate single-species ponds or in sympatry (Fig. 3). Kept together in one pond, the Amago salmon tended to distribute themselves throughout the pond, whereas the Atlantic salmon appeared more restricted in the space use. Based on these observations, we surmise that interspecific competition was limited between these two species at this life stage, although we recognize that Amago salmon tended to dominate the Atlantic salmon in sympatry.

Reared together in one pond, the three Atlantic salmon and three Biwa trout consumed a comparable number of food pellets each day (Fig. 2B). However, the growth of Atlantic salmon exceeded that of Biwa trout (Fig. 4C). Kept together in one pond, the two species tended to divide themselves in the habitat. Hence, we surmised a minimal level of interspecific competition between these species at this life stage, but recognized that Atlantic salmon would possibly dominate Biwa trout with age and growth.

When three individual Atlantic salmon and three Masu salmon were reared together, we observed that Masu salmon tended to occupy the center of the pond and fed easily, whereas the Atlantic salmon tended to stay near the bottom and in shade. Also, kept in sympatry, Masu salmon consumed more food pellets than Atlantic salmon (Fig. 2C) and their body mass increased advantageously (Fig.5C). We conclude that interspecific competition between Masu salmon and

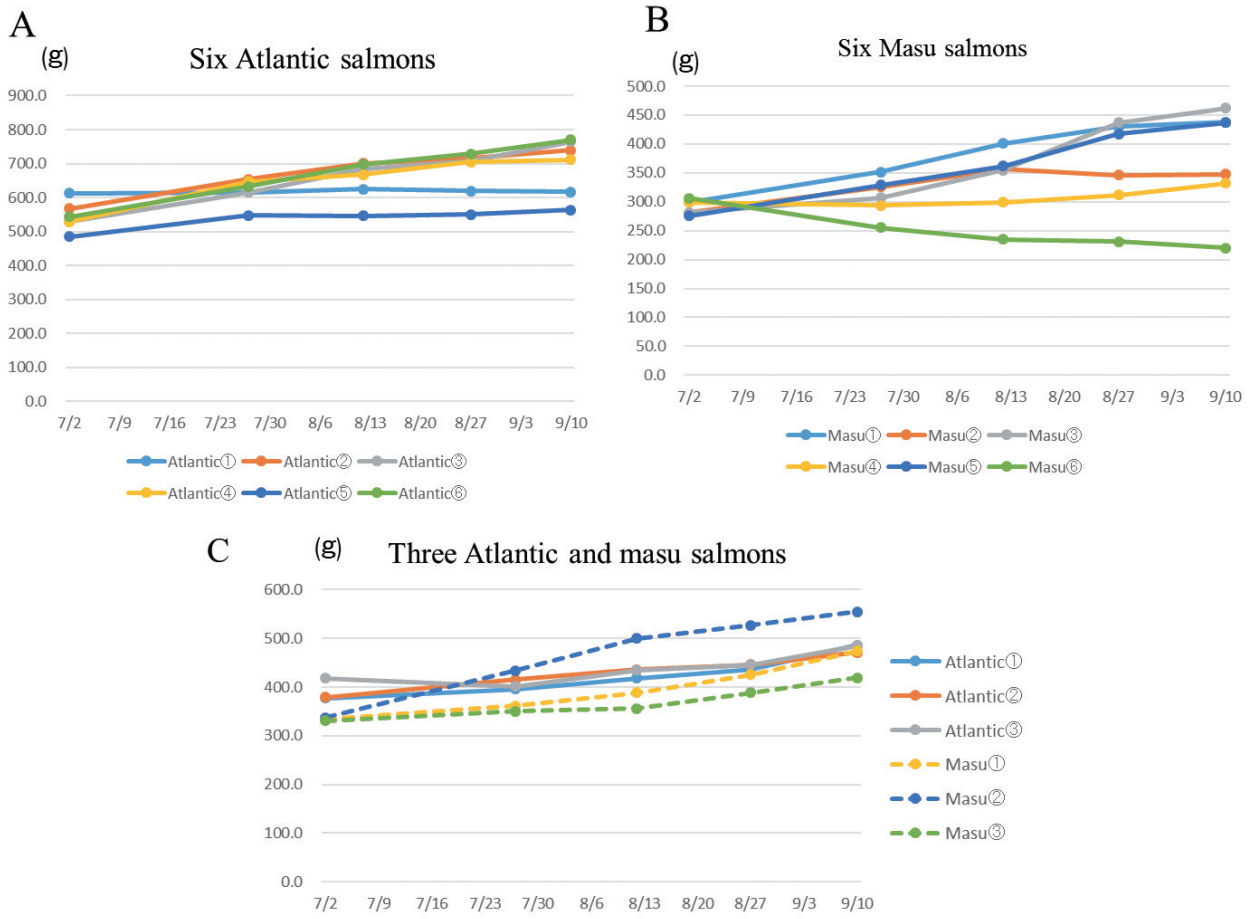


**Fig. 5.** Body weight change in the competition test between Atlantic salmon and Masu salmon. A: The body weight change of 6 individual Atlantic salmon reared in a single-species pool. X-axis: time (day); Y-axis: body weight (g). B: The body weight change of 6 individual Masu salmon reared in a single-species. C: The body weight change of 3 Atlantic salmon and 3 Masu salmon reared and fed together in one pool.

Atlantic salmon was strong in the experimental conditions, and that the level of competition depends on the combination of species at this life stage (sub-adult).

Many accounts of interspecific and intraspecific competition describing dominance, feeding behaviors, and growth rates of Atlantic salmon and other salmonid species exist, particularly as the habitat niche of Atlantic salmon overlaps with that of brown trout and rainbow trout, especially in juvenile stage (Fausch, 1998; Bremset and Heggenes, 2001; Volpe *et al*., 2001b; Stradmeyer *et al*., 2008; Van Zwol *et al*., 2012). Most reports suggest that brown trout dominates and maintain a favorable growth rate regardless of which other salmonid species are present, and also that rainbow trout will dominate Atlantic salmon and consume more food than Atlantic salmon. Habitat segregation is an important means for juvenile Atlantic salmon and brown trout to avoid competition in rivers and lakes (Berg *et al*., 2014), with Atlantic salmon juveniles tending to inhabit deeper waters than brown trout (Heggenes *et al*., 2002). Small, naturally reproducing populations of Atlantic salmon may exist in some rivers in Vancouver Island on the Pacific coast of Canada, where they are believed to segregate within their habit when in sympatry with other salmon species, such as steelhead trout (Maitland and Campbell, 1992; Volpe *et al*., 2001b). In this study, the Atlantic salmon reared in a pond with either Amago salmon or Biwa trout occupied the deeper areas, seemingly to avoid the other species, and ate only food pellets

fallen on the pond floor. Moreover, when reared with Masu salmon, Atlantic salmon were typically chased away from the food pellets.

Our objectives were to determine whether Atlantic salmon could mature in containment conditions in Japan Honshu Island and to evaluate the potential of the species to hybridize and compete with native salmonids. We demonstrated that water temperature was important for the maturation of Atlantic salmon up to the pre-smolt stage, yet small numbers of salmon fry from artificially fertilized eggs survived for two years. Additionally, of four native salmonid species that we attempted to hybridize with Atlantic salmon, only crosses with white-spotted char produced a hybrid that had the potential to survive for at least 2 years. We also observed variable degrees of interspecific competition for food and space in the contained settings, with the level of competition depending on the combination of salmonid species and their life stage. Based on the observational data so far, we propose that escaped Atlantic salmon cultured in Japan would pose a negligible competitive threat to the native salmonid species. These observations can assist the decision making of the government departments and commercial companies in the event that Atlantic salmon, particularly AquAdvantage salmon, are ever commercially cultured in Japan.

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## Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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We have developed an "all fish" growth hormone (GH) chimeric gene construct by using an antifreeze protein gene (AFP) promoter from ocean pout linked to a chinook salmon GH cDNA clone. After microinjection into fertilized, nonactivated Atlantic salmon eggs via the micropyle, transgenic Atlantic salmon were generated. The presence of the transgene was detected by polymerase chain reaction (PCR) using specific oligonucleotide primers. A number of these transgenic fish showed dramatic increases in their growth rate. At 1 year old, the average increase of the transgenic fish was 2 to 6 fold and the largest transgenic fish was 13 times that of the average non-transgenic control.

(2) Deitch E. J., Fletcher G. L., Petersen L. H., Costa I. A., Shears M. A., Driedzic W. R., and Gamperl A. K., 2006: Cardiorespiratory modifications, and limitations, in post-smolt growth hormone transgenic Atlantic salmon *Salmo salar*. *J. Exp. Biol.* 209, 1310–1325

In recent years, there has been a great deal of interest in how growth hormone (GH) transgenesis affects fish physiology. However, the results of these studies are often difficult to interpret because the transgenic and non-transgenic fish had very different environmental/rearinghistories. This study used a stable line of size-matched GH Atlantic salmon (Salmo salar) that were reared in a shared tank with controls (at 10°C, for ~9•months) to perform a comprehensive examination of the cardiorespiratory physiology of GH transgenic salmon, and serves as a novel test of the theory of symmorphosis. The GH transgenic salmon had a 3.6 faster growth rate, and 21 and 25% higher values for mass-specific routine and standard oxygen consumption (MO2), respectively. However, there was no concurrent increase in their maximum MO2, which resulted in them having an 18% lower metabolic scope and a 9% reduction in critical swimming speed. This decreased metabolic capacity / performance was surprising given that the transgenics had a 29% larger heart with an 18% greater mass-specific maximum in situ cardiac output, a 14% greater post-stress blood haemoglobin concentration, 5 – 10% higher red muscle and heart aerobic enzyme (citratesynthase or cytochrome oxidase) activities, and twofold higher resting and 1.7 higher post-stress, catecholamine levels. However, gill surface area was the only cardiorespiratory parameter that was not enhanced, and our data suggest that gill oxygen transfer may have been limiting. Overall, this research: (1) shows that there are significant metabolic costs associated with GH transgenesis in this line of Atlantic salmon; (2) provides the first direct evidence that cardiac function is enhanced by GH transgenesis; (3) shows that a universal upregulation of post-smolt (adult) GH transgenic salmon cardiorespiratory physiology, as suggested by symmorphosis, does not occur; and (4)

supports the idea that whereas differences in arterial oxygen transport (i.e.cardiac output and blood oxygen carrying capacity) are important determinants of inter-specific differences in aerobicity, diffusion-limited processes must be enhanced to achieve substantial intra-specific improvements in metabolic and swimming performance.

(3) Volpe J. P., Anholt B. R., and Glickman B. W., 2001: Competition among juvenile Atlantic salmon (*Salmo salar*) and steelhead (*Oncorhynchus mykiss*): relevance to invasion potential in British Columbia. *Canadian. Can. J. Fish. Aquat. Sci.* **58**, 197–207.

Atlantic salmon (*Salmo salar*) are routinely captured in both freshwater and marine environments of coastal British Columbia (Canada). Recent evidence suggests that this species is now naturally reproducing in Vancouver Island Rivers. Our objective was to quantify the performance of each species in intra- and inter-specific competition by assessing the competitive ability of Atlantic salmon sympatric with native niche equivalent steelhead – rainbow trout (*Oncorhynchus mykiss*). Significant behavioural differences, particularly with respect to agonism, were observed between species; however, the status of an individual as resident or challenger was the best predictor of performance. Resident fish always outperformed challengers, regardless of species. Thus, we suggest that Atlantic salmon may be capable of colonizing and persisting in coastal British Columbia river systems that are underutilized by native species, such as the steelhead.

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# Hybrid Striped Bass National Breeding Program: Research Towards Genetic Improvement of a Non-Model Species

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**Abstract**: The hybrid striped bass (HSB) farming industry at present relies almost totally on wild broodstock for annual production of larvae and fingerlings, and industry efforts to domesticate the parent species of the HSB (white bass: WB, *Morone chrysops*; striped bass: SB, *M. saxatilis*) have been fairly limited in scope. At the USDA-ARS HKD Stuttgart National Aquaculture Research Center (HKDSNARC), multiple areas of research are being pursued, with the end result being to provide HSB producers with a better performing line of broodfish. Among the areas of research that are currently being pursued at HKDSNARC are: 1) the development of genomic resources for WB and SB; 2) the molecular and physiological consequences of alternative production and broodstock diets on HSB and parental species; and 3) the molecular and physiological consequences of exposure to different production environments. An overview of these findings will be discussed.

**Key words**: *Morone*, selective improvement, transcriptome, RNA-seq, performance testing

## Introduction and background

The white bass (*Morone chrysops*) and the striped bass (*M. saxatilis*) are temperate basses with high ecological, recreational, and commercial value in North America. The hybrid striped bass (HSB) was found to exhibit improved performance in captivity with regard to growth, survival, hardiness and disease resistance, presumably through hybrid vigor (heterosis) resulting from the crossing of the two parent species, white bass and striped bass (Kerby and Harrell, 1990; Harrell, 1997). Commercial production of HSB began in the early 1980s with the original HSB cross, or palmetto bass (striped bass female × white bass male), which has been mostly replaced by the more easily spawned reciprocal cross, or sunshine bass (white bass female × striped bass male) (Garber and Sullivan, 2006).

In general, the HSB commercial production cycle is composed of four distinct phases (Hodson, 1995; Harrell and Webster, 1997). Following the hatchery phase (Phase 0), 3 - 5 day-old larvae (fry) are stocked into fertilized, outdoor ponds where they feed on natural zooplankton. Approximately 30 - 45 days after stocking, the fry are recovered as Phase I fingerlings, graded, trained to feed on prepared diets and restocked at approximately 50 mm length and1g body weight. Phase I is the interval from stocking of larvae to harvest of the juvenile fingerlings, which typically extends from the spawning season in April-

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May until harvest of the Phase I fingerlings. Production Phase II begins when the Phase I fingerlings are restocked into ponds for growout until late winter or early spring, typically February or March, when the fish are harvested as Phase II fingerlings, usually 90 - 225 g in body weight. Phase II fingerlings are harvested, graded and restocked into ponds for growout until they weigh 0.68 kg or more, when they are ready for harvest. At harvest size, the fish are considered to be Phase III HSB. Typically, the entire growout time from hatching to harvest is from 18 - 24 months for most HSB producers. In intensive tank culture systems, as opposed to pond systems, seasonal influences are largely irrelevant, and the HSB production phases are defined by mesh size as opposed to the length of time in or season of production. In general, at the end of each production phase, the HSB must be enumerated, measured, graded and restocked or sold to alter total densities, make feed adjustments and reduce variability in fish size.

While HSB is among the most important aquacultured species in the United States, most producers are still reliant on wild-origin broodstock, meaning they are not able to take advantage of genetic gains available from a selectively improved broodstock (Garber and Sullivan, 2006). Given the importance of HSB to American aquaculture, it is critical to continue to develop research results and genetic resources that can be incorporated into a selectively improved broodstock available to American farmers. Following is a brief synopsis of several studies conducted by my lab.

**Effect of amino acid supplementation on gene expression in hybrid striped bass lean muscle**

Fishmeal represents one of the largest influences on the price of feed, leading to an increase in operating expenses for producers that affects the long-term sustainability of the industry. This is because ocean supplies of fishmeal are finite and at the maximum sustainable yield, which limits market availability and results in intense competition among all animal feed industries for this commodity (Naylor *et al*., 2000; Tacon and Metian, 2008; Naylor *et al*., 2009). As research continues to explore minimizing or eliminating fishmeal in commercial aquaculture diets, many of these alternative "replacement" formulations have resulted in poorer fish performance due to an array of reasons, including limited amounts of essential amino acids (EAA) needed for optimum growth (Small and Soares, 1998; Francis *et al*., 2001; Cheng *et al*., 2003; Glencross *et al*., 2007). Muscle is the largest tissue compartment in fish, comprising ~ 60% of the total fish mass and is the desired consumer end product. Hence, it is reasonable to postulate that the muscle amino acid profile provides an ideal model for practical diet formulation and for optimizing muscle growth. In fishmeal replacement diets, three EAAs —lysine, methionine, and threonine — are typically identified as first limiting (Keembiyehetty and Gatlin, 1992; Webster, 2002; Encarnação *et al*., 2004; Gatlin *et al*., 2007).

Two growth differentiation factors that are secreted as part of the regulatory process of myogenesis are myostatin and myogenin. In cattle, myostatin has been shown to act primarily on the muscles by inhibiting myoblast proliferation and differentiation; however, when myostatin is mutated and non-functional, a double muscling effect results (Bass *et al*., 1999; Thomas *et al*., 2000; Langley *et al*., 2002). On the other hand, myogenin is required for the differentiation of myoblasts and fusion of myogenic precursor cells to existing fibers, or to create new fibers during myogenesis (Johansen and Overturf, 2005).

Previously, we conducted a growth trial to test the hypothesis that ideal protein theory accurately predicts first-limiting amino acids and optimum lysine level for a fishmeal-free, commercial-grade diet formulated for hybrid striped bass (Rawles *et al*., 2013). In that work we concluded that even though lysine was not first-limiting in our replacement test diets, lysine concentration in the muscle of hybrid striped bass accurately predicted the appropriate dietary levels (between 2.7 and 4.1 g Lys / 100g) needed for improved fish performance with respect to a number of response variables, including protein accretion and muscle ratio (Rawles *et al*., 2013). The mechanisms by which dietary amino acid supplementation affect nutrient utilization and muscling in fish is unclear, but post-prandial elevations in plasma amino acids have been shown to stimulate protein synthesis in muscle fibers (Frank *et*

*al*., 2006). Moreover, cell culture studies suggest that there are multiple possible levels of crosstalk in the salient signaling pathways mediated by mammalian target of rapamycin (mTOR) – (Proud, 2004; Sarbassov *et al*., 2005; Wullschleger *et al*., 2006). While a significant body of research has focused on establishing the role of amino acids in regulating protein synthesis in livestock (Suryawan and Davis, 2003, 2005; Suryawan *et al*., 2001, 2003, 2004, 2006), very little information exists for agriculturally important finfish. Therefore, our goal in this follow-up study was to elucidate how differential dietary lysine supplementation of a fishmeal-free diet influences the expression of two muscle genes, myostatin and myogenin, in HSB with critical roles in myogenesis.

A practical diet for HSB was formulated without fishmeal and supplemented with Met and Thr and varying levels of Lys to form a series of dose–response diets that were fed to triplicate tanks of juvenile HSB for 12 weeks as described in Rawles *et al*. (2013). The lysine supplementation feeding trial was executed in tanks at the Harry K. Dupree Stuttgart National Aquaculture Research Center (HKDSNARC) using juvenile HSB over an 84 - day period according to Rawles *et al*. (2013). Briefly, the test diets were fed to triplicate tanks of juvenile HSB for 12 weeks. During acclimation, fish were fed a standard commercial diet for maintaining condition with minimal growth. Fish were then pooled, individually weighed (118.4 ± 0.9 g; average initial weight ± SE) and randomly stocked (35 fish/tank) into 27 circular fiberglass tanks (0.63 $m^3$) supplied with flow-through well-water (24 °C; 4 L / min/ tank) and ample aeration from a regenerative blower. HSB were fed to satiation once daily and feed intake was determined as previously described (Rawles *et al*. 2013). On the last day of the trial, feed was withheld and all fish were individually weighed and separated into three size categories within each treatment diet based on final weight – small, medium, and large. Randomly selected fish from each tank were used for determination of whole body composition, nutrient retention and body composition indices that included hepatosomatic index (HSI), intraperitoneal fat (IPF) ratio, and muscle ratio (MR). Muscle tissues were isolated from matching locations on each fish and stored in RNAlater solution (Ambion, Inc, Foster City, CA, USA) at -80 °C until RNA extraction. Real Time RT-PCR methods are described in Childress *et al*. (2015).

Our goal in the current study was to examine how dietary lysine supplementation of a fishmeal-free diet influenced the interplay of two muscle genes, myostatin and myogenin, with critical roles in myogenesis in HSB and to explore the possibility of being able to differentiate growth performance based on the genetic profile of HSB in the study. Based on the criteria we used for genetic variation, i.e., small vs. large growers, we were able to statistically separate differences in myogenin, but not myostatin, expression based on size. Our work showed that with minimal lysine supplementation, myogenin expression was significantly reduced in all fish ($P = 0.010$) as well as in the small growers ($P = 0.042$) as compared to an unsupplemented diet until the "ideal protein theory level" of 3.51 g Lys / 100g diet considered by Rawles *et al*. (2013) was achieved, then myogenin expression increased. Additionally, in all fish ($P = 0.003$) as well the larger growers ($P = 0.010$), when lysine was supplemented over what was considered by Rawles *et al*. (2013) as "ideal" at 3.51 g lysine / 100 g diet, myogenin expression was significantly reduced.

The interplay of myostatin in relation to myogenin and the effect of balanced diets in HSB found in this study contributes towards the goal of achieving marker-assisted selection, which has not yet been widely applied in HSB strains, in order to improve fish performance. To a large extent, this is due to the limited availability of molecular markers for genomic analysis in HSB strains. Compared to other farm animals, including rainbow trout, HSB is still a relatively new commercially produced taxon and has not been thoroughly investigated with respect to breeds or strains with distinct genotypic or phenotypic traits. Hence, the current work contributes towards development of fish that have improved growth performance when fed fishmeal-free diets by showing that dietary limiting amino acid supplementation can influence myogenesis growth factors in HSB.

**Influence of diet on white bass egg fatty acid profile**

Nutrition has significant effects on ovarian growth, fecundity, and progeny robustness (Mourente and Odriozola, 1990; Harel *et al*., 1994; Mazorra *et al*., 2003). Among the dietary constituents of prepared feeds, dietary lipids and their fatty acids are critical for the overall reproductive performance of the female as well as progeny development and survival during yolk sac resorption (Fernandez-Palacios *et al*., 1995, 1997; Navas *et al*., 1997; Bruce *et al*., 1999; Mazorra *et al*., 2003). Although some organisms can synthesize EPA and DHA de novo to satisfy their EFA requirements, many fish lack adequate enzymatic function to produce these fatty acids at a rate sufficient to meet nutrient requirements (Lane and Kohler, 2007). In the case of broodstock, the requirement for dietary EFAs is most likely greatest from a previtellogenic period to ovulation. Therefore, EPA and DHA are often supplemented in diets of broodstock for normal growth and development of progeny (March, 1993; Sargent *et al*., 1995).

Feeding live food sources has proven to be an effective strategy to satisfy the EFA requirements of broodfish. However, an effective formulated feed for broodfish would reduce production costs, simplify feed management, and eliminate a potential pathogen source in culture facilities. These feeds may potentially outperform live foods (nutritionally), ensuring the supply and continuity of viable progeny to the aquaculture industry. Further knowledge of nutritional factors responsible for reproductive success is needed to develop a broodstock diet and improve fingerling production for aquaculture producers. Therefore, the objective of this study was to evaluate the reproductive performance of white bass broodstock fed one of six commercial diets and evaluate the relative fatty acid compositions of their eggs.

Female white bass were held in earthen ponds during the winter and were seined the first week of April when water temperatures reached 15 degrees Celsius. Ninety white bass were evenly stocked into six 600 L fiberglass tanks. The temperature in all six tanks was maintained at 16 degrees Celsius with a water chiller. Lids were placed over the tanks and artificial lights and a timer were used to mimic natural photoperiod. All six tanks of fish were given three weeks to acclimate and were fed Cargill AquaFeed 48-18 (protein-lipid) during this time. Fish were weighed on April 22 to determine initial weight and each tank was then randomly assigned a diet. All tanks were fed to satiation once daily. Each tank was fed its specific diet for 30 days and five fish were randomly selected for samples and weighed on day 31. The remaining fish were fed an additional 28 days and five fish were randomly selected for samples and weighed on day 60. The six diets used included: Zeigler Bass Brood 45-15, Bio-Oregon BioBrood 48-20, Cargill AquaFeed 45-12, Cargill AquaFeed 48-18, Skretting Extruded Steelhead 45-16, Skretting Classic Brood 46-12.

Step-wise discriminant analysis (SDA) was conducted on the fatty acid profiles of eggs from white bass fed six different commercial diets using the SAS 9.3 program STEPDISC (SAS Institute, Cary, North Carolina). Overall, time had an influence in fatty acid content in white bass eggs, with profile of fatty acids changing from the 4 week sampling to the 8 week sampling. Overall, diets grouped by manufacturer regardless of protein or lipid level. A few of the heavy hitters (known to be important in larvae) were different among these diets, including DHA, and EPA. Our next step is to get larval performance data for comparison. We anticipate full results to be completed and published soon.

**Genomic consequences of rearing hybrid striped bass under hypoxic conditions**

Hypoxia is a state of oxygen deficiency that is sufficient to cause impairment of organismal function or in extreme cases, death. Hypoxia is becoming an increasingly important environmental concern (Diaz 2001; Wu *et al*., 2003) and has received extensive attention in the fisheries sciences, particularly aquaculture. Understanding the tissue-specific and temporal changes in gene expression in fishes exposed to hypoxia could reveal new mechanisms of hypoxia tolerance and shed light on this adaptive response in vertebrates. In settings of aquaculture, the intensity and duration of hypoxia depends on a variety of factors including fish biomass, feeding rate, phytoplankton blooms, and installed aeration capacity (Green *et al*., 2015). Previously, we characterized the effects of hypoxia on performance metrics of hybrid

striped bass and found that hypoxia led to reduced feed intake, which resulted in lower nutrient retention and growth (Green *et al*., 2015). However, the precise molecular mechanisms contributing to these disparate phenotypes were unknown. However, recent advancement in genomic resources for striped bass (Li *et al*., 2014; Reading *et al*., 2012) and white bass (Li *et al*., 2014) offer researchers the ability to take a closer look directly at many *Morone* functional pathways that were previously studied via model species. Given the importance of hypoxia on the management and captive rearing of *Morone*, we examine here the transcriptional responses of hybrid striped bass to acute and chronic hypoxia. We highlight unique and shared signatures between hypoxic treatments and the bioenergetic consequences of chronic oxygen deprivation on hepatocellular function. Our findings offer a more comprehensive view of the cellular and molecular consequences of hypoxia and reveal new mechanisms of hypoxia tolerance in teleosts.

The fish used in this study were cohorts of those subjected to hypoxia as previously described by Green *et al*. (2015). Fish were held at DO25 and DO100 levels for 90 d after which nine fish from each treatment were sacrificed and liver samples were collected for RNA-seq. Remaining fish from each tank were removed from their respective treatments using a dip net and immediately placed in the opposite DO saturation DO25 → DO100 (restoration of normoxia) and DO100→DO25 (acute hypoxia) for 6 h, after which, nine fish per treatment were sacrificed and livers collected for RNA-seq. For RNA-seq methods, see Beck *et al*. (2016).

Differential expression analysis was performed between 100% DO saturation (DO100 or normoxia) and 25% DO saturation groups (DO25 or chronic hypoxia), between DO100 and DO100 → DO25 (acute hypoxia), and between DO25 and DO25 → DO100 groups. A total of 1403 unique genes (based on assigned identifies from the NR database) showed significant differential expression in liver (Fig. 1). In detail, there were 91 differentially expressed genes between DO100 and DO25 saturation groups, a meager 39 genes differently expressed between DO25 saturation and DO25 → DO100 saturation groups, and the greatest degree of differential

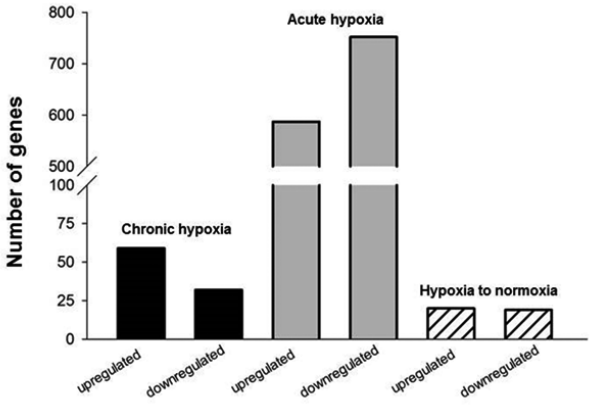


**Fig. 1.** The number of genes that were statistically differentially expressed under each condition and the direction of their expression. Chronic hypoxia (25% oxygen saturation for 90 days) resulted in transcriptional changes to 91 genes (59 upregulated, 32 downregulated); acute hypoxia (25% oxygen saturation for 6 h) altered 1339 genes (587 upregulated, 752 downregulated), and the movement of fish from the chronic hypoxia treatment to normoxia treatment (100% oxygen saturation) changed 39 genes (20 upregulated, 19 downregulated).

expression was observed in DO100 and DO100 → DO25 saturation comparison, with 587 up-regulated genes and 752 down-regulated genes (total 1339 genes). Extracellular flux analysis of liver cells obtained from DO100 and DO25 fish revealed significant differences in oxygen consumption rates (OCR), with DO25 liver possessing around 25% higher OCR (Fig. 2A). Mitotracker Red staining of liver cell suspensions from DO25 and DO100 fish showed that DO25 liver cells showed heightened fluorescence levels, indicative of a greater mitochondrial content (Fig. 2B).

Next-generation sequencing platforms have propelled rates of discovery, particularly in non-model species where few genomic and transcriptomic resources are available. Work by our group showed previously the phenotypic impacts of chronic hypoxia on hybrid striped bass feeding behavior and growth traits (Green *et al*., 2015). Here, we delve further into this area to examine the molecular consequences of oxygen deprivation in an effort to reveal the molecular underpinnings responsible for the overall poorer performance under hypoxemia.

Previously (using the same experimental cohorts of

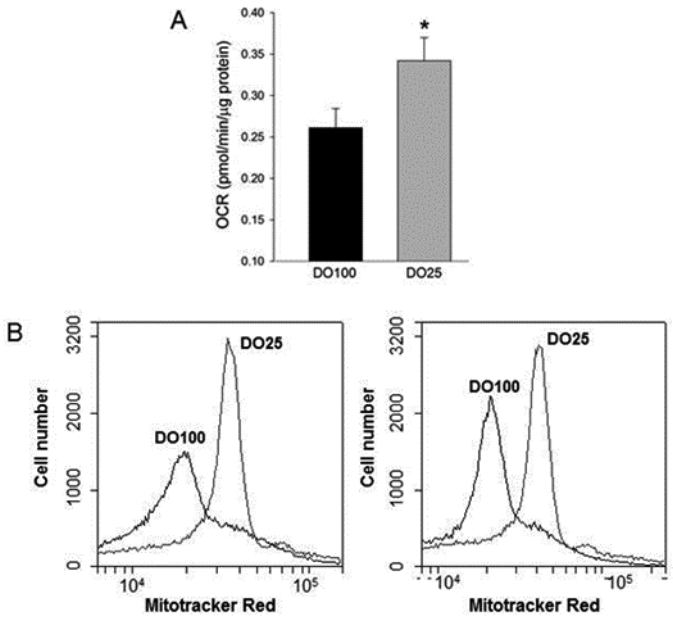


**Fig. 2.** A) Liver tissue isolated from fish subjected to chronic hypoxia show greater oxygen consumption rates (OCR; mean ± SEM; five fish per treatment) and B) increased mitochondrial content (two representative fish from each treatment) as indicated by the fluorescence intensity derived from staining with the mitochondrion-selective dye Mitotracker Red.

the fish employed here), we observed significantly larger livers (as indicated by the hepatosomatic index) in HSB reared in normoxia versus hypoxia and reported significantly higher levels of whole body lipid (Green *et al*., 2015). Qualitatively, the enlarged livers were noticeably pale and soft in texture, while fish exposed to hypoxia exhibited livers that were darkened in appearance, firm, and friable (Green *et al*., 2015). These observations led us to focus on examining the liver transcriptome in the present study. Indeed, the liver plays a central role in metabolic homeostasis and is a major site for the synthesis, metabolism, storage and redistribution of processed carbohydrates, proteins and lipids (Bechmann *et al*., 2012). In tissues such as the heart and liver, lipids provide a rich source of energy via oxidative phosphorylation by mitochondria (Jungermann, 1988; Shohet and Garcia, 2007). In mammals, where hypoxic stress is typically viewed as a pathological state (e.g., fatty liver disease, ischemia), lipid metabolism under low oxygen conditions is reprogrammed to suppress mitochondrial oxidation of lipids as a protective measure against toxic metabolites and oxidative stress. Broadly, hypoxia promotes lipid storage and inhibits lipid catabolism through β oxidation (Whitmer *et al*., 1978; Bostrom *et al*., 2006). In contrast, in aquatic animals, environmental hypoxia is a common challenge that many aquatic organisms experience in their habitat and responding to hypoxia requires metabolic reprogramming so that energy-demanding processes are regulated to match available energy reserves (Gracey *et al*., 2011). Here, we observed changes — with similarities and differences to work in mammals and other teleosts — in multiple genes involved in lipid catabolism and β oxidation, particularly following acute hypoxia. Enrichment analysis pointed us towards a deeper examination of cell death processes. Two pathways related to cellular preservation, apoptosis and autophagy, were clearly influenced by hypoxic insult. In taking together the gross differences in liver size, the alterations in lipid utilization, the suppression of apoptosis combined with the induction of autophagy, our findings suggest that the hepatic tissue of hybrid striped bass may have entered a state of senescence. This may have allowed the animals to liberate, redirect, or shunt resources to other vital tissues to survive hypoxia.

The use of RNA-seq allows for the examination of global transcriptional changes in a tissue at a particular snapshot in time. However, it is widely understood that the level of transcriptional message does not always correlate with protein. With little to no resources (i.e., antibodies) available for HSB, we utilized extracellular flux analysis to quantify aerobic respiration in liver tissue obtained from DO100 and DO25 fish. Unexpectedly, under conditions of reduced oxygen availability, liver cells from DO25 fish showed significant increases in basal oxygen consumption rates, a surrogate measure of mitochondrial function. Curiously, as compared to our previous work with established fish cell lines (Beck and Fuller, 2012), total

aerobic respiration was lower and glycolysis was virtually non-detectable. By using a fluorophore that selectively stains mitochondria, we documented an increase in the mass (linked to number/size of mitochondria) of the mitochondrial compartment, which could have accounted for the increase in oxygen consumption by DO25 liver cells. An increase in mitochondrial number, a process termed mitochondrial biogenesis, is a common cellular response to hypoxia and is thought to improve the efficiency of oxygen consumption and ATP synthesis, and in parallel counteract potential cellular damage brought about by hypoxia (Kopp *et al*., 2014). Consistent with these observations is a greater than 400 fold increase in PGC-1β message in liver tissue from the acute hypoxia treatment. In contrast to mammals, where PGC-1*α* is regarded as the archetypal master regulator of mitochondrial biogenesis, recent evidence from teleosts suggests that PGC-1β may exert more control over mitochondrial gene expression (LeMoine *et al*., 2008). Intriguingly, and related to the above discussion on lipid metabolism, PGC-1β is also known to play a key regulatory role in hepatic lipid metabolism with PGC-1β knockout mice being more susceptible to hepatic steatosis (Sonoda *et al*., 2007) and the transduction of rodent liver with PGC-1β decreased hepatic lipid while plasma triglyceride and cholesterol levels were significantly elevated (Lin *et al*., 2005). Clearly, further research is needed to better understand the interactions between mitochondrial abundance, regulation, and lipid metabolism in hypoxic teleosts.

**Development of moronid genomic resources**

Genetic information has been restricted to a single tissue (ovary) transcriptome and a microsatellite linkage map from striped bass (Reading *et al*., 2012; Liu *et al*., 2012), limiting gene discovery and expression and functional studies in the two species and their hybrid. Here we set out to produce well-annotated transcriptomes for both species to advance future broad-based RNA-seq studies of gene expression as well as aid in more targeted studies of important genes and pathways.

Major tissues and organs (brain, liver, spleen, trunk kidney, ovary, testes, gill, and intestine) from 10 individuals from white bass and 10 individuals from striped bass were harvested, and equal amounts of tissue from each system were pooled prior to RNA extraction. The result was two master pools of RNA, one for each species. Each pool was used for library construction and sequencing in a lane of Illumina HiSeq2000 platform. A total of 262 x $10^6$ total high quality reads were obtained with 135 x $10^6$ reads from striped bass and 127 x $10^6$ reads from white bass. Using the TRINITY de novo assembly software (Grabherr *et al*., 2011), reads were assembled into 203,587 striped bass unique contigs and 185,531 white bass unique contigs. N50 and average contig sizes were 2915 and 1263 bp respectively for striped bass, and 3132 and 1371 bp respectively for white bass (Table 1). These included 166,867 and 185,351 transcripts that were identified for the first time in striped bass and white bass, respectively. Annotation was carried out by BLAST against the UniProt and NR (NCBI non-redundant) databases for both species. At an E-value ≤ 1e-5, 21,186 and 29,624, unigene matches were obtained against the UniProt and NR databases respectively in striped bass, and 21,001 and 28,906 matches were returned in white bass against the same databases. Of these NR matches, 25,902 (87.4%) in striped bass and 25,484 (88.2%) in white bass were predicted to have full-length transcript coverage based on TRINITY analysis. Using more stringent criteria, similar results were obtained from both species, with 18,630 UniProt and 23,605 NR annotated unigenes in striped bass and 18,584 UniProt and 22,354 NR annotated unigenes in white bass (score ≥ 100, E-value ≤ 1e-20; Table 2).

In both species most valuably from a management standpoint, the transcriptomes yielded microsatellite and SNP markers valuable in future downstream

**Table 1.** Summary of de novo assembly results of Illumina RNA-seq data from striped bass and white bass using Trinity assembler

| | Striped Bass | White Bass |
|---|---|---|
| Contigs | 203,587 | 185,531 |
| Largest contig (bp) | 21,100 | 28,262 |
| Large contigs (≥1000bp) | 68,395 | 66,891 |
| Large contigs (≥500bp) | 98,864 | 94,485 |
| N50 (bp) | 2,915 | 3,132 |
| Average contig length (bp) | 1,263 | 1,371 |

**Table 2.** Summary of gene identification and annotation of assembled Striped bass and White bass contigs based on BLAST homology searches against various protein databases (UniProt and nr). Putative gene matches were at E-value ≤ 1e-5. Hypothetical gene matches denote those BLAST hits with uninformative annotation. Quality unigene hits denote more stringent parameters, including score ≥ 100, E-value ≤ 1e-20.

| | Striped bass | | White bass | |
|---|---|---|---|---|
| | UniProt | nr | UniProt | nr |
| Contigs with putative gene matches | 69,134 | 79,062 | 68,312 | 76,884 |
| Annotated contigs ≥1000bp | 54,487 | 58,316 | 54,430 | 57,839 |
| Annotated contigs ≥500bp | 62,602 | 68,876 | 62,158 | 67,682 |
| Unigene matches | 21,186 | 29,624 | 21,001 | 28,906 |
| Hypothetical gene matches | 0 | 1,901 | 0 | 1,858 |
| Quality Unigene matches | 18,630 | 23,605 | 18,584 | 22,354 |

analyses. In striped bass, from a total of 32,111 microsatellites identified by MSATFINDER (Thurston and Field, 2005), 36.05% (n = 11,577) had sufficient flanking regions to allow design of primers. These 11,577 microsatellites were distributed across 10,055 contigs. Similarly, in white bass, from a total of 30,408 microsatellites, 34.53% (n = 10,500) had sufficient flanking regions to allow design of primers. These 10,500 microsatellites were distributed across 9054 contigs. A SNP analysis comparing between species yielded 2220 markers polymorphic in one species but not the other, including 1661 SNPs associated with genes. Additionally, in the future, the reference transcriptomes will serve as an important sequence anchor for short-read genotyping studies using techniques such as RAD-seq or genotyping by sequencing (GBS) (Davey *et al*., 2011).

The TRINITY-based assembly of the white bass and striped bass transcriptomes generated high-quality, gene-length transcripts, which will be of great utility in future expression and functional studies in moronid species. Microsatellite and SNP markers identified at the same time are expected to aid in aquaculture, conservation, and sportfish genetic management and improvement.

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## Annotated bibliography

(1) Li C, Beck B. H., Fuller S. A. , and Peatman E., 2014: Transcriptome annotation and SNP discovery in white bass (*Morone chrysops*) and striped bass (*Morone saxatilis*). *Anim. Genet.* **45**, 885-887.

The authors present the first ever multi-tissue reference transcriptomes for striped bass (*Morone saxatilis*) and white bass (*Morone chrysops*) which are the parental species of the hybrid striped bass, a major U.S. aquaculture species. Being non-model species, this was of critical importance, as prior to this there only existed a medium-density genetic linkage map and a well-annotated ovarian transcriptome. The assembled Moronid reference transcriptomes and identified simple sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs) should advance ongoing studies of reproduction, physiology, and immunology in these species and provide markers for broodstock management and selection.

(2) Childress C. J., Fuller S. A., Rawles S. D., Beck B. H., Gaylord T. G., Barrows F. T., and McEntire M. E., 2015: Lysine supplementation of commercial fishmeal-free diet in hybrid striped bass *Morone chrysops* x *M. saxatilis* affects expression of growth-related genes. *Aquac. Nutr.* DOI: 10.1111/anu.12300

The authors present a follow-up study to a study (Rawles *et al*., 2013) where ideal protein theory accurately predicted first-limiting amino acids and optimum lysine level for a fishmeal-free, commercial-grade diet for hybrid striped bass (HSB). In the current study, authors sought to determine how dietary lysine supplementation of these same diets influences the expression of two genes, myostatin and myogenin, controlling myogenesis in differentially growing groups of HSB. Real-time rt-PCR results in HSB suggest that the levels of lysine added to the diet has an impact on myogenin relative to the unsupplemented diet, but no effect on myostatin. Moreover, presented data also suggests that the amount of dietary lysine supplementation influenced the ratio of myostatin/myogenin expression in HSB and that this pattern mimicked that of most of the growth, composition of growth and nutrient retention data from the authors' previous study and may therefore be a useful marker for selecting fish for improved growth performance.

(3) Fuller S. A., Farmer B. D. and Beck B. H., 2014: White bass *Morone chrysops* is less susceptible than its hybrid to experimental infection with *Flavobacterium columnare*. *Dis. Aquat. Org.* **109**, 15-

22.

The authors present research regarding *Flavobacterium columnare*, the causative agent of columnaris disease, susceptibility differences between hybrid striped bass (HSB) and white bass (WB) in a series of 3 fundamental studies. In the first experiment, the authors sought to determine whether columnaris disease could be developed using a low-water flow experimental challenge in HSB using 3 levels of *F. columnare* (60-, 30-, 10- ml). Each of these treatment groups exhibited significantly different survival rates: 0, 3.3, and 13.3%, with higher survival occurring in treatment groups exposed to less bacteria. In the second experiment (30 ml), both HSB and WB had a 0% survival rate, but the WB took significantly longer to reach 100% mortality. Finally in Expt 3 (10 ml), no HSB survived, whereas 33% of WB survived. Compared to controls, the authors observed extensive gill damage in HSB treated with 10 ml after 24 h, which they hypothesized could have contributed to the higher mortality observed in HSB; an observation not seen on the WB gills. From these series of experiments, it is clear that HSB are more sensitive to *F. columnare*, having lower survival and more extensive histological damage compared to WB following the bacterial challenge.

(4) Beck B. H., Fuller S. A., Peatman E., McEntire M. E., Darwish A. M., and Freeman D.W., 2012: Chronic exogenous kisspeptin administration accelerates gonadal development in basses of the genus Morone. *Comp. Biochem. Physiol. A Physiol.* **162**, 265-273.

The authors present the effects of chronic administration of kisspeptins to immature and mature white bass (WB), striped bass (SB), and hybrid striped bass (HSB) to determine its effects on gonadal development in these species. The authors determined that bi-weekly injections (over 7 weeks) differentially accelerate puberty, as evidenced by increases in the prevalence of spermatozoa in the testes of juvenile fish. Also, in sexually mature fish, kisspeptin treatment led to increased gonad weight, gonadosomatic index, and spermatocrit in some white and striped bass. Additionally, mature white bass treated with kisspeptins showed an advancement in oocyte development as determined by histological examination. Importantly, the gonadal changes occurred in the absence of any photothermal manipulation or hormone injections. This description was the first report of kisspeptin-mediated pubertal initiation in fish, and the first evidence that kisspeptins could modulate gonad maturation.

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# Production of *Benedenia*-resistant Yellowtail (*Seriola quinqueradiata*) Families –A Preliminary Approach to the Broodstock Candidates–

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**Abstract**: The skin fluke *Benedenia seriolae* is a parasite specific to *Seriola* species. It feeds on the epidermal tissues of yellowtail (*Seriola quinqueradiata*) and causes external injuries that render the fish susceptible to bacterial and viral infection. Infection with this parasite is a serious problem for yellowtail aquaculture because it also leads to a reduction in fish growth. A previous study has reported the existence of an inherited *Benedenia* disease-resistance factor in yellowtail. Any yellowtail families resistant to *Benedenia* disease have not yet been produced, although the production of such families for aquaculture would potentially help to reduce not only infectious diseases but also the labor costs to eradicate *Benedenia*. In this study, we investigated time series changes of the number of *Benedenia* parasites on each host yellowtail for the purpose of selecting *Benedenia* resistant broodstock candidates.

In September 2014, the number of individual parasites on 100 fish that were selected randomly from 10,000 wild-caught 0-age yellowtail at an aquaculture farm was investigated and it ranged from 1 to 48 individuals / fish. Thus we selected 961 fish that has the lowest 10% parasite susceptibility (3 or fewer *Benedenia*). The selected fish were then cultured in sea net cages and the number of parasites on each was counted at five times between November 2014 and July 2015. The average number of parasites per fish had a wide range from 0.2 to 39.4 individuals / fish over this period, and the overall mean was 8.9 individuals / fish. One hundred sixty fish with lower than 3 parasites were selected as broodstock candidates.

We are now using these broodstock candidates as parents to produce F1 yellowtail families for *Benedenia*-resistant analysis using DNA marker-assisted-selection breeding methods.

**Key words**: Yellowtail (*Seriola quinqueradiata*), Skin fluke (*Benedenia seriolae*), Breeding, *Benedenia*-resistant family.

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## Introduction

Many fish species are farmed in Japan, and yellowtail is one of the most important species for aquaculture production (Fig. 1). In yellowtail aquaculture, wild- caught juveniles are farmed to marketable size (Nakada 2008). However, fishing season, abundance and size of wild juveniles are unstable. Additionally, fishing pressure on wild juveniles for aquaculture could impact the natural resources of yellowtail. For these reasons, using artificial seed is anticipated to enable a more stable and sustainable aquaculture production. Furthermore, farmers require seedlings with additional value such as disease resistance and rapid growth during culture (Yoshida *et al.* 2012).

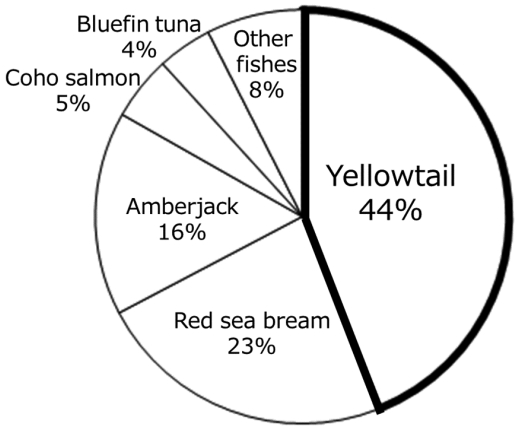


**Fig. 1.** Species composition of marine finfish aquaculture production in Japan 2013. Total production is approximately 240,000 tons (adapted from the 2013 census of fisheries by Fisheries Agency of Japan).

In yellowtail aquaculture, one of the serious problems is the parasitic disease caused by the skin fluke, *Benedenia seriolae*. It parasitizes the fish body surface and feeds on the epidermal tissues. The parasitization causes external injuries and increases the risk of viral or bacterial infections, as well as growth reduction of the host fish. At aquaculture farms, the most common method to eradicate the parasite is the freshwater bathing treatment. However, new parasites resurface within a few weeks after the treatment. Thus, the farmers have to treat the cultured fish many times during the aquaculture period and the treatment requires a great deal of effort, as well as causing stress to the fish via both handling and the freshwater treatment.

A previous study of genetic analysis reported a heritable *Benedenia* resistance factor in yellowtail (Ozaki *et al.* 2013). However, *Benedenia*-resistant yellowtail families have not yet been produced. The production of such families would help reduce both risks of infectious disease and efforts to eradicate the parasite in aquaculture facilities. In the present study, broodstock candidates with a low parasite count were selected for breeding value-added "*Benedenia*-resistant" yellowtail families.

## Materials and methods

### Preliminary selection

The preliminary process focused on selecting yellowtail with the lowest 10% parasite count among 10,000 wild-caught 0-age fish at Shimaura Aquafarm Branch, Maruha Nichiro Corporation, Miyazaki, Japan. Because there was no data available on the parasite intensity for the selection, the initial step of our study was to investigate the infection levels of the yellowtail.

In September 2014, the frequency distribution of the parasite number for 100 randomly selected fish was examined (the procedure of the parasite counts is described later). The parasite count per fish ranged from 1 to 48 individuals / fish, and the lowest 10% of them ranged from 1 to 3 individuals / fish (Fig. 2). From these data, we decided to select fish with 3

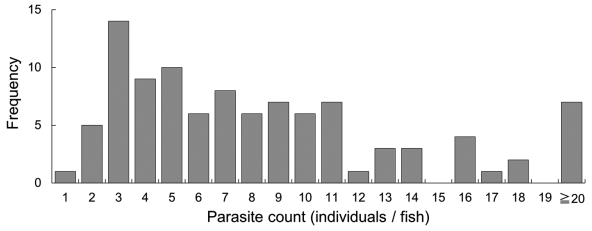


**Fig. 2.** Frequency distribution of the parasite count among the 100 wild-caught 0-age yellowtail. The frequencies from 20 to 48 (the highest value) individuals / fish range from 0 to 2.

or fewer parasites per fish, and 961 fish were preliminary selected from the sample of 10,000 yellowtail at the aquaculture farm. The mean of fork length and body weight of these fish were approximately 30.0 cm and 370 g, respectively.

**Selection for broodstock candidates**

In November 2014, these preliminary selected fish were transferred from the aquaculture farm to floating sea net cages at Goto Laboratory, Seikai National Fisheries Research Institute, Nagasaki, Japan. The mean (± SD) of fork length and body weight of these fish were 36.5 ± 1.5 cm and 828 ± 109 g, respectively. Of all the 961 fish, 800 fish that had no obvious physical damage were selected and passive integrated transponder (PIT) tags (Biomark Inc.) were inserted for individual identification. The fish were reared in 4 groups of 200 individuals in 4 net cages (A–D).

The examinations of parasite count on the experimental fish (detailed in Yoshida *et al*. 2012) were performed at 5 times during November 2014 and July 2015. Because the number of *Benedenia* parasites tends to decrease during winter, the counting of them was not conducted between December and March. At each counting, the experimental fish were individually captured in a mesh bag and then the bags were placed in a freshwater tank for 4 minutes (all *Benedenia* died within 4 minutes). After that, the fish was identified from the PIT tag, and the total number of parasites was obtained by summing the remaining dead parasites on the fish body surface and the removing ones in the bag.

## Results and Discussion

Fig. 3 shows the changes in parasite count for 40 yellowtail with the lowest parasite count and 10 individuals with the highest parasite count in each net cage. Among 5 examinations, the average number of parasites per fish had a wide range from 0.2 to 39.4 individuals / fish over this period, and the overall mean parasite count was 8.9 individuals / fish.

Based on the results, a total of 160 fish (40 fish with the lowest parasite count were selected from each cage) were selected for broodstock candidates with low parasitic susceptibility. The mean (range) of parasite count on these candidates was 3.3 (0.2–7.0) individuals / fish. Additionally, the average parasite count for 9 fish was lower than 1.0 individual / fish during the experimental period, and these fish showed remarkably less-susceptibility.

The purpose of further study is to establish *Benedenia*-resistant yellowtail families. In 2017, F1 yellowtail hatchery juvenile will be produced from these selected broodstock candidates. Moreover, we are developing DNA markers for marker-assisted selection breeding methods. In the F1 generation, we will carry out both trait evaluation and also marker-assisted selection by genetic analyses. The goal of this study is fixation of the trait in the F2 generation and to produce *Benedenia*-resistant yellowtail families for development of sustainable yellowtail aquaculture

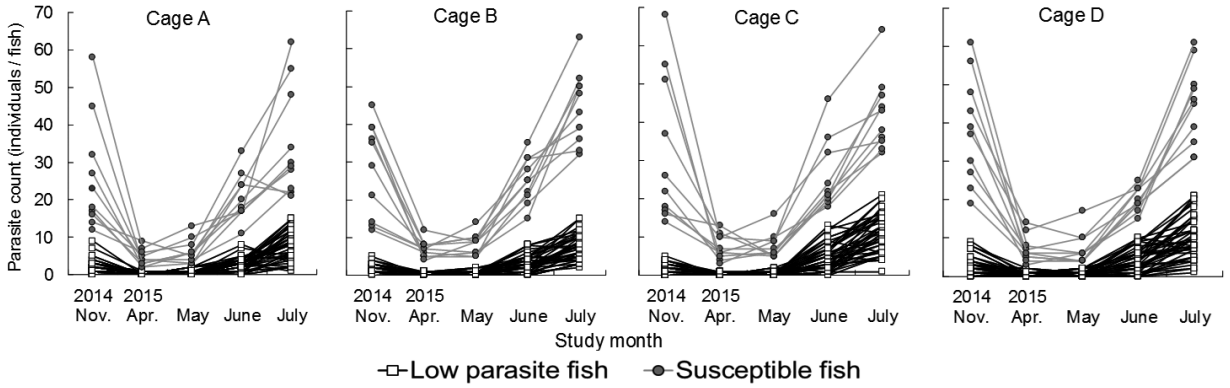


**Fig. 3.** Time series changes of parasite count per yellowtail by the fish cage. The lowest 40 and the highest 10 fish of parasite susceptibility were drawn. Because the number of *Benedenia* parasites tends to decrease during winter, counting of them was nod conducted between December and March.

production.

## Acknowledgment

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

Yoshida K., Ozaki A., Nakagawa M., Hotta T., Aoki J., Koyama T., Araki K., Okamoto N., Sakamoto T., and Tsuzaki T., 2013: Characteristic Evaluation Method Relating to Benedenia Disease of Yellowtail (*Seriola quinqueradiata*). Proceedings of the forty U.S.-Japan aquaculture panel symposium (ed. by Rust M., Olin,P. Bagwill A. and Fujitani M.), NOAA Technical Memorandum NMFS-F/SPO-136, Seattle, pp. 96-99.

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

Benedenia infections caused by the monogenean fluke ectoparasite *Benedenia seriolae* seriously impact marine finfish aquaculture. Genetic variation in host has been inferred to play a significant role in determining the susceptibility to this parasitic disease. To evaluate the genetic basis of Benedenia disease resistance in yellowtail (*Seriola quinqueradiata*), a genome-wide and chromosome-wide linkage analyses were initiated using 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.

(2) Yoshida K., Ozaki A., Nakagawa M., Hotta T., Aoki J., Koyama T., Araki K., Okamoto N., Sakamoto T., and Tsuzaki T., 2013: Characteristic Evaluation Method Relating to Benedenia Disease of Yellowtail (*Seriola quinqueradiata*). Proceedings of the forty U.S.-Japan aquaculture panel symposium (ed. by Rust M., Olin,P. Bagwill A., and Fujitani M.), NOAA Technical Memorandum NMFS-F/SPO-136, Seattle, pp. 96-99.

Benedenia is a parasitic disease caused in Seriola species by *Benedenia seriolae*. This parasite can cause growth reduction and external injuries in yellowtail, increasing the risk of secondary viral or bacterial infection. The main method of parasite removal is to soak the fish in a freshwater bath. However, this method requires a great deal of time,

cost, and effort. We have been studying DNA Marker-Assisted Selection (MAS) breeding, to select for resistance to Benedenia disease. Three components ("Reproduction technology", "Character evaluation", and "DNA analysis") are critically important to promote MAS breeding success. We focus on one of the key components, "Characteristic evaluation method" relating to Benedenia disease in yellowtail.

(3) Mushiake K., Yamazaki H., and Fujimoto H., 2007: Current Situation of Technical Developments in Seed Production of Yellowtail (*Seriola quinqueradiata*) in Japan. Proceedings of the thirty-fourth U.S.-Japan aquaculture panel symposium (ed. by Stickney R., Iwamoto R., and Rust M.), NOAA Technical Memorandum NMFS-F/SPO-85, Seattle, pp. 1-4.

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

(4) Nakada M., 2008: Capture-based aquaculture of yellowtail. Capture-based aquaculture. Global overview (ed. By Lovatelli A. and Holthus P. F.), FAO Fisheries Technical Paper 508, FAO, Rome, pp. 199–215.

The 2004 production of cultured yellowtail (*Seriola* spp.) in Japan from 1,288 enterprises was 150,028 tonnes valued at ¥111.2 billion (US$1.334 billion). Yellowtail mariculture has developed remarkably due to the abundant supply and low price of wild-caught juveniles (Mojako) and sardines used as the main fish feed of fishmeal component. Hatchery produced yellowtail seed are far more expensive. Other critical elements that supported the growth of yellowtail farming include the existence of abundant suitable culture sites along the Japanese coast and innovative technical developments.

The history of yellowtail culture in Japan began over 70 years ago. Before that, fishers cultured undersized fish in ponds and sold them when they reached marketable size. This utilization of bycatch (undersized fish) was accepted by the public, particularly as unmarketable fish were often used as fertilizer or livestock feed. Currently aquaculture production for many species exceeds that landed from capture fisheries.

Some commercial culture trials on amberjack have been undertaken in Taiwan, Province of China, Mexico and Vietnam, but no successes have been achieved with raising yellowtail. The main constraints include diseases and low production costs in tropical areas. In contrast, the culture of *Seriola* spp. is promising due to their strong vitality and rapid growth, and may well expand at the global level through hatchery-produced juveniles.

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# Coho Salmon Broodstock Development: A Case Study of the Domsea Coho Salmon (1977 to 2015)

Robert N. IWAMOTO[*1], Richard TOWNER[*2], James MYERS[*3], Greg HUDSON[*1], and Patricia MUNSELL[*1]

**Abstract**: There are few long-term studies on Pacific salmon that inform on the potential for gains by systematic selective breeding. As a consequence, there is limited data on the potential for inbreeding losses because of constrained population sizes and matings among closely related individuals over a prolonged period of time. In 1977, Domsea Farms Inc., the University of Washington, and the Washington Sea Grant Program initiated a genetic selection program for coho salmon for Domsea's marine net-pen operations. Because little was known at that time regarding the potential for genetic improvement in coho salmon, the program was initially designed with two central goals: 1) collect basic information (heritabilities, genetic and phenotypic correlations) on the potential for genetic improvement in such economically important traits such as smoltification, growth rate to harvest, flesh color, and reproductive fitness; and 2) using that information, develop selection and mating protocols that would maximize selection gains but minimize inbreeding. Despite significant changes in ownership, rearing environment and operations, the program has remained remarkably consistent over the past 38 years or 19 generations of selection. We have demonstrated that selection for improved growth to the smolt stage (7.1-11.3% per generation) and adult phases (43-53 g per generation) can be achieved. Overall, the growth rate of the Domsea coho salmon has improved between 3% and 8% per generation while reproductive traits such as female weight, egg weight, and survival to ponding have remained unaffected by inbreeding. While traditional genetic approaches have been demonstrably successful for this program, it is anticipated that further consideration and application of molecular approaches will help further characterize and advance this broodstock program for coho salmon.

**Key words**: Coho salmon, *Oncorhynchus kisutch*, Domsea, selection gains, inbreeding

## Introduction

Over the past 50 years or so, the application of genetic selection theory to improve strains of fish and shellfish has been an active and, in many cases, successful endeavor (see Quinton *et al.* 2005-Atlantic salmon; Dunham and Smitherman 1984-catfish; Tave 1999-Tilapia). As a result, the genetic bases for trait improvement are now well characterized for many commercially important aquatic species. However, there is less known about the effects of long-term intensive selection and the resulting inbreeding on closed populations because of the lack of well-documented, multi-generational selection programs.

This paper presents the results of a 19-generation long and still on-going selection program on a closed-population of coho salmon (*Oncorhynchus kisutch*). It outlines the general facets of the broodstock (commonly known as the Domsea coho) and some major results including trait characterization and

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improvement as well as inbreeding effects to date.

## Materials and methods

The Domsea coho genetics program was initiated in 1977 as a cooperative effort among Domsea Farms (a then subsidiary of Union Carbide, Inc.), the University of Washington, and the Washington Sea Grant Program. The initial population was founded in 1974 with gametes from the Wallace River Hatchery strain, Skykomish River Basin, in western Washington State. The population had been maintained in captive culture via random mating prior the selection program's start three years later. The initial goals were to develop a population on a two-year spawning cycle and to characterize the genetic bases for several commercially important traits as well as to apply that information to develop a selection program.

Domsea Farms, in the mid to late 1970s, was the first commercial net-pen operation in Puget Sound, WA, and its goal was to produce pan-sized (500 g) coho salmon. Fast growth in fresh and seawater and improved smoltification and subsequent seawater survival were the primary traits of selection interest. Since then, other traits of interest have included fecundity, egg size, and size at maturity.

A breeding design involving 40 full- and half-sib families initially and later just 40 full-sib families was initially used to evaluate growth performance and estimate heritabilities and genetic correlations for and among traits. The selection strategy included between and within-family selection using a selection index and a mating scheme to minimize inbreeding as much as possible. The program utilized multiple selection events at major life history/rearing transition points (Fig. 1). Each year individuals from the top 10 families were crossed in a circular mating pattern to create six full-sib families within each of the top 10 family crosses*1. The 60 families were then reduced to 40 families after incubation based on assessments of egg size, fecundity, and survival to ponding. Each family was reared separately until

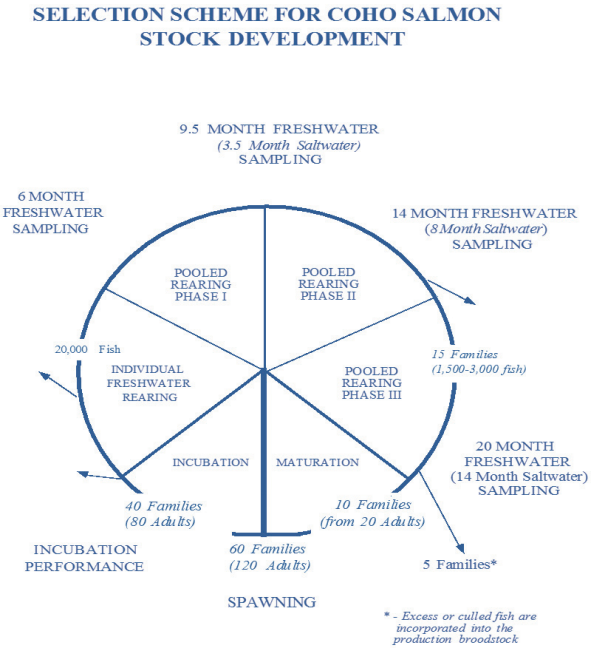


**Fig. 1.** Selection program for Domsea coho salmon broodstock development The program has been virtually unchanged for 19 generations with the exception of the change from partial seawater to total freshwater freshwater rearing.

large enough to externally identify with a freeze brand, then families were combined and transferred to marine net-pens. Fish were sampled during the freshwater growth period, at time of transfer to saltwater and at two other periods during saltwater rearing, at 3.5 and 8.0 months post-transfer to assess saltwater survival and growth. The resulting data were then used in the selection index to reduce the number of families to 10*2. After transfer to freshwater for final maturation, the 6-10 largest males and females from each of the selected families were mated using a circular mating design to avoid mating closely-related individuals.

In 1986-1987, because of high pre-spawning mortality for adults maturing in marine net-pens; the survival, maturation, and reproductive success of adults held in freshwater throughout their entire lives were evaluated. As a result of those trials, the broodstock program was then shifted, and has

*1 Family crosses were adjusted to avoid first-cousin crosses.
*2 In some years the 10 families were selected from the top 15 performing families, due to inbreeding concerns or poor survival post-index selection.

remained ever since, to an all freshwater program. The selection strategy and mating design has remained the same except that the saltwater holding period was then eliminated.

Because of the two-year generational cycle, even- and odd-year lines were established. In 1992, the even-year line was lost due to a hatchery accident, so about half of the odd-year line were subsequently photoperiod-manipulated to spawn two years later, thus re-creating an even-year spawning group. The *de novo* even-year line has been maintained as a separate line ever since.

Pedigrees for both even- and odd-year lines have been maintained from the start, and in combination with population numbers and microsatellite, allozyme, and serum protein (transferrin) data, have been used to estimate inbreeding levels for each generation.

At the initiation of the program, control lines were maintained to assess selection efficacy. However, within two generations, logistics of the program and the space constraints forced the termination of the control lines. Since then, the performance of fish from the founding hatchery population has been compared with the select line to assess changes to the selected lines.

## Results and discussion

### Genetic estimates

The first quantitative genetic parameters for coho salmon traits were reported by Iwamoto *et al.* (1982), Saxton *et al.* (1984), and Iwamoto *et al.* (1990) from the Domsea coho program (see Table 1 for a summary). Overall these genetic estimates for freshwater growth, smoltification and initial seawater survival, seawater growth, carcass-related, and reproductive traits for the Domsea coho salmon indicated that a systematic selection program would be successful (Hershberger *et al.*, 1990).

Selection gains for weight and length at 7, 11, and 14 months post-fertilization were predicted using the heritability estimates and a range of selection intensities (Table 2). The range of predicted selection gains was quite high. For example, the predicted improvement for 7-month weight after the first generation of selection varied from 1.4% to 46%.

**Table 1.** Heritability estimates (standard error in parentheses) for various traits at different generations. Estimates are based on sire (S), pooled sire and dam (S + D), full-sib (FS), or pooled full-sibs/double first cousin (FS + DFC) components. Other abbreviations: SW = saltwater; FW = freshwater.

| | | Generations under selection | | | | |
|---|---|---|---|---|---|---|
| Component | Trait | 1 | 2 | 3 | 5 | 14 |
| $h^2_S$ | 8 m SW Weight | 0.19 (0.11)<br>0.62 (0.21) | | | | |
| $h^2_{S+D}$ | 8 m SW Weight | 0.20 (0.06)<br>0.33 (0.10) | | | | |
| $h^2_{FS}$ | 8 m SW Weight | | 0.40 (0.15) | 0.30 (0.07)<br>0.29 (0.08) | 0.21 (0.09)<br>0.22 (0.09) | |
| $h^2_{FS+DFC}$ | 8 m SW Weight | | | 0.26 (0.07)<br>0.30 (0.07) | 0.20 (0.07)<br>0.24 (0.07) | |
| $h^2_{FS}$ | 24 m FW Weight | | | | | 0.422 (0.146) |
| $h^2_{FS}$ | 24m FW Fecundity | | | | | 0.237 (0.108) |
| $h^2_{FS}$ | 24 m FW Egg Size | | | | | 0.432 (0.135) |
| $h^2_{FS}$ | Carotenoid | | 0.50 (0.16) | 0.30 (0.14) | | |
| $h^2_{FS}$ | Lipid | | 0.18 (0.13) | 0.19 (0.23) | | |

**Table 2.** Prediction of selection gains based on different selection intensities

| Trait | | Mean | Max Estimate | % | Min Estimated | % |
|---|---|---|---|---|---|---|
| 7 Month | Weight | 14.46 g | 6.61 g[a] | 46 | 0.2 g | 1.4 |
| | Length | 101.45 mm | 11.63 mm[a] | 11 | 0.6 mm | 0.6 |
| 11 Month | Weight | 136.71 g | 27.79 g[b] | 20 | 3.3 g | 2.4 |
| | Length | 210.42 mm | 28.97 mm[b] | 14 | 0.7 mm | 0.3 |
| 14 Month | Weight | 374.17 g | 72.00 g[c] | 19 | 7.9 g | 2.1 |
| | Length | 305.37 mm | 21.76 mm[c] | 7 | 3.2 mm | 1.0 |

[a] Based on selecting 60 individuals out of 10,000
[b] Based on selecting 60 individuals out of 7,000
[c] Based on selecting 60 individuals out of 3,000
[d] Based on index results and selecting 10 families out of 40

### Improvements

The 7-month weight of the odd-year line changed from 11.2 g in 1977 to 24.7 g in 2011, while the even-year line increased from 11.2 g in 1977 to 34.1 g in 2012 (Fig. 2). The increase for the odd-year line represented a 7.1% per generation increase, while the increase for the even-year line was equivalent to an 11.3% per generation increase. While the total increase could not be attributed to directed selection alone, it does indicate that there has been substantial improvement for that trait. This is despite changes in level of care and husbandry over the past 38 years due to changes in ownership of the company. The fluctuations in care and husbandry are partially reflected by the yearly variability in performance reflected in Fig. 2.

Although adult weight was not a selected trait until fairly late in the program, the fairly high genetic correlation between spawn weight and 7-month weight, led to increases in spawn weight. The average spawn weight of the odd-year females in 2011 was 2568 g compared with 1669 g for the first generation (Fig. 3). This was an increase of 53 g per generation or an overall increase of 54% over 17 generations.

Because of the changes in ownership, and the differences in husbandry and rearing environment over the many generations, it is difficult to precisely estimate the improvement in growth due to genetics or other causes. The best estimate for this line of coho salmon is that growth rate is improving between 3% and 8% per generation.

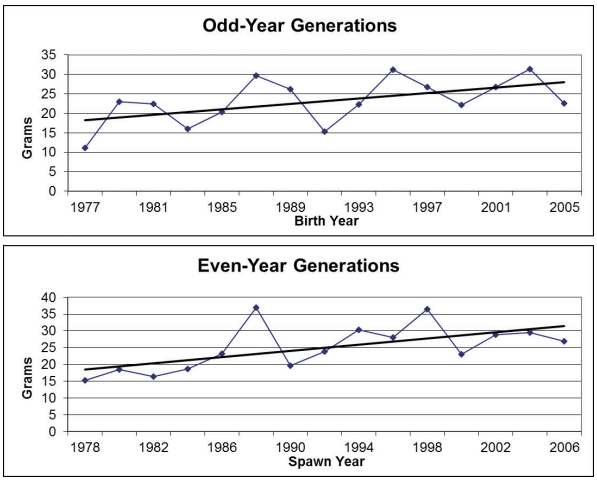


**Fig. 2.** Change in 7-month weight after 14 generations of selection

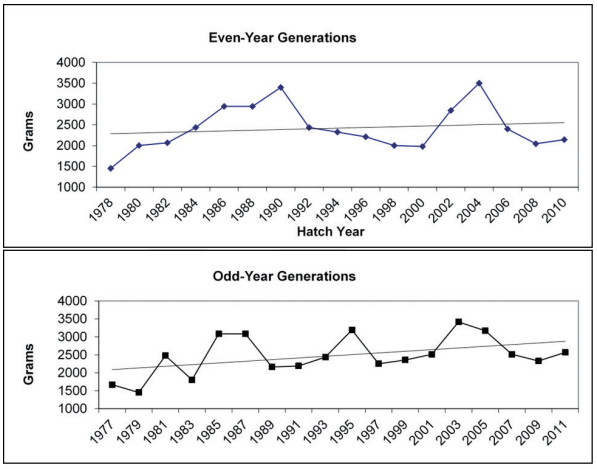


**Fig. 3.** Change in female weight at spawn over the course of 16 and 17 generations for the odd- and even-year broodstocks.

**Inbreeding**

Despite the circular mating scheme, inbreeding could not be avoided but merely delayed because of the intense selection the closed populations were subjected to every generation. Over the life of the program, inbreeding has been estimated in various ways. For example, Myers *et al.* (2001) estimated that after 9 and 10 generations of selection for the even- and odd-year lines respectively, inbreeding had increased by 13 - 48% depending on the method of estimation. Overall, via allozyme analysis, the authors determined that the two lines had undergone little change in average heterozygosity but significant changes in loss of variability at several loci. Pedigree-based inbreeding estimates show a progressive increase in inbreeding of about 1.5% per generation (Fig. 4). The inbreeding estimates for the even-year line shows a striking decrease in inbreeding within the last two generations as a result of deliberate outcrossing with the founder strain.

There have been no obvious indications of inbreeding depression despite the relatively high levels of inbreeding. If the current level of inbreeding is 25% or higher, it would be equivalent to that of full-sib matings – levels at which other studies have indicated can negatively affect reproductive success and early growth and survival of fish (Bondari and Dunham, 1987; Wang *et al*., 2002). Because of the absence of a control group, the effects of inbreeding on overall growth and survival could not be assessed. It is possible that had inbreeding levels been lower over the course of the program, larger improvements per generation might have been observed.

## Conclusion

The Domsea coho broodstock program has and continues to provide important data on the efficacy of a long-term genetic selection program to improve commercially important traits. More importantly, it provides an opportunity to address the question of the effects inbreeding may have on a sustained basis. As the program matures, it will be informative to incorporate genomics theory into the current quantitative genetics-based program to further characterize the genetic basis for commercially important traits and concomitant effects of inbreeding.

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Iwamoto R. N., Myers J. M., and Hershberger, W. K., 1990: Heritability and genetic correlations for flesh coloration in pen-reared coho salmon. *Aquaculture* **86**, 181-190.

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d. Iwamoto R. N., Myers J. M., and Hershberger W. K., 1990: Heritability and genetic correlations for flesh coloration in pen-reared coho salmon. *Aquaculture* 86, 181-190.

The papers cited above provide much of the early quantitative genetic parameters for coho salmon traits. Genetic estimates for freshwater growth, smoltification and initial saltwater survival, seawater growth, and flesh coloration for the Domsea coho salmon were derived from full- and half-sib analyses. In almost every case, the magnitude of the derived heritabilities and genetic correlations indicated that a systematic selection program would be successful.

(2) Myers J. M., Park L. K., Neely K., Swanson P., Elz A., and Hard J. J., 2011: Feeding ration, genetics, and reproductive traits in female coho salmon: Is bigger better? *J. World Aquac. Soc.* **42**, 812-823.

The authors examine reproductive traits and the relationship between environment (feeding ration) and genetics (family) in the Domsea coho salmon stock after 15 generations of selection. Results suggested that phenotypic improvements could be achieved through changes in ration and/or direct selection.

(3) Myers J. M., Iwamoto R. N., Teel D., Van Doornink D., and Hershberger W. K., 1999: Coho salmon broodstock developmet – 1977-1998. Ten generations of systematic selective breeding. *Bull. Natl. Res. Inst. Aquac. Suppl.* **1**, 63-70.

The authors summarize the concept of the Domsea coho salmon broodstock program from the selection scheme to the circular mating design. They also present allozyme comparisons of the founder strain and the broodstock after 10 generations of selection and indicate that significant genetic changes had occurred in the broodstock as a result of genetic selection and drift.

(4) Myers J. M., Heggelund P. O., Hudson G., and Iwamoto R. N., 2001: Genetics and broodstock management of coho salmon. In: Lee, Cheng-Sheng and Donaldson, E. M. (Eds.) Reproductive biotechnology in finfish aquaculture, Aquaculture **197**, 43-62.

This paper summarizes the information collected on the Domsea coho salmon broodstock program after 13 generations of implementation. It discusses the results of sib trials in marine net pens and two freshwater environments and consequently the importance of considering genotype-environment interactions in broodstock programs for specific applications. It tracked theoretical (pedigree data) and genetic changes (allozyme data and individual traits) in the broodstock population.

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# Development of Improved Catfish Germplasm at the Warmwater Aquaculture Research Unit, DSDA - ARS

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**Abstract**: Farm-raised catfish is the largest aquaculture enterprise in the U.S. in terms of both production volume and value. The USDA-ARS Warmwater Aquaculture Research Unit (WARU) mission includes development and release to farmers of catfish germplasm improved for economically important traits. Historically, the channel catfish (*Ictalurus punctatus*) comprised nearly all U.S. farm-raised catfish production, but in the last 10 years, production of the F1 hybrid between the channel catfish and blue catfish (*Ictalurus furcatus*) has increased to about 50% of total production. Therefore, the WARU breeding program is focused on genetic improvement of purebred channel catfish and F1 hybrid catfish performance. The channel catfish improvement program has used selection on BLUP breeding value estimates to improve growth rate and carcass yield. We started with a diverse population of channel catfish derived from 10 commercial farms in 2006 and have evaluated over 21,000 animals from 750 full-sib families produced by 408 sires and 624 dams between 2008 and 2015. Heritabilities for harvest weight and residual carcass weight are 0.29 and 0.36, respectively, and fish are selected based on an index that puts equal weight on individual breeding values for growth and family average breeding values for residual carcass weight. Improvements in growth have been 8 - 10% per generation and are in agreement with expected improvements. Increases in residual carcass weight have been less substantial (-2 g per generation) than those for harvest weight due, at least in part, to the necessity for selection on less accurate family average breeding values for carcass weight.

The hybrid catfish improvement program has focused on evaluation, identification and selection of blue catfish sires that produce superior performing hybrid progeny. We initiated a program to collect several blue catfish populations and evaluate performance of their purebred and hybrid progeny. Initial evaluations suggest the majority of genetic variance for growth in hybrid progeny is additive in nature, and populations and individual sires that produce purebred blue catfish progeny with faster growth also produce hybrid progeny with faster growth. We have ongoing experiments to estimate heritabilities and genetic correlations in the purebred blue and hybrid catfish, which will give us direction in selecting purebred blue catfish for improved hybrid catfish performance. We use DNA markers to identify parentage and establish pedigrees in our populations and are developing a SNP chip to be used for genomic selection to improve our breeding value accuracy and rate of improvement, particularly for carcass yield. We are evaluating other traits for inclusion in our selection index, and are collecting and cryopreserving sperm from superior channel and blue catfish sires for future use. This combination of traditional animal breeding, genomic selection and cryopreservation will result in improved catfish germplasm, improved production efficiency and greater profitability for catfish farmers.

**Key words**: Channel catfish, blue catfish, genetic improvement

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## Introduction

Catfish farming is the largest aquaculture enterprise in the U.S. in terms of both production volume and value (National Agricultural Statistic Service 2012). Approximately 150 million kg of catfish were produced and processed in the U.S. in 2015. Most of the production occurs in the southeastern U.S. and the vast majority of product is consumed domestically. Prior to 2005, nearly all production consisted of purebred channel catfish, *Ictalurus punctatus*, but in the last 10 years, production of hybrid catfish (F1 hybrid between female channel catfish and male blue catfish, *Ictalurus furcatus*) has increased dramatically and represented about 50% of catfish production in 2015. Although catfish aquaculture represents the majority of U.S. aquaculture production, current production is about half of what it was at its peak in 2003. The reduced production of catfish in the U.S. is related to increased production costs (particularly feed), competition from lower-priced imported fillets (*Pangasius* and *Tilapia*) targeted at similar markets, and the subsequent conversion of catfish ponds to more profitable row-crop production. The USDA-ARS Warmwater Aquaculture Research Unit (WARU) mission includes development and release of improved catfish germplasm to U.S. farmers that will improve production efficiency and help U.S. farmers remain competitive in a global seafood market.

The process of genetic selection to develop improved germplasm for release to the industry requires production and performance evaluation of purebred blue catfish, purebred channel catfish and hybrid catfish. Mating designs used for genetic merit estimation require collection of performance data on traits targeted for selection in large, pedigreed populations. Development of improved germplasm is a recurrent process of broodfish selection, offspring production and evaluation, genetic merit estimation and selection of the next generation of broodfish. Breeding projects at WARU are focused on selection to improve performance of purebred channel catfish and selection of purebred blue and channel catfish to improve hybrid catfish performance. The WARU's current catfish breeding program and future directions are summarized in this paper.

## Materials and Methods

### Purebred channel catfish

In 2006, 10 to 12 spawns (full-sib families) were collected from eight commercial farms where farm owners indicated fish were from unique base populations. Spawns were hatched and raised in separate family tanks. Fish were fed commercial diets of appropriate size and composition for their developmental stage. Fish densities in tanks were periodically reduced and equalized in all tanks. When fish averaged greater than 40 g, 100 fish per full-sib family were tagged with individually coded passive integrated transponders (PIT) tags (BioMark, ID, USA). Fish were then transferred to replicate earthen ponds and families reared communally. Fish were fed a 32% protein commercial catfish diet to apparent satiation once daily until the majority of the fish were of market weight (0.5 to 1.0 kg). Fish were then harvested, and measured for total weight and gender. The largest 3 to 6 males and 4 to 7 females from each full-sib family were retained as broodfish and an additional 80 mature broodfish of unknown age were obtained from each of two additional commercial farms and PIT tagged. An initial pedigree file was established with an additive relationship of 0.5 among individuals within full-sib families and assuming all other fish were unrelated. Fish derived from this base population are referred to as the Delta Select strain of channel catfish. Blood samples were collected from all broodfish (835 females and 638 males) for DNA isolation and then broodfish were stocked in the spring of 2008 in earthen ponds at 800 kg/ha and allowed to mate at random. Spawns were collected from ponds every 2 to 3 days and moved to the hatchery from mid-April through early August. Fry were hatched and treated as described above and a sample of 8 to 10 fry were collected from each spawn for DNA isolation. Microsatellite genotypes of fry from each spawn and potential parents were compared to determine parentage of spawns (Waldbieser and Bosworth, 2013). Sixty to 75 fish per family were tagged, stocked communally in earthen ponds and grown to market weight as described previously. Fish were harvested in late October to November when water

temperatures cooled and feeding activity declined. Fish were harvested by seining the ponds, then anesthetized with 200 ppm MS-222 and gender, PIT tag number and weight to the nearest 0.5 g were recorded for each fish. A sample of 4 to 5 males and 4 to 5 females from each full-sib family in the weight range typically processed at commercial facilities (0.4 to 1.0 kg) were electrically stunned, decapitated (Baader 166 heading machine, Baader North America Corporation, Auburn, WA, USA), eviscerated by hand and the carcasses were weighed.

Broodfish used in the 2008 spawning season were held over winter, inventoried, weighed and restocked into spawning ponds spring of 2009. The percentage of male and female channel catfish that are mature and spawn is only about 25% at two years old but increases to over 50% at three years old. Therefore, to increase the number of broodfish that spawn and maintain a higher effective population size, selected broodfish were typically spawned as two year olds and again as three year olds. This process has been repeated with selected broodfish being spawned in 2011 and 2012, and 2014 and 2015.

Phenotypic variance, additive genetic variance, heritablilities and breeding values were estimated for each trait (harvest weight and carcass weight) with linear single-trait animal models using MTDFREML (Boldman *et al*., 1995). The model for harvest weight included fixed effects of pond, year, and gender; age within year*gender as a linear covariate; and animal additive genetic and common environment (confounded effects of full-sib family and fingerling rearing tank) as random effects. Carcass yield (the percentage of whole weight that is comprised of carcass weight) is a trait of high value to catfish processors. However, because of statistical issues related to estimation of variance components for ratios, carcass data was analyzed as residual carcass weight (carcass weight adjusted to a common whole animal weight by using whole weight as a linear covariate). The model for residual carcass weight included fixed effects of pond, year, gender and day of processing; whole weight within year*gender as a linear covariate; and random effects of animal additive genetic and common environment. Genetic correlations between harvest weight and carcass weight were not analyzed using a multi-trait model because animals measured for carcass weight were selected based on a size range required to fit the processing equipment and therefore were not a random sample, which would have biased the correlation estimate. Instead, the genetic correlation between harvest weight and carcass weight was estimated as the correlation between full-sib family average breeding values for harvest weight and residual carcass weight.

Breeding values for harvest weight and residual carcass weight were estimated with MTDFREML and approximately the top 10% of fish from each year-class were selected as broodfish based on an index placing equal weight on individual's breeding value for harvest weight and full-sib family value for residual carcass weight. Response to selection for harvest weight and residual carcass weight was evaluated by estimating the correlation between mid-parent breeding value (average breeding value of a sire and dam) and the phenotypic means of their corresponding full-sib progeny family means after adjustment for relevant fixed effects (year, pond, sex). Genetic trends (changes in mean breeding value of the population over time) were also estimated for harvest weight and residual carcass weight.

**Blue and hybrid catfish**

There is little commercial production of blue catfish and little data relevant to genetic effects of blue catfish on purebred or hybrid catfish performance. Therefore, our initial goal was to gather blue catfish germplasm from diverse sources and evaluate the effects of these populations on harvest weight and carcass yield in their purebred and hybrid progeny. Because blue catfish mature at a late age (typically five years or older) and shipping large fish is costly and difficult, we obtained fish from some sources as larvae, some as juveniles and some as mature adults. Our initial evaluations of blue catfish focused on comparisons on the population level rather than evaluations of individual males within populations due to the fact that obtaining sperm from blue catfish males to produce hybrids requires killing the male and storage of fresh sperm is limited to about five days.

We conducted a series of studies comparing effects of blue catfish populations on purebred blue catfish

and hybrid catfish progeny growth. Typically, blue catfish are pond- spawned, reared in family tanks, PIT-tagged and then stocked in ponds and reared communally as described previously for channel catfish. Hybrids are produced by hormone (LHRHa or pituitary extract) induced ovulation of female channel catfish and fertilization with blue catfish sperm obtained by maceration of testes (Bosworth *et al*., 2005). Hybrid catfish larvae are reared in family tanks, PIT-tagged and evaluated for performance in earthen ponds as described previously.

Data from five growth trials are presented. The first trial (2009 year-class) compared effects of 4 to 5 sires from each of two blue catfish populations on harvest weight of their hybrid catfish progeny; the second trial (2010 year-class) compared effects of 7 to 10 sires from each of five blue catfish populations on harvest weight of their hybrid catfish progeny; the third trial (2012 year-class) compared effects of 5 to 15 full-sib families from each of three blue catfish populations on harvest weight of their purebred blue catfish progeny; the fourth trial (2014) compared effects of 3 to 15 full-sib families from each of four blue catfish populations on fingerling weight of their purebred blue catfish progeny; and the fifth trial compared effects of 9 to 27 full-sib families from each of three blue catfish populations on fingerling weight of their purebred blue catfish progeny. Two trials (2014 and 2015 year-classes) are ongoing and, therefore, only weights of fingerlings at stocking are presented.

Mean harvest weight of hybrids and purebred blues produced from blue catfish populations were compared within each trial using the Mixed Procedure of SAS (SAS Institute, Cary, NC, USA). Models for harvest weight included pond and sex as fixed effects, age as a covariate and full-sib family within population as a random effect. Models for fingerling weight included age as a covariate and full-sib family within population as a random effect. Full-sib family was used as the error term in comparisons of population means. Correlations between mean weight of purebreds and hybrids produced using each population were estimated for each trial and the mean of these correlations was determined and provided an estimate of the relationship between purebred and hybrid growth of the various populations tested.

## Results

### Channel catfish

A total of 21,055 progeny from 750 full-sib families produced by 624 dams and 408 sires have been measured for harvest weight; 4,060 progeny from 585 families produced by 494 dams and 342 sires have been measured for carcass weight (Table 1). The heritability estimates for harvest weight and residual carcass weight are 0.29 (± 0.03) and 0.35 (± 0.05), respectively. Values for phenotypic, additive genetic and common environmental variance and heritabilities for harvest weight and residual carcass weight are listed in Table 2. The correlation between full-sib family mean breeding values for harvest weight and carcass yield was -0.11. Correlations among mid-parent breeding values and corresponding full-sib family means were 0.50 ($p < 0.0001$) for harvest weight and 0.41 ($p < 0.0001$) for residual carcass weight. The mean breeding values for harvest weight were 0 g for the base population, 78 g for the 2011 and 2012 year-classes, and 140 g for the 2014 year-class; mean breeding values for residual carcass weight were 0 g for the base population, 2.2 g for the 2011 and 2012 year-classes, and 4.8 g for the 2014 year-class.

### Blue and hybrid catfish

Blue catfish population had a significant effect on purebred blue catfish and hybrid catfish progeny growth in each of the five trials presented (Table 3). Purebred and hybrid catfish progeny produced from Rio Grande population parents consistently had the highest progeny harvest weight. The overall mean correlations across studies between mean harvest weight for hybrid and purebred progeny produced by the various populations tested was 0.98 ($p < 0.001$), indicating the effect of blue catfish population on progeny growth is consistent across hybrid and purebred progeny.

## Discussion

Heritabilities for harvest weight (0.29) and residual carcass weight (0.35) in the Delta Select strain of

**Table 1.** Number of full-sib families, sire and dams and means (+ SD) and number of fish measured for harvest weight and carcass yield for 2008, 2009, 2011, 2012 and 2014 year-classes of Delta Select channel catfish

| Year-class | Full-sib families | Sires[1] | Dams[1] | Harvest Weight | | | Carcass Yield % | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Mean | SD | n | Mean | SD | n |
| 2008 | 161 | 107 | 149 | 777.4 | 284.9 | 4,762 | 58.3 | 1.7 | 829 |
| 2009 | 186 | 113 | 181 | 614.7 | 225.3 | 5,686 | 58.0 | 1.8 | 1,352 |
| 2011[3] | 170 | 89 | 161 | 702.2 | 263.7 | 1,982 | -- | -- | -- |
| 2012 | 104 | 67 | 90 | 814.6 | 281.0 | 4,484 | 64.4 | 1.7 | 924 |
| 2014 | 113 | 73 | 109 | 881.4 | 287.5 | 4,141 | 64.3 | 1.7 | 955 |
| Total n | | | | | | | | | |
| Harvest weight | 750 | 408 | 624 | | | 21,055 | | | |
| Carcass yield | 585 | 342 | 494 | | | | | | 4,060 |

[1] The total number of sires and dams is less than the sum of sires and dams across years because some sire and dams spawned in consecutive years.

[2] Carcass data were analyzed as residuals carcass weight after covariate adjustment to a common whole weight, but values presented in Table 1 are carcass yield (100*carcass weight/whole weight) to be more meaningful to the reader. 2008 and 2009 carcass yield data were calculated based on skin-off carcass weights, 2012 and 2014 carcass yield data were calculated based on skin-on carcass weight.

[3] A severe outbreak of proliferative gill disease resulted in substantial mortalities in 2011 and, therefore, no fish were processed.

**Table 2.** Phenotypic, additive genetic and common environmental (tank and full-sib family) variance and heritabilities (+ SE) for harvest weight and residual carcass weight in the Delta Select strain of channel catfish

| Trait | Variance Component | | | | |
|---|---|---|---|---|---|
| | Phenotypic | Additive Genetic | Common Environment | Heritability | SE |
| Harvest weight g | 58,302 | 16,965 | 4,797 | 0.29 | 0.03 |
| Residual Carcass weight g | 114.4 | 40.5 | 6.5 | 0.35 | 0.05 |

**Table 3.** Effect of blue catfish sire population on least square means1 for harvest and fingerling weight of purebred and hybrid catfish offspring

| Year-Class | Hybrid/ Purebred | Trait | Blue Catfish Population | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Rio Grande | D&B | MS River | MO River | Kentucky | SE |
| 2009 | Hybrid | Harvest Weight g | --- | 362.0[a] | --- | --- | 278.3[b] | 23.1 |
| 2010 | Hybrid | Harvest Weight g | 572.2[a] | 525.0[a,b] | 506.7[b] | 459.6[c] | 442.0[c] | 26.2 |
| 2012 | Purebred | Harvest Weight g | 489.2[a] | 309.9[b] | 286.2[b] | --- | --- | 50.5 |
| 2014 | Purebred | Fingerling Weight g | 45.2[a] | 38.9[a,b] | 31.5[b] | 16.1[c] | --- | 5.5 |
| 2015 | Purebred | Fingerling Weight g | 143.8[a] | 95.4[b] | 91.3[b] | --- | --- | 8.1 |

[1] Means within row are significantly different at $P < 0.05$

channel catfish are similar to those for growth and carcass yield observed in other farmed fish species (Navarro *et al*., 2009; Nguyen *et al*., 2010) and terrestrial livestock (Hermesch *et al*., 2000; Aslam *et al*., 2011). The positive correlation between mid-parent estimated breeding values (EBVs) and mean harvest weight of their full-sib progeny (r = 0.5) and increased genetic trend over time indicate that the response to selection for harvest weight is in agreement with expectations. Data from the first two generations of selection demonstrated an increase of about 8 to 10% in average breeding values for harvest weight each generation, similar to response reported for farmed carp (Dong *et al*., 2015), tilapia (Hamzah *et al*., 2014), trout (Kause *et al*., 2005) and salmon (Gjedrem, 2000). Response to selection for carcass weight was positive but less than expected. The lower response to selection for carcass weight relative to harvest weight is due, at least in part, to the necessity to select on full-sib family mean EBVs for carcass weight, which are less accurate than the individual EBVs used to select for harvest weight. However, the correlation between mid-parent EBVs and offspring carcass weight (r = 0.41) and increased genetic trend over generations suggest positive gain in carcass weight is being made. Even small increases in carcass yield have a large effect of profitability of catfish processors.

The high correlation between mean growth of blue catfish and hybrid catfish progeny produced by the same blue catfish sire populations, along with earlier published data demonstrating much higher variation for general combining ability than for specific combining ability for effects of blue catfish sires on hybrid progeny growth (Bosworth and Waldbieser, 2014), suggest that much of the genetic influence of blue catfish on growth in hybrid progeny is additive. This may be due to genes acting in an additive genetic manner or common dominance deviations between the blue catfish populations and channel catfish we have evaluated. The current data suggests that purebred blue catfish population performance is predictive of hybrid catfish performance and that we can substantially improve hybrid growth simply by selecting for increased purebred blue catfish growth. We have a large on-going project with 120 blue catfish sires from four populations each mated to two blue catfish females and 2 to 3 channel catfish females in a series of factorial matings. Offspring from these matings are being evaluated for growth and carcass weight and the data will be used to estimate heritabilities of these traits in purebred blue catfish and hybrid catfish, genetic correlations among traits in purebred blue and hybrid catfish, and relative importance of additive and dominance genetic variation for traits. This information will provide details on the optimal approach to selecting purebred catfish for improvement of hybrid catfish.

Current germplasm development has focused on improving catfish growth and meat yield because these traits are economically important to catfish farmers and processors and because they can be measured accurately and relatively inexpensively on the large numbers of animals required for accurate estimation of heritabilities and breeding values. However, we have been collecting data on reproductive traits, disease resistance and meat quality and may include additional traits in future selection indices, if the heritabilities and economic values of the traits indicate that it would benefit catfish farmers and processors.

The use of microsatellite DNA polymorphisms to identify parentage in channel, blue and hybrid catfish populations has played an important role in our genetic evaluations by providing pedigree information required for heritability and breeding value estimation (Waldbieser and Bosworth, 2015). However, we are now planning to expand use of DNA technology in our breeding program by evaluating the use of SNP markers for genomic selection in the Delta Select channel catfish population and in evaluations related to improvement of hybrid catfish performance. We are near completion of a 50 K SNP chip (Affymetrix, Santa Clara, CA, USA) based on markers that segregate in the Delta Select population and will be evaluating approaches to using genomic selection to improve accuracy of breeding value estimates. Genomic selection should be particularly beneficial for improving accuracy of breeding value estimates and the rate of genetic gain for traits like carcass yield, which have high heritability and large economic value but cannot be measured on live animals (Daetwyler *et al*., 2012).

Ultimately, one of our primary missions at the WARU is to release improved catfish germplasm to U.S. catfish farmers for commercial production. The role of development and release of commercial germplasm is a unique situation for a research agency and requires considerable planning to allow equitable distribution of germplasm, evaluation of performance and determination of impact after release. We are currently evaluating Delta Select channel catfish from our third generation of selection and are discussing options for a commercial release with industry stakeholders. The data collected to date indicate that the selected population should have substantially faster growth and higher carcass yield than the base population, which represented fish from hatcheries providing greater than 50% of industry production at the time the base population was formed. However, at this time, no direct comparisons of performance of the Delta Select and channel catfish currently being used by the industry have been conducted. Attempting to replicate the multitude of management strategies employed by farmers (stocking densities, feeding strategies, aeration management, etc.) and compare the Delta Select and industry fish in a research setting is likely impossible. Therefore, a scenario in which the fish are initially released to a small number of producers willing to provide accurate feedback on commercial performance, including growth, survival, feed conversion and processing yield, prior to large scale release may be the best approach. If performance data from the initial release is favorable, the high reproductive capacity of catfish would allow rapid expansion and additional widespread release of the Delta Selects to commercial producers. Releases could be made every couple of generations to distribute fish with additional improvements to farmers if the initial release is favorable.

The blue catfish breeding program is not as advanced as the channel catfish program for various reasons, including a lack of domesticated populations, the time involved in testing blue catfish due to the later age at maturity of blue catfish compared to channel catfish, and the requirement to kill blue catfish males to obtain sperm for hybrid catfish production. However, the potential for industry impact through development and release of improved blue catfish germplasm is tremendous. Calculations based on discussion with commercial hybrid hatchery operators suggest 3,000 to 4,000 male blue catfish would suffice for current levels of hybrid catfish fry production. Even if hybrid catfish production doubles, that is still less than 10,000 males a year, which is substantially less than the male offspring produced from a single large blue catfish full-sib family. Therefore, identification and development of improved blue catfish germplasm, with subsequent rapid expansion to commercial industry production, has the potential to have a rapid and dramatic positive benefit on hybrid catfish performance.

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(2) Henderson C. R., 1975: Use of all relatives in intraherd prediction of breeding values and producing abilities. *J. Dairy Sci.* **58**, 1910–1916.

Commonly used cow evaluation methods apply principles of the selection index to herdmate deviations on the cow and close relatives. In contrast, best linear unbiased prediction adjusts records by best linear unbiased estimates of all fixed effects in the model and simultaneously weights those adjusted records by selection index principles. It would be advantageous to utilize all known relationships among animals in the herd in the latter method, but computations have been too laborious, requiring the inverse of the numerator relationship matrix. By a method of writing this inverse rapidly without computing the relationship matrix itself, all relationships can now be used in intraherd cow evaluation. Further, tests of progeny by artificial insemination on sires used in the herd can be incorporated.

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A combined crossbred and purebred selection (CCPS) method, i.e. using crossbred and purebred information, was proposed to achieve genetic response in crossbred animals. Selection index theory was applied to establish a CCPS index. The CCPS was compared with pure-line selection (PLS) and crossbred selection (CS) methods. The genetic correlation between purebred and crossbred performance ($r_{pc}$) and crossbred heritability ($h_c^2$) are crucial factors in the comparison. The CCPS is always

better than PLS or CS when a fixed number of purebred progeny is tested. With a fixed total number of purebred and crossbred tested progeny, CCPS is only worse than PLS for very high values of rpc (> 0.8). Superiority of CCPS over PLS increases and over CS decreases with decreasing $r_{pc}$. The larger $h_c^2$ is relative to purebred heritability,the more response CS and CCPS will achieve. The robustness of CCPS against inappropriate assumptions on $r_{pc}$ and $h_c^2$ values was investigated. The expected response is always an overestimate, and the actual response is smaller than the optimal response when $r_{pc}$ is assumed one but the true $r_{pc}$ is smaller. The difference between actual and optimal response increases as $r_{pc}$ decreases, but it is small for large $r_{pc}$ values (e.g. < 3% for $r_{pc}$ > 0.7). The expected response is smaller than the actual response when $r_{pc}$ is large and $h_c^2 > h_p^2$. Finally, the actual response to CCPS is larger than the optimal response to PLS for positive values for $r_{pc}$. The main conclusions are: (1) CCPS method is optimal for obtaining genetic response in crossbreds and (2) CCPS with inappropriate assumptions on $r_{pc}$ and $h_c^2$ values (e.g. recognizing crossbreds as purebreds) achieves more genetic response than PLS for common values of $r_{pc}$ and crossbred heritability.

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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 z50,000 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 (Ne 5 100), 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.

(5) Legarra A. O.F., Christensen O.F., Aguilar I., and Misztal I., 2014: Single Step, a general approach for genomic selection. *Livest. Prod. Sci.* **166**, 54–65.

Genomic evaluation methods assume that the reference population is genotyped and phenotyped. This is most often false and the generation of pseudo-phenotypes is uncertain and inaccurate. However, markers obey transmission rules and therefore the covariances of marker genotypes across individuals can be modelled using pedigree relationships. Based on this, an extension of the genomic relationship matrix can be constructed in which genomic relationships are propagated to all individuals, resulting in a combined relationship matrix, which can be used in a BLUP procedure called the Single Step Genomic BLUP. This procedure provides so far the most comprehensive option for genomic evaluation. Several extensions, options and details are described: compatibility of genomic and pedigree relationships, Bayesian regressions, multiple trait models, computational aspects, etc. Many details scattered through a series of papers are put together into this paper.

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# Abstracts of Poster Presentations

## 1: Whole genome re-sequencing of fugu populations

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**Key words**
Fugu, *Takifugu rubripes*, conservation, genetic diversity, single nucleotide polymorphism, *Fst*, linkage disequilibrium, genomic selection

### Abstract

Historically, the prevalence of intraspecific diversity, both neutral and adaptive, in many marine species has been unclear. However, the recent advent of new technologies, in particular, high-throughput DNA sequencing is making it feasible to evaluate genetic variability at the whole-genome level. Fugu (tiger pufferfish *Takifugu rubripes)* is one of the important cultured marine fish species in East Asia. The yield of cultured fugu accounted for approximately 5% of total marine fish aquaculture in Japan, and ranked 3rd-4th by value. In addition, this species is becoming an important target for stock enhancement because of severe declines in the wild populations. In recent years, approximately two million hatchery-born seedlings have been released each year. Tagging and releasing experiments have suggested that fugu shows homing behavior to the natal site for spawning following long-range migration, and by extension, that distinct local populations may exist. As expected, recent study using 21 microsatellite loci revealed a shallow divergence between two wild populations from Japan Sea and Pacific Ocean. Nonetheless, the detailed degrees of population and adaptive structuring remain unclear. Moreover there is virtually no information regarding the extent and magnitude of linkage disequilibrium (LD) that play essential roles in choosing marker loci for the management of brood stocks and conservation of wild populations. In this study, we resequenced wild individuals of fugu from Wakasa Bay (Japan Sea) and Mikawa Bay (Pacific Ocean) to compare the genetic diversity within and between populations. Two libraries each containing ten individuals from either of the populations were constructed for paired-end sequencing (2 x 101bp) on the Illumina Hiseq2000. We obtained 43.2M reads per sample yielding coverage of 11.4 per genome, on average. We mapped these reads on the fugu reference genome (Fugu v.5) and called single-nucleotide polymorphisms (SNPs) using BWA, Samtools and GATK software. The number of SNPs detected per individual was about 700 thousand and the SNP frequency was about 480bp per SNP. Missing SNPs because of the shallower depth were estimated as 2 to 2.5 per cent of the total SNPs for each sample. Multidimensional scaling plot clearly separated the two populations, and individuals from Ise Bay were genetically closer than those from Wakasa Bay. However, the global *Fst* (= 0.0057) was small and no outlier locus was detected by BayeScan software. These results suggest that the genetic divergence between the two populations is shallow. Linkage disequilibrium analysis was done using PLINK software. The two populations were similar in the LD state. We detected putative 3,000 LD blocks from each population but 90 per cent of them were smaller than 1kb. The mean $r^2$ value was 0.48 between two SNPs at 100bp distance whilst that was less than 0.20 at 1kb distance. This rapid LD decay indicates these populations have maintained at relatively healthy states until recently. The expected SNP size for the implementation of genomic selection program for fugu breeding was 300k.

### Annotated Bibliography

Katamachi D., Ikeda M., Sato T., Suzuki S., Kikuchi K, and Ojima D. 2014: Development of a multiplex PCR assay for population genetic analysis of the tiger puffer *Takifugu rubripes* using 16 microsatellite DNA loci. *Aquaculture Sci*., **62**, 55−63

The tiger *puffer Takifugu* rubripes is a marine fish species economically important to East Asia, particularly Japan. To evaluate the genetic variability and population structure of the tiger puffer in detail, we generated a multiplex PCR assay of microsatellite DNA loci, a fast and cost-effective technique that allows high-throughput genotyping. In this study, we report the development of four multiplex PCR assays for this species using 16 microsatellite DNA loci located on independent chromosomes. We ensured quality control throughout all steps of the multiplex PCR assay development, i.e., exclusion of loci detected with stuttering, allele dropouts, or null alleles. We evaluated this set of microsatellite DNA loci for polymorphisms using 113 fishes collected from three different locations in the sea around Japan. This combination of loci will prove useful for future investigations of the fine-scale population genetic structure of this species.

## 2: Incipient transition of a sex-determining gene among closely related species of fugu

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### Abstract

Sex determination in teleosts fish is often genetic, where segregation of sex-determining loci assign the phenotypic sex. In these species, sex-linked polymorphic markers can be used for sex identification, potentially providing important information regarding the management of cultured species and conservation of wild species. However, the different master sex-determining (SD) genes in different fish lineages appear to have evolved independently and have been frequently replaced by new genes. Therefore, the sex-linked markers are often specific to a few stock population of the focal species, greatly limiting use of such markers for the management of aquaculture species. To gain more insights into the transition between SD systems in teleosts, we study closely-related species of fugu belonging to genus *Takifugu*. This genus has undergone an adaptive radiation in the last 2-5 million years, resulting in about 20 extant species including fugu (tiger pufferfish). Fugu is one of the most economically important food fish in Japan and also is the first fish with a fully sequenced genome. Previously, we have shown that sex in fugu appears to be determined by a missense single nucleotide polymorphism (SNP) in the *Amhr2* gene. In this study, we have taken advantage of this finding and the rich genomic resources of fugu to explore the genetic basis of sex determination in closely-related species of fugu. We found that while sex in the majority of *Takifugu* species is likely determined by the SNP in the *Amhr2* gene, it is clearly not the case in a few species. To confirm this, we performed genome-wide linkage analysis and identified novel SD loci distinct from the *Amhr2* locus in these species. Interestingly, the transition of the SD system appears to be in progress at least in one species, as a small percentage of males still retains the "sex-determining SNP" on the *Amhr2* gene. These results indicate that fugu and its closely-related species can be an excellent model group for investigating the transitions between alternative master SD genes.

## 3: Estimation of breeding value in model fish, guppy (*Poecilia reticulata*) and its application for selective breeding in aquaculture

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**Key words**
Guppy (*Poecilia reticulate*), selective breeding, body size, breeding value

## Abstract

Selective breeding is one of the most important methods for the genetic improvement in not only livestock animals, but also aquatic organisms. Many varieties and strains were produced in aquatic organisms; however the most succeeded example of selective breeding in aquatic organisms is ornamental fish. In the case of quantitative traits, the example of succeeded selective breeding is scare. In the case of the selective breeding based on phenotypic value, many cases showed inconsistent result. This is due to its inability to remove environmental and dominance effect. Therefore the method to evaluate the accurate abilities of parent is necessary. The breeding value was contrived from such circumstances. Though the breeding value possesses such importance, the applications of the breeding value in the aquaculture are not so much. The reasons why the application in aquaculture is little are 1) the large number of offspring can obtain from small number of parental fish; 2) short life cycle of target species caused difficulties to estimate breeding value in parental fish and their offspring. Although, it is hard to obtain the breeding value, the role of breeding value is very important for the efficient selection. From the above mentioned reason, it is necessary to identify the breeding value for the effective selective breeding in aquaculture. In this study, the breeding values were estimated from selection experiment for body size in the guppy, *Poecilia reticulata*, and examined the efficiency of the selection between used phenotype and breeding value. Comparison of breeding value in parental and offspring indicated significantly positive correlation. Positive correlation also observed between breeding values of parental and body size of their offspring. Selection based on breeding value showed 5% larger in body size compared with selection using phenotype. Selection based on breeding value showed lesser increment in coefficient of inbreeding compared to the selection based on phenotype. These results suggest that the breeding values are effective to the evaluating parental fish and useful for selective breeding. It is expected that the application of breeding value in the industrially important fish, such as Bluefin tuna and Japanese eel.


### 4: The life table demography and population growth of the rotifer *Brachionus angularis* Gosse, from Kenya; the influence of temperature and food density


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**Key words**

Rotifera, alga, life table parameters, fecundity, *Brachionus angularis*



## Abstract

The nature of reproduction of the Kenyan strain of *Brachionus angularis* was investigated using individual and small batch culture approaches. First, the Kenyan rotifer was identified using morphological and molecular techniques. The life-table demography and the population growth studies were conducted at three temperatures (i.e. 20, 25 and 30°C) using *Chlorella vulgaris* at three densities (i.e. $2.5x10^5$, $2.5x10^6$ and $2.5x10^7$ cells $mL^{-1}$). The lorica length (85.6 ± 3.1 μm) and width (75.4 ± 3.6 μm) of the Kenyan sample were smaller than those of similar species cited in the literature. The phylogenetic tree grouped the Kenyan sample together with *Brachionus caudatus* and *Brachionus angularis*. However, with additional morphological data e.g. presence of two median occipital spines with either reduced or lacking sub-median spines, *Brachionus angularis* was identified as the most likely match for the Kenyan sample. The rotifers were most fecund (2.11 ± 0.07 offspring $female^{-1}$ $day^{-1}$) and reproductive (8.43 ± 0.24 offspring $female^{-1}$) at 25°C with $2.5\times10^6$ cells $mL^{-1}$ of *C. vulgaris*. The highest intrinsic rate of

natural increase (0.74 ± 0.02 $d^{-1}$), specific population growth rate (0.49 ± 0.01), longest life expectancy at hatching (12.41 ± 0.28 d) and shortest generation time (2.87 ± 0.03 d) were also observed at 25°C with $2.5\times10^6$ cells $mL^{-1}$ of *C. vulgaris*. However, the duration of hatching to first egg spawning was shortest (2.86 ± 0.21 h) at 30°C with $2.5\times10^7$ and longest (8.83 ± 0.39 h) at 20°C with $2.5\times10^5$ cells $mL^{-1}$ of *C. vulgaris*. In the batch cultures, the highest population density (255.7 ± 12.6 ind $mL^{-1}$) and lowest (122.0 ± 3.6 ind $mL^{-1}$) were realized at 25°C with $2.5\times10^6$ and at 20°C with $2.5\times10^5$ cells $mL^{-1}$ of *C. vulgaris* on day 8 respectively. There was earlier population density peaks at higher food densities ($2.5\times10^7$ cells $mL^{-1}$ of *C. vulgaris*) regardless of temperature. In conclusion, the Kenyan strain of *B. angularis* seems to have favorable morphological and reproductive features making them suitable for aquaculture activities. The life table demography of this strain is optimal at 25°C with $2.5\times10^6$ cells $mL^{-1}$ of *C. vulgaris*. The results of this study are relevant for improvement of the freshwater aquaculture activities. Further studies on the population growth of the rotifer are recommended using other different food types.

## 5: Effect of dissolved organic matter on electrochemical removal of ammonia in recirculating aquaculture systems

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**Key words**
Recirculating aquaculture system, electrochemical oxidation, ammonia, dissolved organic matter, seawater

**Abstract**

Improvement of water treatment systems in closed recirculating aquaculture systems (RAS) is necessary for reducing total volume of culture water, which determine the size of the whole system as well as running costs. New RAS using electrochemical oxidation for removal of ammonia in culture seawater are being developed by Nagasaki Prefectural Institute of Fisheries. In this system, hypochlorous acid, which produced by electrolysis of seawater, oxidizes the bromide ions, yielding hypobromous acid. Both hypochlorous and hypobromous acids react with ammonium ion and oxidize it to nitrogen gas. Since these free chlorine and free bromine also react with dissolved organic matter (DOM), efficiency of electrochemical ammonia removal could be affected by quantity and reactivity of DOM in culture seawater. In this study, removal of total ammonia nitrogen (TAN) and DOM by additions of hypochlorous acid was investigated using culture seawater of two RAS for tiger puffer *Takifugu rubripes* (RAS-1) and kelp grouper *Epinephelus bruneus* (RAS-2). The RAS-1 comprised of a culture tank (20 $m^3$), a solid settler, a circulation pump, a foam fractionator, an electrolysis unit, a reaction tank (for ammonia removal), and activated charcoal tank (for removal of residual chlorine). The RAS-2 has a biofiltration tank in addition to the units provided in the RAS-1. The culture seawater was filtered through Whatman GF/F filter and dispensed into seventeen replicate 250-ml amber glass bottles. Additions of chlorine (Sodium hypochlorite solution) were conducted to achieve 17 steps chlorine doses of 0~16 (or 70) mg/L as $Cl_2$. After 20 min contact period at 25°C, free available residual chlorine and combined available residual chlorine in the sample were analyzed by the DPD method. TAN was measured using an autoanalyzer. DOM was determined as humic-like and protein-like fluorophores based on the three-dimensional excitation emission matrix spectroscopy. Concentration of TAN in the culture seawater consistently decreased along with the increase in chlorine dose and then disappeared (what is called breakpoint). In the bottles with higher levels of chlorine dose, free available residual chlorine was detected according to the excess amount of chlorine

dose. Concentrations of combined available residual chlorine were very low but small increase was observed around the breakpoint. Humic-like and protein-like fluorophores showed large decrease by the addition of 1 mg/L $Cl_2$ (the lowest chlorine dose) and then kept relatively constant concentrations until the breakpoint. In the bottled added with excess amount of chlorine (higher than the breakpoint), further decreases in humic-like and protein-like fluorophores were observed. These results suggest that DOM consists of highly reactive fraction and semi-labile fraction, and the former could reduce efficiency of electrochemical ammonia removal in the culture seawater. Compare to the chlorine demand to achieve the breakpoint in DOM-free artificial seawater, the culture seawater from RAS-2 required 17~42% more chlorine to oxidize TAN, but the difference between the artificial seawater and RAS-1 culture seawater was not clear. Skin mucus of kelp grouper seems to be one of the sources of highly reactive DOM in RAS-2.

### Annotated Bibliography

Ohwaki H., S. Yamamoto, A. Okamoto, and Y. Kurokawa. 2011. Development of a large-scale closed recirculating aquaculture system for saltwater fish. Report of Industrial Technology Center of Nagasaki, 40, 52-55 (in Japanese)

For the constraction of a large-scale closed recirculating system for aquaculture of saltwater fish on land, we have been developing a new seawater treatment unit using hypochlorous acid produced by electrolysis of seawater with platinum modified titanium electrode, and a closed recirculating aquaculture system with the electrochemical water treatment. Acidic water, which is a byproduct of electrolysis of seawater, can be used for the removal of dissolved $CO_2$ that accumulated in the culture water. Our computer simulation model could reproduce the flow pattern in the electrolysis unit and the estimated pH value of the out-flow water agreed well with the observation. We found that dissolved $CO_2$ in the culture water can be easily removed by bubbling of the acidic water.

## 6: Effect of tetrodotoxin-containing diet on feeding, digestion and growth of tiger puffer *Takifugu ruburipes* juveniles

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**Key words**
Tiger puffer, *Takifugu rubripes*, tetrodotoxin, feeding trial, appetite

### Abstract

Marine pufferfish contain tetrodotoxin (TTX), an extremely potent neurotoxin. We recently clarified that a tiger puffer *Takifugu rubripes* juvenile detects TTX by olfactory organ, and actively ingests and accumulates TTX not only in liver and skin but also in central nervous system. We further revealed that gene expression of some appetite peptides in the brain of hatchery-reared non-toxic fish changed by TTX-sensing and TTX-administration. In the present study, feeding trial using non-toxic and toxic diets was conducted with hatchery-reared *T. rubripes* juveniles in order to examine the relation between the appetite and TTX ingestion. A total of 120 non-toxic cultured juveniles (mean body weight of 3.6 g) were randomly divided into two groups where one group was fed with non-toxic commercial diets and the other was fed with TTX-containing diets (5.3 MU/g feed). Fish were maintained in 3 tanks (20 fish/200 l) for each group with flow through system (2 l/min) and vigorous aeration. The fish were fed with non-toxic and toxic diets to apparent satiation three times a day at 08:00, 13:00 and 17:00 hours for 28 days. All fish survived until the last day of feeding trial. There were no significant differences in the growth performance between fish fed non-toxic diets and toxic diets: total length (85.8±1.2 vs 85.1±2.7 mm), standard length (81.1±1.0 vs 80.1±1.5 mm), degree of loss of caudal fin (77.7±0.6 vs 75.5±5.5%), body weight (18.4±1.7 vs 17.6±1.1 g), feed

intake (11.7±0.6 vs 11.3±0.8 g), assimilation rate (91.1±1.6 vs 86.3±3.0%), weight gain (14.9±1.6 vs 14.0±1.1 g), feed efficiency (127.3±7.1 vs 123.7±5.3%). Total amount of administered and accumulated TTX in the fish fed toxic diets are 59.9±4.1 MU/fish and 33.6±2.0 MU/fish (56.4±7.1% of administered), respectively. These results indicate that TTX at this high dose may not have function as feeding stimulant for *T. rubripes* juveniles, but that feeding activity and growth of the juveniles are not inhibited by potent neurotoxin. We will further perform quantitative analysis and investigate the immunohistochemical localization of appetite peptides in the brain of TTX-sensed and TTX-administered hatchery-reared *T. rubripes* juveniles.

### 7: Temperature tolerance in two clonal strains of mangrove killifish, *Kryptolebias marmoratus*

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**Key words**
Mangrove killifish, *Kryptolebias marmoratus*, thermal tolerance, hybrid

**Abstract**

Mangrove killifish *Kryptolebias marmoratus* is the only known self-fertilizing vertebrate. This unique species broadly distributes in coastal mangrove habitats from southern Brazil through the Caribbean Islands and Central America to North Florida. They are capable of synchronous self-fertilization, producing homozygous clones as a consequence. Our laboratory has two clonal strains, PAN-RS and DAN, which were originally collected from near Bocas del Toro, Republic of Panama and Dangriga, Belize, respectively. PDHy strain which is the hybrid of PAN-RS and DAN was produced by artificial insemination (Nakamura et al. 2008). The descendants of PDHy are divided into 4 strains, PDHyI, PDHyII, PDHyIII and PDHyIV according to the growth rate. Since Panama and Belize show different climate, we hypothesized that PAN-RS, PDHy and DAN show different temperature tolerance.

We used two clonal strains (PAN-RS and DAN) and hybrid strains (PDHyI, II, III, and IV). Fish were kept in plastic containers filled with 60 mL of 17 ppt artificial brackish water under 25 ℃ and photoperiod of 14L:10D. "Upper and lower thermal acclimation limits" were quantified for each strain using chronic thermal tolerance methodology (Fangue et al. 2006). All fish were kept under 25 ℃ for one week, and 20 fish from each strain were subjected to either increasing or decreasing water temperatures of 0.5 ℃ per day. This experiment was continued until all fish died. The respective chronic thermal maximum or minimum value was taken as the high or low temperature at which 50% morbidity was observed. The respective chronic thermal maximum was significantly higher in DAN (36.6 ℃) than PAN-RS (35.7 ℃), (Log-rank test, $p<0.01$). The respective chronic thermal maximum was 30.7 ℃, 33.6 ℃, 33.7 ℃ and 32.5 ℃ for PDHyI, PDHyII, PDHyIII and PDHyIV, respectively. PDHyI showed the lowest respective chronic thermal maximum among all strains. Respective chronic thermal maximum of each PDHy strain was lower than their parents. The respective chronic thermal minimum was 9.4 ℃ for PAN-RS and 9.6 ℃ for DAN, with no significant difference (Log-rank test, $p=0.59$).

### 8: Effects of coexistence of protozoa *Euplotes* sp. coexistence on the population growth of minute monogonont rotifer *Proales similis*

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**Key words**
*Proales similis, Euplotes* sp., monoculture, mixed-culture

Abstract

The rotifer *Brachionus rotundiformis* (SS-type) is commonly used as starter food for rearing small-mouthed marine fish larvae. However, several tropical marine fishes have much smaller mouth gap, hence cannot feed on the *B. rotundiformis*. To solve this problem, the minute monogonont rotifer *Proales similis* is preferred due to its smaller size than *B. rotundiformis*. However, the culture of *P. similis* is easy to be collapsed due to inability to withstand the handling stresses and environmental changes in the culture medium. In this study, we investigated the interaction between *P. similis* and the protozoa *Euplotes* sp. in the rotifer culture water. We independently cultured *P. similis, Euplotes* sp. and a combination of *P. similis* and *Euplotes* sp. in 144 ml glass jar containing 50 ml of seawater (22 ppt) at an initial density of 1 ind./ml at 28°C in darkness for 14 days. All the treatments were triplicated and daily fed with *Nannochloropsis oculata* at 8.0 x $10^5$ cells/ml without water exchange. A similar experiment was conducted separately with Chlorella vulgaris as food at 2.9 x $10^5$ cells/ml daily without water exchange. When fed with *N. oculata*, population density increased in *Euplotes* sp. in the monoculture and mixed-cultures. However, the population growth of *P. similis* in the mixed-culture decreased after 8 days and *P. similis* disappeared after 14 days of culture. There was a significant difference in the population density of either *P. similis* or *Euplotes* sp. between the monoculture and mixed-culture on day 14. When cultured with *C. vulgaris*, population density of *P. similis* decreased from day 6, while that of *Euplotes* sp. reached its highest peak on the same day. *P. similis* dissapeared completely on day 14. These results suggest that the presence of *Euplotes* sp. in the *P. similis* cultures significantly suppressed the population growth of *P. similis* presumably due to stressful interactions. Furthermore, the competition between *P. similis* and *Euplotes* sp. for bacteria may also have existed. Even though we did not observe a behavioral interaction of *Euplotes* sp. and *P. similis*, the swimming speed of the *Euplotes* sp. was higher than the *P. similis*.

## 9: Distribution of larval and juvenile greater amberjack (*Seriola dumerili*) around the Penghu Islands, Taiwan

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**Key wards**
Greater amberjack, *Seriola dumerili*, early life, spawning ground, otolith

Abstract

The greater amberjack *Seriola dumerili* (family Carangidae) widely distributes around the world. Because of its commercial importance, rapid growth and good adaptation to captivity, *S. dumerili* is a very important species for aquaculture in Japan. Juveniles of *S. dumerili* associate with floating objects such as drifting seaweeds. However, there is very limited knowledge about larval and early-juvenile stages of this species in the wild. In order to investigate the early life history of *S. dumerili*, firstly we validated the otolith daily increments using artificially-raised fish (11-51 days after hatching). Then, field surveys were made by R/V Hai-an, Fishery Research Institute, Council of Agriculture, Taiwan around the Penghu Islands, Taiwan, from May to August 2015. Frontal zone and drifting seaweeds were visually observed during survey, and drifting seaweeds were scooped together with associated fishes by a hand net (Φ45 cm, 3 mm mesh). Surface tows of plankton net (Φ1.3 m, 0.33 mm mesh) were conducted for 10 minutes with towing speed of 2 knots in frontal zones and other areas. At each sampling station, vertical

profile of water temperature and salinity were measured by a CTD (SBE-19 plus, Sea-Bird Electronics Inc.). Fish species were identified and zooplankton abundance (mg DW·m$^{-3}$) and species composition (%) were calculated. We also measured four *S. dumerili* samples caught in 2014 deposited at Penghu Marin Biology Research Center, Fishery Research Institute, Council of Agriculture, Taiwan. Increments of sagittal otolith of reared fish showed the same number as their age in days after hatching (ANCOVA, df=1, $p$=0.32). Relationships between otolith diameter ($y_{dia}$, $\mu$m) and age ($x_{day}$, days), and between otolith diameter ($y_{dia}$, $\mu$m) and TL ($x_{TL}$, mm), were described as following equations: $y_{dia}=0.0003 \cdot x_{1.93}^{day}$ (r=0.991), and $y_{dia}=0.1937 \cdot x_{TL}-0.1018$ (r=0.981), respectively. We caught a total of four larval and juvenile *S. dumerili* by surface towing, but not from drifting seaweeds. Total length of *S. dumerili* ranged from 7.4 to 42.5 mm, and age ranged from 18 to 56 days after hatching. All *S. dumerili* were caught in open water and in frontal zone, however we could not detect significant differences in zooplankton abundance between frontal zone and other station ($t$-test, $p$=0.87). Thecostraca was significantly abundant in the stations away from the frontal zones ($t$-test, $p$<0.01). In 2014, larval and juvenile *S. dumerili* were caught in January and May, and body length ranged from 5.8 mm in NL to 54.2 mm in TL. Our study indicates that *S. dumerili* spawns from April to June in 2015, and frontal zone in open water is nursery ground for *S. dumerili* around the Penghu Islands, Taiwan.

## 10: Effect of starvation on mixis induction in offspring and genetic mechanism of the monogonont rotifer *Brachionus manjavacas*

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### Abstract

Short time starvation of neonates hatched from rotifer resting eggs induces active mixis up to the 10th generation. However, this phenomenon remains unexplained whether it can last for further generations. Also, the mode of heredity and the acquired parental characteristics inherited by the subsequent generations are not clear. In this study, we used the rotifer *Brachionus manjavacas* and *Brachionus plicatilis* to investigate the heredity of mixis induction in rotifers up to the 80$^{th}$ generation and the inheritance of the acquired traits by methylation of DNA. The maternal rotifers hatched from resting eggs were starved for 12 hours before determination of the acquired characteristics. The control group was not starved. A set of 8 individual rotifers were randomly selected from starved and control group. Rotifers were individually cultured in 0.2 ml of sea water (22 ppt.) in 8 well microplate at 1 individual per well untill the 80$^{th}$ generation. The rotifers were daily provided with *Nannochloropsis oculata* suspension at 6.0 × 10$^{6}$ cells/ml. The mixis induction in each generation was determined. We determine the genetic factors that changed the mixis induction rate of the parent and future generations. First, we designed the primer using partial base sequence of the methyltransferase, which was the DNA methylase which EST analysis of *B. plicatilis* performed PCR as template cDNA of *B. manjavacas*. The mixis induction of the offspring from starved parents increased until 38$^{th}$ generation. In addition, mixis induction during accumulated generations peaked at the 17$^{th}$ generation. We observed repetitions of increase and decrease of beyond the 17$^{th}$ generation. The amplification of the DNA fragment of the predicted size was confirmed through PCR analysis using the primer designed from the DNA methyltranferase (DNMT) gene of *B. plicatilis*. This phenomenon could be explained by the epigenetic inheritance involving methylation of the DNA. It was estimated that the DNMT gene fragment by BLAST analysis. We have been investigating this by comparing the *DNMT* gene expression level among generations.

## 11: Body size, culture and fish larval ingestion on a minute rotifer *Colurella* cf. *adriatica*

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**Key words**
Minute rotifer, *Colurella* cf. *adriatica*, body size, population growth, fish larvae, ingestion

### Abstract

Current procedure of rearing small mouthed marine fish larvae is using the Super Small (SS) type of rotifer *Brachionus rotundiformis* as starter food during first days of larval first feeding. The *B. rotundiformis*, however, is ineffective or even unsuitable for larvae of several marine tropical fish with even smaller mouth size including, Napoleon fish (*Cheilinus undulatus*), groupers (genus *Epinephelus*), angelfishes (family Pomachantidae). In present study, we examined the feasibility of a minute rotifer *C.* cf. *adriatica* as live food by measuring its body size, analyzing population growth and fish larval ingestion on the rotifer.

Rotifer *C.* cf. *adriaticawas* isolated using a plankton net (45 $\mu$m mesh size) from an estuary in Mangket, North Minahasa, North Sulawesi, Indonesia. Water temperature and salinity at the time of sampling were 28±1 °C and 30±1 ppt, respectively. Sixty adults of the rotifer were measured for its body length and width. As comparison, body length and width of a local strain *B. rotundiformis* were also measured. Population growth of *Colurella* cf. *adriatica* was assessed by culturing the rotifer under four densities (3, 6, 9, 12 x $10^6$ cells/ml) of *Nanochloropsis oculata* as food source. The rotifer was cultured at salinity of 20 ppt and placed in a controlled room temperature at 25±1℃. Water volume of the culture was 4 ml (using 3x4, multiwell plate) and the initial density of the rotifer was 1 ind./ml. Observation was made daily by counting the numbers of rotifer in each well until the density decline. Larval ingestion on the rotifer was investigated in Gondol Research Institute for Mariculture, Bali-Indonesia. Approximately 10 ind./l eggs of humpback grouper (*Cromileptes altivelis*) were transferred to four 200-l larval rearing tank. The first two tanks were fed with 10 ind./ml of rotifer *C.* cf. *adriatica* from day 2 till day 5 after hatching, while the other two tanks were left without any addition of food. All surviving larvae were harvested on day 5 and the numbers of remaining larvae were counted. Gut content of the surviving larvae was analyzed to see the presence of rotifer. Body length and width of *C.* cf. *adriatica* were distributed from 82.8-103.2 and 46.8-61.7 $\mu$m, respectively. The mean body length (95.9±3.8 $\mu$m; mean± standard deviation) and width (46.8-61.7 $\mu$m) of *C.* cf. *adriatica* were significantly smaller/narrower than *B. rotundiformis* (175.2±9.2 and 123.5±7.7 $\mu$m, respectively) (*t-test*, $p<0.05$). Rotifer *C.* cf. *adriatica* grew well in all *N. oculata* treatments. The rotifer attained highest population densities on day 16 (774±167 ind./ml) and 18 (656±139 ind./ml) at *N. oculata* densities of 6 and 9x$10^6$ cells/ml, while it was reached on day 26 (646±85 ind./ml) and 34 (560±58 ind./ml) at *N. oculata* densities of 3 and 12x106 cells/ml, respectively. Humpback grouper larvae show higher survival (1.5%) on *C.* cf. *adriatica* treatment than control (0.3%) (*t-test*, $p<0.05$). By analyzing gut content of the remaining larvae, it was found that individual of rotifer *C.* cf. *adriatica* presence in gut of the larvae indicating that larvae of humpback grouper ingested the rotifer.

## 12: Production of myostatin gene-knockout Japanese anchovies (*Engraulis japonicus*) using TALEN-based genome editing

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**Key words**
Japanese anchovy, *Engraulis japonicus*, myostatin, knockout, TALEN, genome editing

**Abstract**

Genome editing techniques such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR) have attracted considerable attention in recent years since these technologies can mediate targeted and efficient genetic modifications (knockout, knock-in, and gene modification) in various organisms. Thus, targeted genome editing, which enables researchers to tailor genomic loci of interest, is one of the most promising approaches for plant, animal, and fish breeding. Nevertheless, the application of the techniques to teleost fishes have been limited almost exclusively to popular experimental fish models, such as zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*). There are no literature reports on the application of these techniques to marine fish, which includes many important species for the fisheries industry. In this context, we regard the Japanese anchovy (*Engraulis japonicus*) as a most suitable candidate for genome-editing experiments in marine fish, because they have the following advantages: 1) easy rearing and breeding in a small-scale fish tank. 2) year-round and multi-year spawning under photoperiod and temperature control. 3) quick growth into mature individuals, which produce another generation of eggs, about three months. Myostatin (MSTN), previously referred to as growth differentiation factor 8 (GDF8), is a negative regulator of skeletal muscle growth. In mammals, MSTN-deficient animals resulted in an increase of skeletal muscle mass with both hyperplasia and hypertrophy. Likewise, recent studies revealed that the MSTN gene inhibits skeletal muscle growth even in fish. Thus, to produce a fish breeding model generated by genome editing, we performed targeted gene disruption of the MSTN gene in Japanese anchovies using TALEN technology. We constructed three TALEN pairs targeting the first intron of the MSTN gene and the in vitro transcribed RNAs of the pairs were injected into the yolk of embryos at the one-cell stage. As a result, mutant F0 embryos were obtained with a very high insertion and/or deletion (indel) mutation rate (~96.9%), and thus the F0 founders were mated with each other to produce MSTN-knockout anchovies at the F1 generation. To our knowledge, this is the most advanced study for genome editing in marine fishes. The rearing of F1-individuals and their genotyping is now in progress.

## 13: Isolation and screening of novel probiotic lactic acid bacteria for aquaculture

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**Key words**
Probiotics, lactic acid bacteria, *Lactobacillus* sp., pathogen, antagonistic activity, tolerance in gastrointestinal juice

**Abstract**

Most of probiotics used in aquaculture are considered as alternative therapies for the use of antibiotics to prevent diseases in aquatic animal. Among of probiotic bacteria, lactic acid bacteria are one of potential candidate due to several strains have been isolated from fish gut as beneficial microflora and acted antagonistic against Gram-negative fish

pathogens. Therefore, in this study we conducted to isolate lactic acid bacteria from fermented food as candidate strains which may be used to reduce the antibiotics using for sustainable aquaculture. The probiotic properties of isolates were surveyed. Lactic acid bacteria were isolated from samples such as fermented foods by an agar plating method using GYP and MRS media. The antagonistic activity test against fish pathogens *Lactococcus, Streptococcus* and *Edwardsiella* was carried out according to the method of the double layer agar method. The tolerant ability of isolates on NaCl (0%, 3%, 5%, 10%), pH (from 2 to 9), artificial gastric juice (at pH range 2-4) with pepsin and intestine juice (at pH 8) with gall powder were evaluated. Isolates were identified based on the sequences of 16 S rRNA gene (~700bp). Totally 55 strains of lactic acid bacteria were isolated from rice bran and several kinds of fermented vegetables. In antagonistic test, three isolates showed positive results against three strains of *Edwardsiella tarda*, three strains of *Streptococcus disgnactie*, three strains of *S. iniae* and three strains of *Lactococcus garvie*. These three strains of GYP 31, GYP 69 and GYP 4-20 were identified as Lactobacillus sp.. The relative growth of GYP 31 strain at pH range 5-8 was from 100% to 80%, at pH 2-4 and pH 9 were below 20%; while GYP 69 strain and GYP 4-20 strain only grew well at pH 5, pH 6 with 100% of the relative growth. Three strains grew well at NaCl concentration from 0-5%, 3-5% and 0-3% with the relative growth from 80% to 100%, respectively. In tolerance test on acid and artificial gastrointestinal juices, GYP 31 strain expressed the sustained ability and survival itself better than GYP 69 strain and GYP 4-20 strain. GYP 31 strain showed the highest viable count as $1x10^8$, $1.4x10^8$, $5.6x10^7$cfu/ml in acid solution (at pH 3,5-4), artificial gastric juice at (pH 4) and intestine juice (at pH 8), respectively, although the viable count was lower than those in the control group ($2.2x10^9$cfu/ml at pH 7). From these results, *Lactobacillus* sp.. GYP 31 strain is considered as a potential probiotic candidate in aquaculture due to its ability competition with pathogens and high tolerance in the gastrointestinal tract of fish.

## 14: Effect of protease addition to EP diet on the growth of amberjack, *Seriola dumerili*

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**Key words**
*Seriola dumerili*, protease, digestibility, growth rate, feed, low temperature, aquaculture

### Abstract

In aquaculture, the late growth of the cultured fish in winter season is serious problem for economical aquaculture. The extruder pellet (EP) has been used as an composed feed for the culture fish because EP can be produced efficiently. However, the EP is very hard to decompose it by digestive enzymes as compared with raw diet. The activities of digestive enzymes in the gastrointestinal tracts of the cultured fish significantly decrease due to the low temperature during winter season. Therefore, it is very difficult that the digestive enzymes with low activities decompose the EP diet efficiently. To dissolve this problem, in this study, the application of proteolytic enzymes from microorganisms was examined to accelerate the digestion of the EP diet for the cultured fish. Acid and alkaline proteases from microorganisms was used. The effect of pH and temperature on enzyme reaction was also investigated. Alkaline and acid protease were added to the EP diets in a 0.1M Tris-HCl buffer and 0.1M glycine-HCl buffer, respectively. The reaction mixtures were incubated at 15, 20, 25, 30 and 35°C for 180 min. The degradation of the EP diet was evaluated by weighing the solid bodies. The nitrogen concentration of the centrifugal supernatant in the reaction mixtures were determined according to the

Kjeldahl method. The alkaline proteases showed stable activities at 20-35°C, and the activity at 15°C significantly decreased by 44%. The decomposition of the EP diet was enhanced by addition of both proteases at 20°C. These results indicated that the addition of proteases from microorganisms is effective to enhance the decomposition of the EP diet at low temperature. Amberjack, *S. dumerli* was fed with EP diet with or without alikaline protease (control group) for 60 days. After 60 days of rearing, the average fish body weight was higher in the group with alikaline protease as compared with that in the control group. These results showed that it is possible that the addition of protease enhance the growth rate of *S. dumerili* in winter season.

### Annotated Bibliography

Satoh K. 2005. Study on improvement of composed diet for yellowtail culture. Bulletin of Oita Institute of Marine and *Fisheries Science*, **6**, 19-77 (in Japansese)

To date, Japanese aquaculture, mainly for yellowtail Seriola quinqueradiata, has been developed using raw-fish as their feedstuff, such as sardine and mackerel obtained from adjacent sea, that used to be abound and available with low cost. The reduction of sardine resources definitely caused the nessesity of composed diet in yellowtail culture. Therefore, the studies examined to improve and to improve accommodate the composed diet for yellowtails as to feedstuff, protein digestibility, feed additives, and feeding regime.

## 15: Amino acid profile of thraustochytrids cells and potential of application to aquafeed

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**Key words**

Aquaculture, feeds, fishmeal, protein source, thraustochytrid, amino acid composition, byproducts

### Abstract

Fishmeal is used as protein sources for aquaculture feeds. However, the price is drastically increased to around 170,000/ ton in 2013 during recent 10 years because of the decrease of fish resources, anchovy. This is serious problem for sustainable aquaculture. Therefore replacement of fishmeal to another resource is urgently needed. Thraustochytrids are marine protists and classified to one of Stramenopiles. They are widely distributed in marine environment and accumulate large number of lipids in cell bodies. Therefore, thraustochytrids have attracted strong interests for production of valuable lipids as single cell oils (SCOs) such as biodiesel and omega-3 fatty acids. In the process of lipid extraction from the cultured cells, some solids (extract residue) are produced as byproducts. It is considered that this extract residue except for lipids is mainly consists of protein. From the viewpoints of industrial application of thrautochytrid cells as protein sources, we have planned to use the byproducts as resources instead of fishmeal for aquafeeds. In this study, we evaluated the recovery rate of protein in a thrausotchytrid, *Aurantiochytrium limacinum* strain mh0186 known as a docosahexaenoic acid (DHA) producer under the lipid extraction process. For selection of adequate strains for protein production, thraustochytrids were isolated from marine environment. *A. limacinum* strain mh0186 was cultured in a GY broth. The cultured cells were collected by centrifugation and lyophilized for proximate analysis. The content of protein, lipid and ash in the lyophilized cells was determined according to Kjeldahl method, Folch method and heat-ashing method, respectively. The composition of amino acid and fatty acid were analyzed by liquid chromatography-mas spectrophy (UF-Aminostation, Shimazu Co. Ltd., Japan) and gas chromatography (GC-2014, Shimazu Co. Ltd., Japan), respectively. In the process of lipid extraction, the extract residue was collected and re-lyophilized. The protein content and amino acid composition were analyzed by same method described as above. Seawater, sands, leaves, seaweed were collected for

the isolation of thraustochytrids from coastal area in Miyazaki, Kumamoto and Oita, Kyushu, Japan. Thraustochytrids were isolated on a B12 Culture Agar "*Nissui*" plate medium by pine pollen-baiting method. The isolates were cultured in a GY broth, and the cultured cells were collected by centrifugation for analysis of the composition of amino acid and fatty acid. Isolates were identified at genus level based on the 18S rRNA sequence analysis. The content of crude protein lipid and ash per g of the cultured cell of mh0186 strain were 333 mg, 440 mg and 42 mg. On the one hand, the protein content of the extract residue obtained from 1g of the cultured cells was 226 mg (recover rate, 68%). In both samples of the cultured cells and the extra residue, glycine, leucine, isoleucine, glutamate and arginine were mainly detected. One hundred twenty thraustochytrids strains were isolated. In some isolates including *A. limacinum* strains SR21 and mh0186, *T.aureum* ATCC34304, *Schizochytrium aggregatum* ATCC28209, glycine, leucine glutamate, arginine and were detected as major amino acids. Strain Tak2 specifically accumulate 25% cystathionine to total amino acids.

## 16: Diurnal changes in frequency of the burst swimming behavior of adult Pacific bluefin tuna (*Thunnus orientalis*) in a land-based tank

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**Key words**
Pacific bluefin tuna, collision death, burst swimming behavior, diurnal change, broodstock tank

### Abstract

In aquaculture of Pacific bluefin tuna, *Thunnus orientalis* (PBT), development of a stable system of artificially-reared fingerlings is needed to ensure the sustainability of aquaculture through a reduction on the reliance on wild captured juveniles. Therefore, we constructed two large indoor land-based PBT broodstock tanks at Seikai National Fisheries Research Institute, FRA, Japan, and are examining the environmental cues such as water temperature and photoperiod which are essential for successful and stable spawning of PBT. However, heavy mortality of adult PBTs occur in the tank due to collisions with the tank wall. It is considered that the collision deaths are associated with the burst swimming behavior. In this study, to clarify the process of the collision death, we examined diurnal changes in frequency of the burst swimming behavior of three-year-old PBTs in the land-based tanks. Nineteen three-year-old PBTs were reared in the land-based tanks (20 m in diameter, 6 m in depth) and their swimming behaviors were recorded using a video camera for six days. A day was compartmentalized into six periods defined as dawn (7:00 to 9:00), daytime-I (11:00 to 13:00), daytime-II (15:00 to 16:00), dusk (16:00 to 18:00), night-I (20:00 to 22:00) and night II (0:00 to 2:00) according to changes in illumination. We counted the frequency of the burst swimming behavior at each period from the recorded video imagery. As a result, the frequency of the burst swimming behavior at dawn was significantly higher than that in other time periods. Notably, the burst swimming behaviors were frequently observed in 30 min just after illumination (from 32 to 85 lux) during dawn. Additionally, the frequency of the burst swimming behavior of multiple PBTs in the tank like "a panic" were often observed during dawn, whereas solitary burst swimming behaviors were observed at the other time periods. These results suggest that collision deaths of PBT in the tank were caused by burst swimming behavior associated with sudden increases of light intensity during dawn.

## 17: Interval and spawning frequency of Pacific bluefin tuna *Thunnus orientalis* in a land-based tank

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**Key words**
Pacific bluefin tuna (*Thunnus orientalis*), spawning frequency, mtDNA *D-loop* region, haplotype

Abstract

Recently, the exploitation of natural stocks of Pacific bluefin tuna *Thunnus orientalis* (PBT) has increased to dangerous levels though increased fishing pressure. The scarcity of this species and its high commercial value, together with its very high growth rates, makes it a potential candidate for marine aquaculture. Therefore, the artificial propagation and seed production techniques for PBT represent objective for sustainable utilization of this resource. Fundamental information on the spawning ecology of PBT is essential for the development of production techniques. However, this information is still largely lacking. In this study, we investigated spawning frequency of PBT by comparing mitochondrial DNA *D-loop* region haplotypes of broodstock fish, with those of fertilized eggs and hatched larvae. Broodstock PBT, fertilized egg and hatched larval samples were obtained from a land-based tank at Research Center for Tuna Aquaculture, Seikai National Fisheries Research Institute in Nagasaki, Japan. Spawning activity in a land-based tank was observed over a 98 day period during May 16 to August 28 in 2014. The sampling of fertilized eggs and hatched larvae was conducted for a total of 15 days within the 98 days. A total of 15 samples were collected for an initial consecutive 3 day period and then at approximately 1 week intervals from May 16 to August 28 in 2014. The 36 broodstock individuals and 659 eggs and hatched larvae were observed to have 3 haplotypes. These haplotypes were named A, B and C, respectively. Among the broodstock fish, haplotype B was detected at high frequencies, secondly haplotype C, and thirdly haplotype A. The number of each broodstock individuals was 32, 3, and 1, respectively. Each haplotype of eggs and hatched larvae was detected on 14 days, 1 day and 8 days, respectively, during the 15 sampling days. Haplotype B occurred in the 3 days of consecutive spawning. This is consistent with genetic or histological observations that other wild *Thunnus* species spawn multiple times and on consecutive days. These results demonstrate that PBT in a land-based tank may have the potential to spawn consecutively and multiple times.

## 18: Effect of timing of restricted feeding on sexual maturation in the yellowtail, *Seriola quinqueradiata*

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**Key words**
Yellowtail (*Seriola quinqueradiata*), sexual maturation, restricted feeding, broodstock management

Abstract

In aquaculture of large sized marine fish such as bluefin tuna and yellowtail, extensive amounts of fish feed are needed for the broodstock management because of their large body size. In order to save on the feeding cost for their broodstock management, development of a restricted feeding technique without affecting reproductive performance is required. However, available information about the

effect of food supply on reproduction is limited in fish. In this study, we examined the effect of restricted feeding during the gonad immature and vitellogenic phases on sexual maturation in females of the yellowtail, *Seriola quinqueradiata*. Two-year-old cultured yellowtail females, which the average body weight was 5.0 kg, were divided randomly into three sea cages on November 2012 and reared until the next spawning period (April 2013) under natural conditions at the Goto station, Seikai National Fisheries Research Institute, FRA, Japan. The feeding regimes in each cage were defined as follows: control group fed to satiation three times a week throughout the experimental period, two restricted groups fed 30% of the amount of feed given to the control group (100%) during the immature (from November to January) or vitellogenic phase (from February to April), respectively. At the end of the experimental period, the average body weights were 6.6 kg in the control group, 5.8 kg in the restricted group during the immature phase and 5.9 kg in the restricted group during the vitellogenic phase, which shows that the restricted feeding reduced approximately 50% of somatic growth throughout the experimental period. Interestingly, the gonad weights in the restricted feeding group during the vitellogenic phase were low as compared with the control group and the restricted feeding group during the immature phase. Histological observations revealed that females in all groups had oocytes that completed the accumulation of yolk globules at the spawning period. However, the mean diameter of most advanced ovarian follicles in the restricted feeding group during the vitellogenic phase was significantly smaller as compared with the other groups. Furthermore, plasma estradiol-17$\beta$ levels in the restricted feeding group during the vitellogenic phase were significantly lower as compared with the other groups at the spawning period. These results indicate that the restricted feeding during the vitellogenic phase alters the gonadal development in relationship with the plasma estradiol-17$\beta$ levels in the yellowtail females.

## 19: Evaluation of nitrogen excretion in young, immature and adult Pacific bluefin tuna (*Thunnus orientalis*) measured in the land-based tank

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**Key words**
Pacific bluefin tuna (*Thunnus orientalis*), nitrogen, ammonia excretion rate, ration size

### Abstract

Ammonia excretion in marine teleosts accounts for 70 to 90% of their total nitrogen excretion. Ammonia is toxic to fish and is a major factor limiting fish biomass and stocking density in intensive culture systems and aquariums. Quantification of ammonium nitrogen is important for estimating stocking biomass/density, water flow and size of the biological filter in the culture system. Several studies have suggested that ammonia excretion is affected by several factors such as species, body weight, water temperature and ration size. Although several tuna species such as Pacific bluefin tuna (PBT) and yellowfin tuna have been reared in aquariums and research facilities including Seikai National Fisheries Research Institute, there is a lack of studies about ammonia excretion in tunas. In this study, we investigated the effects of body weight and ration level on nitrogen excretion for PBT in captivity.

Young (0.67 ± 0.14 kg, n = 50), immature (14.4 ± 1.88 kg, n = 2) and adult (42.9 ± 6.5 kg, n = 2) PBTs were introduced to experimental land-based tanks from net cage or rearing tank, and thereafter they were acclimated to running sea water conditions. Experimental tank size varied according to fish size (20 kl for young fish; 65 kl for immature fish and 150 kl for adult fish). The PBTs were fed raw fish or artificial feed until the experiments. Before the fasting and postprandial experiments, the fish were deprived of food for 48 hours. Rearing water was sampled every 2 h for the first 12 h, and at 4 h intervals from 12 to 24 h. In the postprandial experiment, the experimental fish were fed bait fish (chub mackerel, *Scomber japonicas*, or sandlance, *Ammodytes personatus*) or artificial feed. Fish feces were collected from the tank bottom after 24 h. Determination of fecal nitrogen and concentration of ammonium, nitrite and nitrate nitrogen was carried out on each fecal and water samples. Weight-specific ammonia excretion rates of fasted fishes showed an inverse relationship with body weight (W). The relationship for total ammonia nitrogen (TAN) was: TAN (mg N $W^{-1}$ $d^{-1}$) = 297.4·$W^{-0.36}$ ($r^2$=0.99). The ammonia excretion rate at 10 kg in PBT was twice as much as it is for red seabream (unpublished data). Although postprandial ammonium excretion rate was in relation to the ration size ($R$, mg feed-N $W^{-1}$ $d^{-1}$), linear regression analysis indicated that TAN excretion rates increased with ration size: TAN (mg N $W^{-1}$ $d^{-1}$) = 332.4·$R$−179.6 ($r^2$=0.87). There was no significant difference in the rates between PBT fed the bait fish and the artificial feed. Postprandial fecal nitrogen was positively correlated with ration size. Fecal nitrogen was excreted at a level of 0.9-1.9% for the baitfish and 0.3-1.3% for the artificial feed. Comparing the results of PBT with other fish, these values were lower than that of red seabream (12.8%) and pufferfish (16.1%). This study highlights the need for effective evaluation of nitrogen loading and water quality management in PBT rearing facilities and aquaculture grounds.

## 20: A high density genetic linkage map for yellowtail (*Seriola quinqueradiata*) containing 6,275 EST-based SNPs

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### Abstract

The marine products industry has developed as majority of the fishery, which are captured and directly using of aquatic resources. Only recently the breeding are considered as important research because available of aquatic resources are restricted gradually. The expectation of aquaculture research is getting higher in response to the prediction of aquatic resources depletion. Also the genetic improvement of economic traits are needed, it hopes apply to superior fish breeding, because artificial juvenile have a possibility to improve the phenotype for suited to aquaculture condition in every generation. We are researching practical application about selection of economic important traits from natural genetic resources using yellow tail (*Seriola quinqueradiata*) as target species. High density SNP arrays have become the tool of choice for QTL mapping, Genome-wide association studies, marker-assisted selection (MAS) and genomic selection (GS). 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. Earlier mapping studies

have identified QTL for important commercial traits including parasite disease resistance, and combining the resources of a high density genetic map with genome sequence data will facilitate the fine mapping of these loci and the identification of candidate genes. In this study, Affymetrix SNP array was used to genotype 460 samples collected across five families from wild population in coastal waters of Goto Fukue-island. To establish 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 Roche/454 GS FLX platform generated 1,353,405reads. The sequencing of SNP identification produced 570,846 raw reads derived from the full-length library and 456,482 raw reads derived from the 3'-anchored library derived from 5 hundred juveniles. Quality – based variant calling using CLC Genomics Workbench detected 9,356 biallelic putative 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 originated alleles from male or female. In order to avoid the error of genotyping, the accuracy of genotypes in their progenies was checked from parental male and female alleles. Genotype data were converted to a backcross format as though the grandparent genotype was unknown. Pairwise analysis was performed, and markers were sorted in linkage group at a minimum LOD threshold of 5.0. Linkage phases were determined retrospectively by examining the assortment of alleles among linked markers. A total of 6,275 EST-based SNPs were mapped to 24 linkage groups. The total distance covered by the male and female maps were 1,230cM and 1,031cM. This map is currently being used to map QTL for a number of commercially important traits, and will be used to improve the assembly of the yellowtail genome. It is possible to rapidly develop domesticated strains having commercially important traits in yellowtail aquaculture.