ISSN2758-3953
水研機構研報
Bull.Jap.F ish.Res.Edu.Agen.

水産研究・教育機構
研　究　報　告

53
令和８年３月

BULLETIN OF
JAPAN FISHERIES RESEARCH AND
EDUCATION AGENCY

No. 53
March 2026

国立研究開発法人 水産研究・教育機構
神奈川県横浜市
JAPAN FISHERIES RESEARCH AND EDUCATION AGRNCY
Yokohama, Kanagawa, Japan

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 52nd Business Meeting of the UJNR Aquaculture Panel was conducted at the Sinphonia Technology Hibiki Hall Ise, Mie Prefecture, Japan on November 5, 2024, and the Scientific Mini-Symposium was held at the same venue from November 5 to 6. The symposium theme was "Next Step for Sustainable and Resilient Aquaculture", which was under the 12th Three-Year Plan, "A New Era for Sustainable Aquaculture – The Next 50 years of Research, Education and Collaborations", commenced in 2024. Nineteen oral presentations were made on topics such as alternative feed, genetics/selective breeding, health management, seaweed culture, and ecosystem management during the two-day symposium.

The proceedings of the 52nd UJNR Aquaculture Panel Scientific Mini Symposium "Next Step for Sustainable and Resilient Aquaculture" 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 improve the aquaculture environment 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 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.

Natsuko Miki, Ph.D.
Chair of UJNR Aquaculture Panel
Executive Director
Japan Fisheries Research and Education Agency

Participants in the 52nd UJNR Aquaculture Panel Scientific Symposium,
held in Sinfonia Technology Hibiki Hall Ise, Mie, Japan, November 5 - 6, 2024

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Contributed Papers and Abstracts

Program

52nd Scientific Symposium of UJNR Aquaculture Panel

Next Steps for Sustainable and Resilient Aquaculture

Date:

November 5 13:00 - 17:15
November 6 9:00 - 17:30

Venue:

Sinfonia Technology Hibiki Hall Ise, Ise, Mie

Aim of the Symposium

The Japan Fisheries Research and Education Agency will host the 52nd UJNR Aquaculture Panel Scientific Symposium in Ise, Mie, Japan. The UJNR Aquaculture Panel is a cooperative research exchange between the U.S. and Japan, jointly addressing environmental and technical issues that affect the aquaculture industries of both nations.

The UJNR Aquaculture Panel has been interacting with each other for more than 50 years. The global aquaculture industry has continued to develop and expand during this time. Making aquaculture a sustainable industry has become an important issue in recent years. Therefore, in the 12th Three-Year Plan, we have decided to discuss research, education, and collaborations over the next 50 years to make aquaculture sustainable regarding fishery resources and energy and to ensure a permanent supply of animal protein to humanity. This long-term vision inspires hope and a sense of purpose as we embark on this journey. The 52nd UJNR Aquaculture Scientific Symposium is the first year of the current Three-Year Plan, and we will discuss how to make the aquaculture industry sustainable through alternative feeds, breeding, health management, seaweed culture, and ecosystem management.

Tuesday, November 5, 2024

Welcome and aim of the symposium

Natsuko Miki (Japan Panel Chair, Japan Fisheries Research and Education Agency)
····· 13:00 - 13:15

Plenary Lecture

(Moderator: Kazumasa Ikuta)

Building capacity for land-based aquaculture production in the US-national academia-industry-federal partnerships
Yonathan Zohar (University of Maryland, Institute of Marine and Environmental Technology & Department of Marine Biotechnology) ····· 13:15 - 13:45

Alternative Feeds

(Moderators: Caird Rexroad and Akiyuki Ozaki)

Next-generation sustainable aquaculture systems employing insect protein-based feeds
Kazumasa Ikuta (Japan Fisheries Research and Education Agency) ····· 13:45 - 14:00

Multifunctional utilization of insects in aquaculture
Takeshi Miura (Ehime University) ····· 14:00 - 14:30

Validation of the suitability of full fat and defatted black soldier fly meals in diets for rainbow trout
Wendy Sealey (Bozeman Fish Technology Center, Agricultural Research Service, United States Department of Agriculture) ····· 14:30 - 15:00

Protein assimilation from larvae of black soldier fly *Hermetia illucens* in diets for red seabream *Pagrus major*
Tadashi Andoh (Fisheries Technology Institute, Japan Fisheries Research and Education Agency) ····· 15:00 - 15:30

Break ····· 15:30 - 15:45

Omics analysis of red seabream (*Pagrus major*) fed a soybean meal-based diet
Hazuki Yoshinaga (Fisheries Technology Institute, Japan Fisheries Research and Education Agency) ····· 15:45 - 16:15

Frass from black soldier fly larvae as an aquafeed ingredient: Nutritional value and potential health benefits
Mediha Aksoy (Aquatic Animal Health Research Unit, Agricultural Research Service, United States Department of Agriculture) ····· 16:15 - 16:45

Effect of feeding black soldier fly larvae diets on growth and culture condition of Kuruma prawn
Katsutoshi Ito (Fisheries Technology Institute, Japan Fisheries Research and Education Agency) ····· 16:45 - 17:15

Wednesday, November 6, 2024

Genetics/Selective Breeding and Monosex Breeding

(Moderator: Luke Gardner and Shohei Takuno)

Current status of artificial seed production and selective breeding in the Japanese yellowtail *Seriola quinqueradiata*: The progress achieved by FRA
Kenta Adachi (Fisheries Technology Institute, Japan Fisheries Research and Education Agency) ····· 09:00 - 09:30

Population structure and selective breeding program for the growth of farmed rainbow trout (*Oncorhynchus mykiss*) in Japan
Tsubasa Uchino (Fisheries Technology Institute, Japan Fisheries Research and Education Agency) ····· 09:30 - 10:00

Advancing monosex breeding technology for sablefish (Gindara) aquaculture
Adam Luckenbach (Fisheries Northwest Fisheries Science Center, NOAA) ·· 10:00 - 10:30

Break ····· 10:30 - 10:45

Health Management

(Moderators: Caird Rexroad and Tomofumi Kurobe)

Developing vaccination strategies for prevention of atypical furunculosis in sablefish (*Anoplopoma fimbria*)
Kenneth D Cain (Northwest Fisheries Science Center, NOAA Fisheries) ····· 10:45 - 11:15

Hygiene management is important to prevent red sea bream iridovirus transmission between net pens: Insights from a case study that assessed cross-contamination
Yasuhiko Kawato (Fisheries Technology Institute, Japan Fisheries Research and Education Agency) ····· 11:15 - 11:45

Disease control measures in hirame juvenile hatchery: The case of hirame aquareovirus
Tomoki Maeda (Fisheries Technology Institute, Japan Fisheries Research and Education Agency) ····· 11:45 - 12:15

Lunch break ····· 12:15 - 13:30

Seaweed Culture and Ecosystem Management

(Moderators: Luke Gardner and Satoshi Watanabe)

Opportunities and challenges for Alaska kelp aquaculture
Jordan Hollarsmith (Alaska Fisheries Science Center, NOAA Fisheries) ····· 13:30 - 14:00

The present status and future scope of seaweed aquaculture in Japan
Hiromori Shimabukuro (Fisheries Technology Institute, Japan Fisheries Research and Education Agency) ····· 14:00 - 14:30

Production improvement of Nori aquaculture using biostimulants
Mahiko Abe (National Fisheries University, Japan Fisheries Research and Education Agency) ····· 14:30 - 15:00

Seaweed seedling culture technique using LEDs and feeding behavior of herbivorous fish to suppress fouling seaweed - In the case of "*hiziki*" *Sargassum fusiforme*-
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Break ····· 15:30 - 15:45

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Tatsuru Kadota (Fisheries Technology Institute, Japan Fisheries Research and Education Agency) ····· 15:45 - 16:15

Pacific oyster condition and mortality in a U.S. Pacific coast estuary: Can relationships with climate, food and reproductive state be utilized to sustain future production?
Brett Dumbauld (Pacific Shellfish Research Unit, Agricultural Research Service, United States Department of Agriculture) ····· 16:15 - 16:45

Image analysis for estimating soft body mass from shell morphology in the Pacific oyster, *Crassostrea gigas*
Junpei Shinji (Fisheries Technology Institute, Japan Fisheries Research and Education Agency) ····· 16:45 - 17:15

Scientific symposium closing

Janet Whaley (US Panel Chair, NOAA Fisheries Office of Aquaculture) ····· 17:15 - 17:30

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Bull. Jap. Fish. Edu. Agen. No. 53, 9 - 13, 2026

Multifunctional use of insects in aquaculture

Takeshi MIURA*[1, †] and Chiemi MIURA*[1]

Abstract: Insects are being explored as sustainable alternatives to fishmeal, a conventional component in aquaculture feed, due to their environmental advantages. However, the higher production cost of insect-based meals compared to fishmeal remains a significant challenge. The wider adoption of insect meals depends on effectively highlighting their functional benefits beyond sustainability, as well as reducing production costs.

Our research has shown that housefly (*Musca domestica*) pupae can enhance disease resistance in fish, suggesting the presence of immunostimulatory compounds in the pupae. These compounds, which activate phagocytic cells, are not unique to housefly but are found in various insect species. Studies have successfully isolated immunostimulatory substances from several insects, including melon fly (*Bactrocera cucurbitae*), black soldier fly (BSF; *Hermetia illucens*), Japanese oak silk moth (*Antheraea yamamai*), and silkworm (*Bombyx mori*). One such compound, “Silkrose”, derived from the silkworm, has been studied extensively and has been shown to improve not only disease resistance in fish but also muscle structure, stress tolerance, and heat resistance.

Gene expression analysis in fish treated with Silkrose has revealed changes in the expression of genes associated with immune function, redox balance, lipid metabolism, and protein processing. These genetic changes suggest that Silkrose reduces stress, enhances disease resistance, improves thermal tolerance, and may contribute to better flesh quality in fish. Notably, similar gene expression patterns have been observed in other animals, including mice, crustaceans, and bivalves, indicating that the effects of Silkrose extend beyond fish and apply to a wide range of vertebrates and invertebrates.

Other insect species, such as BSF, crickets, and mealworm, also contain similar bioactive compounds, suggesting they may offer comparable benefits. Insects, already recognized as promising protein sources for food and feed, are receiving increasing global attention. Their biofunctional properties are expected to further enhance their values as feed ingredients, potentially positioning them as ingredients superior to conventional fishmeal. Future research will aim to maximize the use of these bioactive compounds to fully realize the potential of insects as alternative protein sources in aquaculture feeds.

When insect-based meals become more cost-effective and their functional benefits are better understood, they could play a pivotal role in advancing sustainable aquaculture. By promoting fish health, improving stress resilience, and enhancing product quality, insect-derived ingredients may surpass fishmeal in both performance and environmental compatibility, supporting the growing demand for eco-friendly and nutritious aquaculture solutions.

Key words: insect for feed, functional property, fish culture, immunostimulant

Accepted on Feb 26, 2026
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Introduction

Many fish species commonly farmed in Japan, such as red sea bream, yellowtail, greater amberjack, eels, and Pacific bluefin tuna, are carnivorous and rely on fishmeal derived from wild edible fish such as anchovies. These species have higher fishmeal requirements; approximately five kilograms of fishmeal are needed to produce one kilogram of red sea bream, seven to ten kilograms for yellowtail, and fourteen to seventeen kilograms for Pacific bluefin tuna. Therefore, producing high-value carnivorous fish through aquaculture requires a substantial amount of wild fish biomass. From a food sustainability standpoint, feeding these species with such feeds is both contradictory and unsustainable.

To address this issue, alternative protein sources to fishmeal are needed. In recent years, insect-based meals have gained attention as potential substitutes (Huis *et al*. 2013). Although approximately 950,000 insect species have been identified, only a few are currently utilized as protein sources. Given the growing concerns about the sustainability of fishmeal supply, it is essential to seriously consider the use of insects as sources of animal protein for aquafeeds.

Characteristics of insect meal as an aquafeed ingredient

Not all insect species are suitable for use in aquafeeds. Candidate species must be safe, available in stable quantities, and reared on substrates that do not compete with human food sources. Cost is also crucial; to compete with fishmeal, which is priced at around USD 1.70/kg, insect meal must be affordable below this level. Based on these criteria, three species of insect larvae currently stand out: the housefly (*Musca domestica*), the black soldier fly (BSF; *Hermetia illucens*), and the mealworm (*Tenebrio molitor*). Housefly larvae can be reared on livestock manure or food waste; BSF larvae thrive on food waste; and mealworm can be raised on cereal by-products or vegetable scraps. These characteristics make all three species strong candidates for inclusion as ingredients in aquafeeds.

However, insect meals may contain compounds that are toxic to fish. One such compound is catechol, a melanin precursor formed via catecholamines, which is present in relatively high concentrations (25 mg/kg in housefly, 16 mg/kg in mealworm, and 140 mg/kg in silkworm). Feeding catechol-supplemented diets to red sea bream resulted in suppressed growth and increased mortality. Because catechol is lipophilic, removing lipids from housefly meal effectively reduces its levels. A diet formulated with defatted housefly meal instead of fishmeal supported the red sea bream growth and attained nearly equivalent to that achieved with a fishmeal-based control diet. Therefore, with appropriate detoxification, housefly meal represents a viable alternative to fishmeal (Hashizume *et al*. 2019).

Furthermore, in some cases, insect meals even outperform fishmeal. For example, replacing 100% of fishmeal in a red sea bream diet with mealworm meal resulted in weight gains of 1.4- and 1.8-fold without and with mealworm lipid removal, respectively. Thus, when processed properly to eliminate toxic compounds such as catechol, insect meals can match or exceed the performance of fishmeal (Ido *et al*. 2019).

Field trial of aquaculture using insect meal

Promising results in controlled laboratory experiments do not always guarantee the success in the field. Therefore, a field trial was conducted at a red sea bream farm in Uwajima, Ehime, in collaboration with a farmer. Mealworm meal, which had shown the most favorable results in laboratory tests, was used. From July to the following March, approximately 8,000 fish per cage (with an initial average weight of about 700 g) were fed a diet containing 10% mealworm meal. On March 16, the fish grew to an average weight of 1.63 kg, matching the growth of fish fed a control diet without mealworm.

Functional properties of insect meal

Replacing merely 5% of fishmeal with housefly meal can enhance feed intake, growth, and disease resistance in red sea bream (Ido *et al*. 2015). This immunostimulatory effect has been observed in several insect species, suggesting that insect meals offer an additional value beyond simply providing protein.

To identify the compounds responsible for this effect, we screened 13 mass-reared insect species for activity on RAW264 mouse macrophages by measuring nitric oxide (NO) production (Fig.1), a common biomarker of immune activation and function in fish. While housefly extracts showed moderate activity, pumpkin fly (*Bactrocera cucurbitae*) pupae demonstrated stronger NO-inducing effects. Using the pupal extracts, two-stage chromatography enabled the isolation of a novel acidic polysaccharide (molecular weight ~1 MDa) named “Dipterose BC” (Ohta *et al*. 2014). Similar polysaccharides were later purified from the silk moth (*Antheraea yamamai*, “Silkrose AY”) (Ohta *et al*. 2016), the silkworm (*Bombyx mori*, “Silkrose

BM") (Ali *et al*. 2018), and the BSF ("Dipterose BSF") (Ali *et al*. 2019).

Aquaculture practice often adopts high-density stocking, which increases the risk of disease outbreak. Approximately 10% of production costs are related to fish disease management. Although vaccines can be effective, species diversity and small market sizes hinder their development. Antibiotics pose risks such as resistance and environmental impact. Therefore, natural immunostimulants such as Dipterose and Silkrose could offer an eco-friendly and promising approach to disease control.

Silkworm pupae, a by-product of silk production that is estimated to be produced at around 520,000 tonnes annually in the world, are a readily available and cost-effective source of functional polysaccharides, making them well-suited for large-scale immunostimulant production.

The effects of insect-derived functional compounds on fish

We investigated the molecular mechanisms underlying Silkrose's immunostimulatory effects using the medaka fish (*Oryzias latipes*). After feeding medaka Silkrose-enriched diets for one week and then performing an intraperitoneal challenge with *Edwardsiella tarda*, we observed reduced infection rates and significantly lower mortality (Fig.2). Gene expression profiling revealed modulation of innate immunity

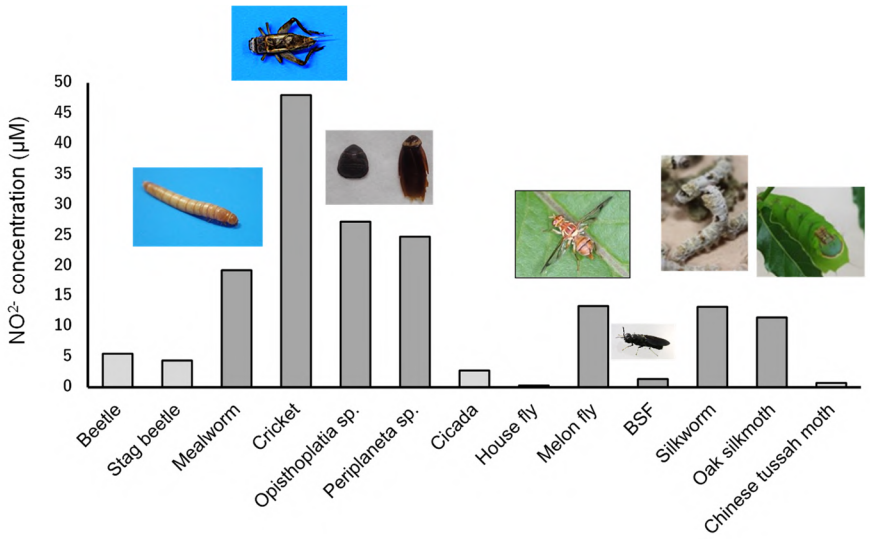

Fig.1 Search for insect species with high levels of immune-activating substances

The immune-activating capacity of insect species is indicated by the amount of nitric oxide (NO) produced by macrophage-derived RAW264.7 cells.

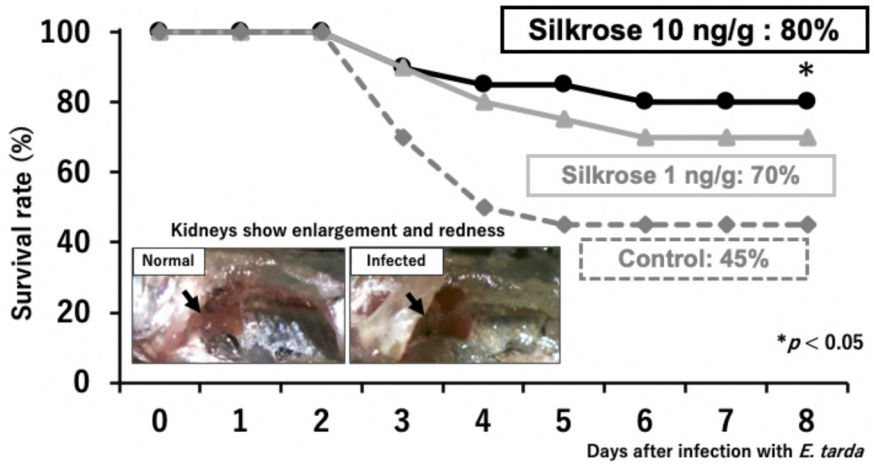

Fig.2 Survival rate of medaka following oral administration of Silkrose and then immersion exposure to *Edwardsiella tarda* (Ali *et al*. 2021)

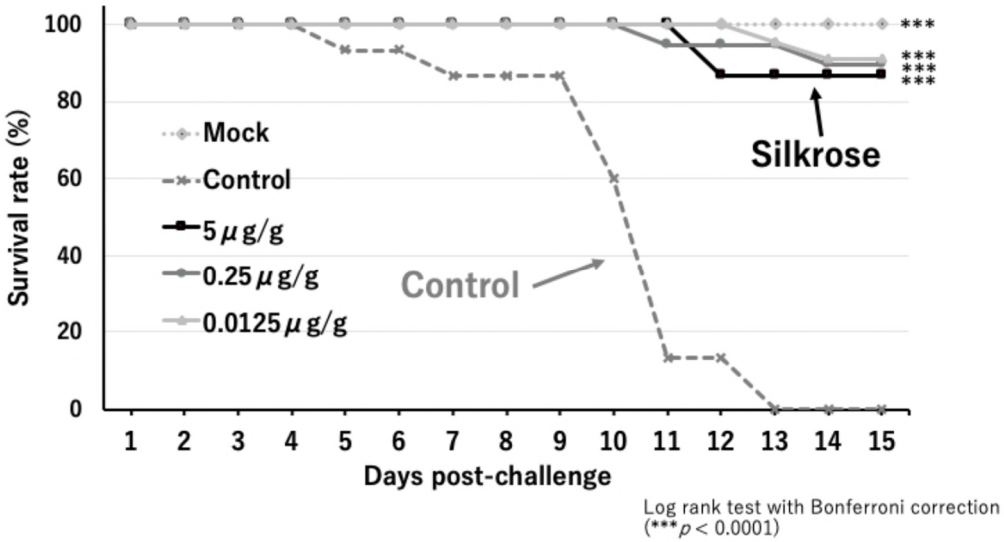

Fig.3 Survival rate of *Litopenaeus vannamei* following oral administration of Silkrose and then immersion exposure to the *Vibrio penaeicida* IAYKG13-1 strain (Ali *et al*. 2018)

(including complement activation, NO synthesis via Toll-like receptors, and antimicrobial peptide production), acquired immunity (antigen presentation and killer T-cell pathways), and mucosal barrier enhancement (Ali *et al*. 2021).

In red sea bream, feeding a 0.1% Silkrose diet for one week followed by an *E. tarda* challenge (5×10^6 cells via injection) resulted in a survival rate of 63.6%, compared to 22.7% in the control group. This demonstrates a significant improvement in disease resistance linked to systemic immunomodulation. Silkrose also reduced mortality in shrimp (both kuruma and Pacific white) through oral administration (Fig.3) (Ali *et al*. 2018). Because shrimp rely solely on innate immunity and antibiotic overuse is common in world shrimp aquaculture (6,560,000 tonnes produced in 2019), Silkrose offers a safer alternative for disease management.

Additionally, Silkrose reduced ectoparasite loads (*Benedenia* and *Caligus*) on yellowtail and white trevally, while promoting epithelial cell proliferation and likely strengthening skin barrier defenses (Fig.4) (Miura *et al*. 2022).

Gene expression analysis in medaka revealed that Silkrose modulates pathways beyond immunity, including lipid metabolism, redox processes, and oxygen transport. These physiological effects are likely significant in aquaculture species and warrant further molecular investigation.

Recent data indicate that Silkrose enhances thermal tolerance in zebrafish exposed to heat stress; while control fish died, treated fish survived. The upregulation of heat-shock proteins in the liver suggests a protective mechanism that may help mitigate the risks associated with ocean warming.

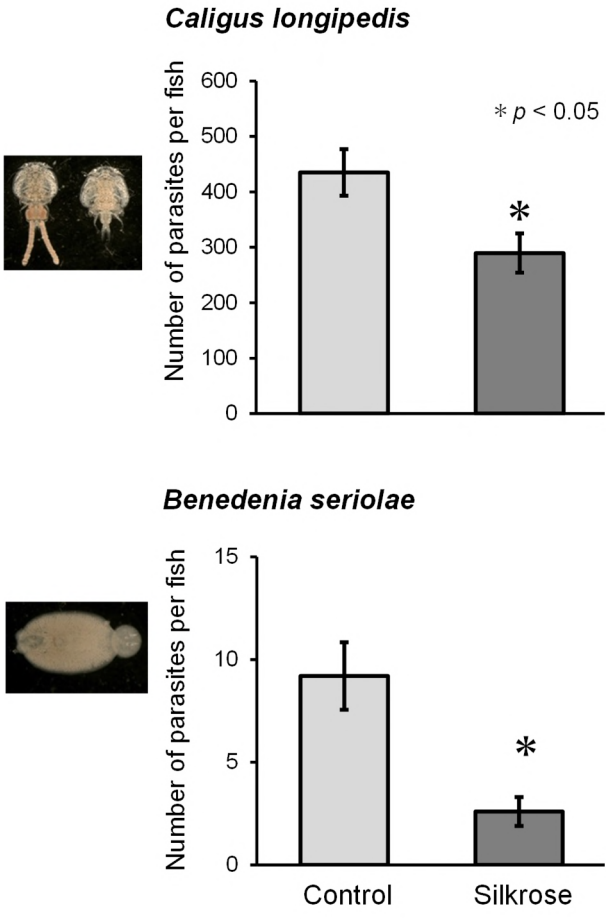

Fig.4 Effect of Silkrose treatment on the load of *Caligus longipedis* and *Neobenedenia girellae* parasites on white trevally at an aquaculture field site (Miura *et al*. 2022)

Effects on non-aquatic animals

Silkrose also benefits other animal species. In mice, oral administration lowered serum low-density lipoprotein cholesterol and reduced weight gain on a high-fat diet. In broiler chickens, it promoted the growth during heat stress. These findings suggest that Silkrose may have applications beyond aquaculture, including in livestock, pets, and possibly even humans.

Insect meal: "beyond the protein"

In summary, both silkworm- and BSF-derived products contain bioactive polysaccharides that enhance immunity, disease resistance, thermal tolerance, and more. Mealworm and housefly likely harbor similar molecules. Although insect meals currently struggle to compete with fishmeal on price due to the lower cost of marine-derived fishmeal, their functional properties elevate them to a "beyond the protein" category. Scientifically characterizing these bioactive compounds and quantifying their modes of action could enable insect meals to rival—or even surpass—fishmeal in aquaculture.

Although the use of insects in animal feed has been discussed for over a decade, and BSF products are now commercially available worldwide, Japan continues to lag behind in industrial adoption, largely due to persistent negative perceptions. As more companies enter the market, the production costs will fall. A rich, nutritious animal feed should not come at the cost of environmental destruction. Insects offer a sustainable solution by converting human-generated waste into valuable fertilizer and high-quality, functional protein for aquaculture. Growing awareness of the environmental and social benefits of insect-based feed is also essential to drive domestic uptake of insect products. Often overlooked, they could become the unsung heroes of tomorrow's food systems.

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水研機構研報, 第 53 号, 15 – 29, 令和 8 年
Bull. Jap. Fish. Edu. Agen. No. 53, 15-29, 2026

Validation of the suitability of full fat and defatted black soldier fly larvae meals in diets for rainbow trout (*Oncorhynchus mykiss*)

Wendy M. SEALEY*[1, †], Ken OVERTURF*[2], Gary BURR*[3], Jesse TRUSHENSKI*[4], Patrick CROWLEY*[5], and Kari MADDEN*[5]

Abstract: Improved insect stocks, culture methods, and mitigation of safety concerns have increased availability and use of commercial black soldier fly larvae (BSFL) meals as animal feedstuffs. Both full-fat and defatted BSFL meals can have beneficial applications in aquatic animal feeds. The protein fraction in both meal types can serve as a high-quality amino acid source that compliments feeds with reduced fish meal and increased amounts of plant ingredients. The lipid fraction of full-fat BSFL meal is rich in medium-chain fatty acids, a readily utilized energy source, and has been associated with immunostimulation in fish. However, differences in BSFL cultivation and processing methods can lead to variation in BSFL meal composition, digestibility, and quality. Thus, to more effectively utilize BSFL meals in rainbow trout (*Oncorhynchus mykiss*) feeds, it is necessary to thoroughly characterize the nutrient content, digestibility, and functional attributes of different types of BSFL meals to validate their suitability.

To accomplish this goal, an *in vivo* digestibility trial was conducted in juvenile trout to determine the available nutrient content of commercially sourced full fat and defatted BSFL meals. Based on the resulting digestibility data, a growth trial was conducted using practical-type rainbow trout diets formulated to contain 0, 5, or 10% full fat or defatted BSFL meal; an additional test diet was created by top-coating the 0% BSFL meal with BSFL lipid at a level equivalent to the 10% full-fat BSFL diet. All diets were formulated to contain 44.8% digestible protein and 15% crude lipid, and balanced to available lysine, methionine, threonine and phosphorus targets of 3.82, 1.30, 2.14 and 0.6, respectively, prior to cooking extrusion. For the growth trial, fifteen rainbow trout (10.4 ± 0.2 g, initial weight) were randomly stocked into quadruplicate tanks (400 L each) and fed their respective diets for 12 weeks to assess effects on growth performance and immune function.

Growth results indicated no significant effects of BSFL inclusion level, type, or their interaction on final fish weight (184-196 g), weight gain (1,678-1,756 %), feed conversion ratio (0.84-0.87), feed intake (1.76-1.86 % body weight/d), hepatosomatic index (1.2-1.3 %), viscerosomatic index (8.4-10.0 %), or fillet ratio (54.0-59.6 %).

Evaluation of expression of the immune related genes, IL-10, IL-1β, TNFα, HSP70, Defensin β3, IL-4 like, and UDP glucose 6-dehydrogenase (UDPG6D) in the intestine revealed no significant changes in the fish fed full-fat or defatted BSF meal. However, expression of IL-10, IL-1β, TNFα, IL-4 like gene, HSP70, and UDPG6D was significantly upregulated in the intestine of fish fed the 0% BSFL meal diet with added BSFL lipid.

These data suggest that both full-fat and defatted BSFL meals are suitable for rainbow trout feeds when diets are formulated on an available nutrient basis that accounts for differences in protein availability and lipid content. The effects of BSFL lipid, but not full-fat BSFL meal, on immune gene expression requires further study but suggests that the immunomodulating components of BSFL lipid may be sensitive to degradation during extrusion.

Key words: rainbow trout, black soldier fly, insect proteins

Accepted on Feb 26, 2026

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Introduction

In aquaculture, feeding constitutes approximately 60% of overall production costs, making it critical for economic viability to utilize low-cost, nutritionally balanced diets (NRC 2011). The efficiency of fish production relies heavily on achieving an advantageous feed conversion ratio while addressing the high costs associated with protein inclusion in fish feed. Primary sources of protein, such as fish meal, offer high digestibility and a superior amino acid profile but are costly with inconsistencies in available supply, prompting the exploration of alternative protein sources (Glencross *et al.* 2007).

In addition to the multitudes of plant protein products that have been evaluated (Gatlin *et al*. 2007), unconventional protein sources such as industrial by-products, insects, seaweed, and former food products have gained attention in recent years for their potential to replace traditional feed ingredients. Insects are part of the natural diet for many fish species, and thus including insect-based ingredients in the diet is promising (Glencross 2020). Several different insect species and product types have been investigated including housefly maggot meal, mealworms, locust meal, silkworm meal, and black soldier fly larvae (BSFL, *Hermetia illucens*) meal (Makkar *et al.* 2014; Henry *et al.* 2015). Among these, utilization of BSFL as both a protein and lipid source is appealing due to their ability to convert organic waste into protein- and lipid-rich biomass with a favorable nutrient profile (English *et al.* 2021; Weththasinghe *et al.* 2022). Concurrently with the acknowledgement of the sustainability value of insects as alternative ingredients, improved insect stocks, modernized culture methods, and mitigation of safety concerns have increased availability of commercial BSFL meals as animal feedstuffs (English *et al*. 2021). BSFL meals have been investigated in various aquaculture species including tilapia (Ylidrium-Askoy *et al.* 2020), Atlantic salmon (Belghit *et al.* 2019), largemouth bass (Peng *et al*. 2021), and others (Mohan *et al.* 2022). However, incorporation of BSFL meals in aquaculture diets has uncovered several challenges related to the level of dietary inclusion (Weththasinghe *et al*. 2022). Specifically, initial processing and treatment of BSFL affects the final quality of the meal and thereby the amount that can be used in feeds (Gasco *et al.* 2024).

Nonetheless, BSFL meals can have beneficial applications in aquatic animal feeds when included in the diet at appropriate levels. Defatted BSFL meal can serve as a high-quality amino acid source that compliments feeds with reduced fish meal and increased amounts of plant ingredients (English *et al*. 2021), while full-fat BSFL meal are rich in medium-chain fatty acids that are readily utilized as an energy source (Weththasinghe *et al*. 2021) and can upregulate fish immune function (Koutsos *et al.* 2022). Differences in BSFL meal processing methods and BSFL feedstock (i.e., what the BSFL are provided as a growth medium/food source) play key roles in the lipid quality and profile of the resultant BSFL meal (Lalander *et al.* 2019).

To more effectively utilize BSFL meals in aquaculture feeds, it is necessary to thoroughly characterize the nutrient content, digestibility, and functional attributes of different types of BSFL meals to validate their suitability. Accordingly, the current study determined the digestibility of full-fat or defatted BSFL meal in feeds for rainbow trout (*Oncorhynchus mykiss*) and examined the performance and immune function of rainbow trout fed diets containing these ingredients at two different inclusion levels. Additionally, the effect of dietary BSFL lipid inclusion, independent of BSFL meal, was evaluated.

Materials and Methods

1. Digestibility trial

(1) Experimental design Two novel, commercially sourced BSFL meals were assessed in an *in vivo* digestibility trial to determine their available nutrient content. The products were full-fat BSFL meal and defatted BSFL meal obtained from Chapul, LLC. (McMinnville, OR, USA). The methods of Cho *et al.* (1982), Bureau *et al.* (1999), and Forster (2001) were used, with modifications for manual feces collection and reference diet formulation, to estimate apparent digestibility coefficients (ADCs) (Table 1). Yttrium oxide was the inert maker. A complete reference diet (Table 1) meeting or exceeding all known nutritional requirements of trout was blended with the test ingredients in a 70:30 ratio (dry-weight basis) to form the test diets.

(2) Diet manufacturing All diets were mixed and co-ground without fish oil using an air-swept pulverizer (model 18-H, Jacobson, Minneapolis, MN, USA) to a particle size of less than 250 μm. Diets were manufactured using a twin-screw cooking extruder (DNDL-44, Bühler AG, Uzwil, Switzerland) with six-barrel sections and 18-s transit time through the barrel. The material was not steam conditioned prior to extrusion and water was added to barrel section 2. The 3.5 mm floating pellets were dried in a pulse-bed drier (Bühler AG) at 102˚C until moisture levels were below 7% and cooled prior to

vacuum oil infusion top-coating (A.J. Mixing, Ontario, Canada). Diets were stored in a cool dry environment until utilized.

(3) Fish culture and fecal sample collection Fish weighing an average of 230 g were stocked at a rate of 25 fish per 400-L tank connected to a common bio-filter with spring water inflow of approximately 20% makeup. Water temperature was maintained at 15ºC. Lighting was maintained on a 13 :11 h L: D cycle. Each diet was randomly assigned to triplicate tanks of fish. Fish were fed to apparent satiation twice daily for seven days prior to fecal collection.

(4) Sample collection and analyses Fecal samples were obtained by manual stripping, 16-18 h post-feeding. For each collection, all fish in the tank were sedated with 50 ppm tricane methanesulfonate dissolved in a water bath, gently dried, and stripped by applying light pressure to the lower abdominal region, taking care to exclude urinary excretions from the fecal samples. To collect adequate sample volumes for laboratory analyses, feces were collected three times with a minimum recovery period of one day between collections. Fecal samples were pooled by tank, freeze-dried and stored at -20ºC until chemical analyses were performed.

Dry matter analysis of ingredients, diets, and feces was performed according to standard methods (AOAC 2012). Yttrium and phosphorus were determined in diets and feces by inductively coupled plasma atomic absorption spectrophotometry (Anderson 1996). Crude protein (N x 6.25) was determined in ingredients, diets and feces by the Dumas method (AOAC 2012) on a Leco TruSpec N nitrogen determinator (LECO Corporation, St. Joseph, MI, USA). Total energy was determined by adiabatic bomb calorimetry (Parr1281, Parr Instrument ADM Inc., Moline, IL, USA). Amino acid analysis was performed by the University of Missouri-Columbia Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA) according to in-house protocols. Apparent digestibility coefficients (ADCs) of each nutrient in the test diets were calculated according to established equations (Kleiber 1961; Forster 2001).

2. Growth trial

(1) Experimental design Based on digestibility data generated in the trial described above, a growth trial was conducted using practical-type rainbow trout diets formulated to contain 0 (Control), 5, or 10% full fat (“FF”) or defatted (“DF”) BSFL meal; an additional test diet was created by top-coating the Control diet with BSFL lipid sourced from the same commercial supplier at a level equivalent to the 10% FF diet (“BSFL Lipid”). All growth trial diets (Table 2) were formulated to contain 44.8% digestible protein and 15% crude lipid, and balanced to available lysine, methionine, threonine and phosphorus targets of 3.82, 1.30, 2.14 and 0.6, respectively. All other micronutrients were provided at levels above NRC requirements (NRC 2011) and at levels proven beneficial at the Bozeman Fish Technology Center (Bozeman, MT, USA) in the evaluation of alternative proteins. For the feeding trial, 2.5 mm pellets were extrusion manufactured as previously described for the digestibility trial.

Table 1 Composition of digestibility trial reference diet

Ingredients	(% dry-weight)
Wheat flour[1]	28.3
Squid meal[2]	25.0
Soy protein concentrate[3]	17.1
Fish oil[4]	13.5
Corn gluten meal[5]	8.3
Soybean meal[6]	4.3
Vitamin premix ARS[7]	1.0
Chromic oxide[8]	1.0
Choline chloride[8]	0.6
Taurine[9]	0.5
Stay-C 35[10]	0.2
Trace mineral premix[11]	0.1
Yttrium oxide[8]	0.1

[1] Archer Daniels Midland (Decatur, IL, USA), 14 g/kg protein.
[2] Rangen Inc. (Buhl, ID, USA), 810 g/kg protein.
[3] Solae Profine VP (St. Louis, MO, USA), 693 g/kg crude protein.
[4] Omega Proteins Inc (Houston, TX, USA).
[5] Cargill Animal Nutrition (Minneapolis, MN, USA), 601.0 g/kg protein.
[6] Archer Daniels Midland (Decatur, IL, USA), 480 g/kg protein.
[7] Vitamin premix ARS 702 DSM Nutritional Products, Basel, Switzerland. Provided per kg diet: vitamin A (as retinol palmitate), 30,000 IU; vitamin D3, 2160 IU; vitamin E (as DL-%-tocopheryl-acetate), 1590 IU; niacin, 990 mg; calcium pantothenate, 480 mg; riboflavin, 240 mg; thiamin mononitrate, 150 mg; pyridoxine hydrochloride, 135 mg; menadione sodium bisulfate, 75 mg; folacin, 39 mg; biotin, 3 mg; vitamin B_{12}, 90 µg.
[8] Sigma-Aldrich Company (St Louis, MO, USA).
[9] Archer Daniel Midlands (Decatur, IL, USA).
[10] Rovimix Stay-C 35 (DSM).
[11] Contributed, per kg of diet; zinc, 40 mg; manganese, 10 mg; iodine, 5 mg; copper, 9 mg; selenium, 0.4 mg.

(2) Fish culture For the growth trial, fifteen rainbow trout (10.4 ± 0.2 g, initial weight; Troutlodge Inc, Sumner, WA, USA) were randomly stocked into quadruplicate 400-L tanks in a recirculating system connected to a common bio-filter with

Table 2 Composition (% dry weight) of growth trial diets containing different levels of full-fat (FF) or defatted (DF) BSFL meals or BSFL lipid

Ingredients	Control	BSFL Lipid	5% FF	10% FF	5%DF	10%DF
Fishmeal[1]	13.32	13.32	12.54	11.76	12.35	11.40
Poultry by-product meal[2]	13.90	13.90	13.09	12.28	12.89	11.90
Full-fat BSFL meal[3]	0	0	5	10	0	0
Defatted BSFL meal[3]	0	0	0	0	5	10
BSFL lipid[3]	0	3.72	0	0	0	0
Menhaden fish oil[1]	4.69	2.83	3.90	3.11	4.47	4.25
Poultry fat[4]	3.84	1.98	3.05	2.25	3.63	3.42
Wheat flour[5]	25.10	25.10	23.26	21.43	22.46	19.79
Lysine HCl[5]	1.29	1.29	1.29	1.28	1.31	1.32
DL-Methionine[5]	0.43	0.43	0.44	0.45	0.44	0.46
Threonine[5]	0.30	0.30	0.30	0.31	0.32	0.33
Blood meal[2]	5	5	5	5	5	5
Feather meal[2]	5	5	5	5	5	5
Corn protein concentrate[6]	5	5	5	5	5	5
Soybean meal[5]	15	15	15	15	15	15
Lecithin[5]	1.5	1.5	1.5	1.5	1.5	1.5
Stay-C 35[7]	0.2	0.2	0.2	0.2	0.2	0.2
Vitamin premix ARS 702[8]	1	1	1	1	1	1
Monocalcium phosphate[5]	2.5	2.5	2.5	2.5	2.5	2.5
Choline Cl 50%[5]	1	1	1	1	1	1
Taurine[5]	0.5	0.5	0.5	0.5	0.5	0.5
TM ARS 1440[8]	0.1	0.1	0.1	0.1	0.1	0.1
Astaxanthin[7]	0.08	0.08	0.08	0.08	0.08	0.08
Calcium propionate[2]	0.15	0.15	0.15	0.15	0.15	0.15
Yttrium oxide[9]	0.1	0.1	0.1	0.1	0.1	0.1

[1] Omega Protein Corp., Menhaden fish meal, special select (700 g/kg protein), Hammond, LA, USA; Menhaden fish oil, Virginia Prime, Reed, Virginia, USA.

[2] Rangen Inc. (Buhl, ID, USA), Poultry by-product, pet food grade, 670 g/kg protein; Blood meal, 960 g/kg protein; calcium proprionate.

[3] Chapul, LLC. (McMinnville, Oregon, USA).

[4] Tyson Foods, Springdale, AR, USA.

[5] Archer Daniels Midland Alliance Nutrition (Twin Falls, ID, USA), wheat flour 14 g/kg protein; L-lysine hydrochloride; DL-methionine; threonine; soybean meal, 460 g/kg protein; lecithin - Yelkinol AC dry lecithin; monocalcium phosphate; choline chloride 50%, taurine.

[6] CPC Empyreal 75, Cargill Corn Milling, Blair, NE, USA.

[7] Ascorbyl polyphosphate Rovimix Stay-C 35; Carophyll pink, DSM Nutritional Products Ltd., Basel, Switzerland.

[8] Star Milling, Peris, CA, USA. ARS 702 contributed per kilogram of diet: vitamin A (as retinol palmitate), 10,000 IU; vitamin D3, 720 IU; vitamin E (as DL-%-tocopheryl-acetate), 530 IU; niacin, 330 mg; calcium pantothenate, 160 mg; riboflavin, 80 mg; thiamin mononitrate, 50 mg; pyridoxine hydrochloride, 45 mg; menadione sodium bisulfate, 25 mg; folacin, 13 mg; biotin, 1 mg; vitamin B12, 30 µg; ARS 1440 contributed in mg/kg of diet: zinc, 37; manganese, 10; iodine, 5; copper, 3; selenium, 0.4.

[9] Sigma-Aldrich Company, St Louis, MO, USA.

spring water inflow of approximately 20% makeup. Water temperature was maintained at 15°C. Fish were fed twice daily to apparent satiation. Apparent satiation was defined as all the feed the fish would consume in a 30-min period. Feed consumption was recorded weekly. All fish in each tank were counted and group-weighed every three weeks and fed their respective diets for 12 weeks to assess effects on growth performance and immune function.

(3) Sample collection and analyses At the termination of the 12-week trial, all fish were counted and weighed. Three fish per tank were randomly selected, euthanized by submersion in buffered tricane methanesulfonate (350 mg/L) for 10 min and then dissected to collect tissue samples and determine the

following organosomatic indices:

Fillet Ratio (FR) = fillet mass with ribs (g) * 100/fish mass (g)

Hepatosomatic Index (HSI) = liver mass (g) * 100/fish mass (g)

Viscerosomatic Index (VSI) = viscera mass (g) * 100/fish mass (g)

Fillet samples from these three fish per tank were analyzed to determine dietary effects on fillet fatty acid profile. Approximately 0.2 g of freeze-dried tissue from each sample was added to a 2 mL microcentrifuge tube containing 3 stainless-steel balls. A 3:1 chloroform: methanol solution was then added to the tubes in sufficient volume to fill them. The tubes were shaken on a bead beater (Mini-Bead Beater 24, BioSpec Products, Bartlesville, OK, USA) for three 30 s intervals, allowing 2 min between each shake. The tubes were then transferred to a microcentrifuge (Microfuge 18, Beckman Coulter, Brea, CA, USA) and spun down at 15,000 *g* for 20 min. Standards for calibration (CRM18918, CRM47570, CRM47571, Supelco, Bellefonte, PA, USA) were prepared in similar solvent and combined into a single fatty acid mixture. Pasteur pipettes were used to transfer the supernatant from the 2 mL tubes into labeled 10 mL round bottom glass tubes. An auto-pipette was then used to add 2 mL 1.25 M HCl-methanol solution to the supernatant in the 10 mL tubes which were then incubated (Isotemp 11-715-125D, Fisher Scientific, Pittsburg, PA, USA) for 0.5 h at 70°C. Following incubation, the tubes were allowed to cool to room temperature before 4 mL of distilled water was added to each. The tubes were then vortexed for 2-3 s and spun down for 5 min in a centrifuge (Sorvall ST16, Thermo Scientific, Waltham, MA, USA) at 500 g to separate the aqueous and non-aqueous layers. The upper aqueous layer from each tube was removed using suction and discarded. The lower chloroform layer was transferred to pre-labeled GC vials containing inserts. The methylated samples were analyzed using a Trace 1300 GC system (Thermo Scientific) supplied with a flame ionization detector set at 325°C and an Omegawax 250 capillary column (30 m x 0.25 mm i.d., 0.25 µm film thickness). Helium was used as the carrier gas. Detector gas flows were as follows: Air, 350.0 mL min^{-1}; Hydrogen, 35.0 mL min^{-1}; makeup gas, 45.0 mL min^{-1}. Total flow rate at the split vent was 480 mL/min, and flow rate through the column was 2.4 mL min^{-1}. The split/splitless injector was set at 235°C with a split ratio of 200:1, and 0.20 µL injections were made. The following oven temperature program was used: initial temperature 170°C, ramp 1.3ºC min^{-1} to 200°C followed by ramp 1.3°C min^{-1} to 225°C. The total run time was 42.31 min. Results were calculated on a relative percent concentration basis by dividing the response factor (RF)-corrected areas of individual fatty acid peaks by the total peak area. The identity and elution order of additional fatty acids were determined similarly utilizing a mass spectrometer detector in electron ionization (70 eV) mode with the following settings: MS transfer line temperature of 250°C, ion source temperature of 200°C, mass range of 50-400 amu, scan time of 0.25 s and start time of 2.1 min. The FAME calibration standards eluted in the following order: methyl octanoate (C8:0), methyl decanoate (C10:0), methyl laurate (C12:0), methyl tetradecanoate (C14:0), methyl palmitate (C16:0), methyl palmitoleate (C16:1 n-9z), methyl octadecenoate (C18:0), cis-9-oleic methyl ester (C18:1 n-9z), methyl linoleate (C18:2 n-6,9zz), methyl linolenate (C18:3 n-3,6,9zzz), methyl eicosapentaenoate (EPA) (C20:5 n-3,6,9,12,15zzzzz), methyl arachidate (C20:0), methyl docosanoate (C22:0), methyl erucate (C22:1 n-9z), methyl lignocerate (C24:0), and methyl docosahexaenoate (DHA) (C22:6 n-3,6,9,12,15,18).

An additional three fish per tank were randomly selected and euthanized as described above to determine whole body composition. Moisture, crude protein, gross energy, and crude lipid of whole-body samples were determined according to the standard methods (AOAC 2012) described for the digestibility trial. Protein retention efficiency values were calculated as follows:

Protein retention efficiency (PRE)
= protein gain (g)* 100/protein fed (g)

To assess the effects of BSFL meal and BSFL lipid inclusion on rainbow trout immune responses, a panel of genes known to undergo changes in expression during inflammation were measured in the intestine of three fish from each replicate tank. Briefly, a section of the distal intestine was dissected from each fish and frozen in liquid nitrogen. Total RNA was isolated from the samples using TRIzol according to the manufacturer's protocol (Invitrogen, Rockville, MD, USA). RNA quantity and quality were determined spectrophotometrically using a nanodrop (Thermofisher, Waltham, MA, USA). Trace amounts of DNA were removed from all samples by treatment with DNase I (Invitrogen) and samples were assessed by quantitative real time RT-PCR as previously described by Johansen and Overturf (2005) using a Quantstudio 6 (Thermofisher). Pro-inflammatory and immune-responsive genes examined included IL-10, IL-1β, TNFα, HSP70, Defensin β3, IL-4 like, and UDP glucose 6-dehydrogenase (UDPG6D). All the treatment values were standardized to a ß-

actin control for both CT values and relative numerical values providing a numerical value for the control and each treatment from which the relative change was calculated (Johansen and Overturf 2005). Accession numbers for genes analyzed along with primers and probes used and efficiency for each gene are provided in Table 3.

3. Statistical analyses

Pooled SEMs from tank means are considered as the best estimate of variation inherent in this type of experiment and were used as an indicator of variation among treatments. Results were tested for normality (Shapiro-Wilk test) and homoscedasticity (Bartlett test). Normally distributed data was examined for significance of diet using ANOVA. Differences with $P < 0.05$ were considered significant for all analyses. Means were compared with the control (Menhaden fish meal for the digestibility trial and the control diet for the feeding trial) using Dunnett's test. All data analyses were performed with JMP 15.1 (SAS Institute, Cary, NC, USA).

Results and Discussion

BSFL meals have previously been reported to contain approximately 50-60% crude protein and 30-35% crude lipids for full-fat meals on an as-fed basis (Tomberlin *et al*. 2002). However, nutrient bioavailability of BSFL meals is less consistent due, in part, to their variable chitin content, which salmonids cannot digest (Olsen *et al*. 2006), and different processing methods that are used during lipid extraction and drying that can compromise amino acid availability (English *et al.* 2021).

In the current study, nutrient and energy ADCs for full-fat and defatted BSFL meals were not significantly different from each other or menhaden fish meal, with the exception of phosphorus (Table 4). Phosphorus digestibility was significantly higher for BSFL meals (57.9 - 58.0%) compared to menhaden fish meal (33.4%). Similarly, ADCs of aspartic acid, proline and tyrosine were significantly higher than menhaden fish meal while ADC of ornithine was significantly lower. The ADC

Table 3 Gene names, accession numbers, probe and primer sequences and PCR reaction efficiencies for the quantitative PCR analysis performed on the fish samples

Gene Name	Accession Number	Probe and Primer Sequences (Listed 5'-3')	PCR reaction efficiency
B-actin	AF254414	BactinF: CCCTCTTCCAGCCCTCCTT BactinR: AGTTGTAGGTGGTCTCGTGGATA BactinMGB: 6FAM-CCGCAAGACTCCATACCGA-NFQ	97.4
Defensin β3	NM_001195183	Def β3F: ACGGAGGGTCATATTCATCAAATCAAA Def β3R: GCAATGACTAAAAGAGCCACTAGCA Def β3MGB: CTGCCTGATGATCTTC-NFQ	94.6
HSP70	AB062281	HSP70F: ACAGAGACAGGCCACTAAGGA HSP70R: CTCGTTGATGATCCTCAGCACAT HSP70MGB: TCAGCCCAGCGATCAC-NFQ	97.2
Interleukin-1β	AJ004821	IL1βF- GTAATATTTCCTCTTCCCCTGTGTGT IL1βR- CCCTGAGGCAGCTTGGA IL1βMGB- CATGCTGCACTTTCAG-NFQ	94.7
Interleukin-4	XM_036961927	IL4F- TCTTTCACAATTCCAGATTTCAGCTGTA IL4R- GCCCAGCAAAATTAGATGACAACT IL4MGB- ACACGCACATTAATTC-NFQ	93.0
Interleukin-10	AB118099	IL10F: AGTAGCTCAACGGGTAGAGAGAA IL10R: AATTCCTTGTCGTCAGTGAGTGT IL10MGB: TCTGGTCCCCAAGATC-NFQ	95.5
TNFα	NM_001124357	TNFαF: GAATACAATCCTAATCTTTCCGCTGACA TNFαR: GAACCCGCCCTGGGAAAA TNFαMGB: CTGGCCGTCATCCTTT-NFQ	92.0
UDP glucose 6-dehydrogenase	XM_036934411	UDPG6DF: CCAGGGTTGAAGGAGGTAGTG UDPG6DR: TCTCTGATGGCGGAATCTATATCTGT UDPG6DMGB: TCCCTCGGCATGACTC-NFQ	95.6

Table 4 Apparent digestibility coefficients (ADCs, %) for menhaden fish meal, full-fat BSFL meal, and defatted BSFL meal

	Menhaden Fish Meal	Full fat BSFL Meal	Defatted BSFL Meal	*Pooled SEM*	*Prob > F*
ADCs					
Dry matter	72.1	71.2	65.6	3.5	0.1279
Crude lipid	93.1	89.4	89.0	2.5	0.6505
Crude protein	72.9	71.4	70.4	1.9	0.3639
Gross energy	78.5	77.3	73.0	2.9	0.1187
Phosphorus	33.4	57.9*	58.0*	7.4	0.0098
Amino Acids					
ALA	81.0	84.2	85.4	1.9	0.0741
ARG	85.7	88.0	88.7	1.9	0.2193
ASP	70.1	79.1*	78.9*	3.8	0.0432
CYS	57.7	52.7	54.7	9.4	0.8151
GLU	82.3	78.5	80.5	3.2	0.4021
GLY	70.0	75.1	76.4	2.9	0.0796
HIS	79.7	80.2	80.7	2.6	0.8965
HYD-LYS	57.9	88.6*	84.3*	6.8	0.0342
HYD-PRO	52.9	68.4	69.1	9.5	0.5330
ILE	83.3	83.9	83.9	3.0	0.9686
LEU	84.2	80.0	81.1	2.5	0.1901
LYS	85.6	88.0	86.4	2.1	0.4091
MET	81.4	85.6	84.8	4.6	0.5323
ORN	75.1	37.4*	37.6*	3.9	<0.0001
PHE	81.8	80.6	80.3	2.6	0.7497
PRO	72.3	80.4*	83.5*	2.4	0.0029
SER	76.8	78.5	79.3	3.1	0.6131
THR	79.2	80.4	79.7	2.5	0.8472
TRP	85.2	88.6	87.7	2.5	0.2968
TYR	85.0	89.6*	89.8*	1.5	0.0134
VAL	83.2	88.0	90.3	3.4	0.1055

Values are means (n = 3). ADCs in the same row with an asterisk are significantly different from ADC of menhaden fish meal as determined by Dunnet's test ($P < 0.05$).

values reported here are consistent with Dumas *et al.* (2018), who reported the ADCs of protein and amino acids in defatted BSFL meal varied between 87 and 93%, whereas digestibility of lipid and dry matter in BSFL meal were relatively low at 73 and 75%, respectively. Dry matter and gross energy digestibility in the current study was lower for defatted BSFL meal but lipid ADCs were higher for BSFL meals (Table 4) than those previously reported. The current digestibility findings also fall on the upper end of the ranges reported for other BSFL meals in rainbow trout in a recent literature review by Mohan *et al.* (2022). Taken together, these data indicate the novel BSFL meals investigated in the current study are high quality products, and the bioavailability of the nutrients present in these products were not damaged during fat extraction or drying.

The growth performance observed in the current trial also points the suitability of the tested BSFL meals as ingredients in rainbow trout feeds, achieving approximately 1,700% weight gain during the trial (Table 5). Additionally, the feeds were all readily accepted, and no mortality was observed during the 12-week feeding study (Table 4). Statistical analysis of the growth results indicated no significant effects of BSFL meal or BSFL lipid inclusion on final fish weight ($P = 0.7321$), weight gain as a percent increase ($P = 0.9513$), feed conversion ratio ($P = 0.8333$), or feed intake ($P = 0.5885$; Table 5). These results are in alignment with early research examining the potential of black soldier fly meal as feed ingredients in rainbow trout at inclusion levels of 15 to 18% that found no significant effects on growth and feed efficiency (St-Hilaire *et al.* 2007b; Sealey *et al.* 2011). More recent work has recapitulated the successful incorporation of BSFL meal at levels up to approximately 15% in feeds for rainbow trout (Renna *et al.* 2017; Dumas *et al.* 2018; Caimi *et al.* 2021).

Organosomatic indices are valuable indicators of general health and the nutritional adequacy of a fish's diet (Hoque *et al*. 1998). In the current study, hepatosomatic index ($P =$

0.6808), viserosomatic index (P = 0.5200), and fillet ratio (P = 0.3959) were not significantly affected by BSFL meal or BSFL lipid inclusion (Table 5). These results are aligned with other studies that have examined the effects of BSFL on rainbow trout organosomatic indices (Renna *et al.* 2017; Caimi *et al.* 2021) and fall within previously reported ranges for rainbow trout of this size (Sealey and Gaylord 2025).

Proximate composition data provides insight into an animal's growth efficiency and, by proxy, nutrient utilization. In the current study, BSFL meal or BSFL lipid inclusion did not affect rainbow trout whole body moisture (P = 0.3282), lipid (P = 0.4544) or protein (P = 0.3280) content (Table 6). In contrast, a significant effect of diet level was observed for fillet moisture content (P = 0.0298), where in rainbow trout fed 10% BSFL fillet moisture decreased by approximately 0.5% (Table 7). Although statistically significant, the difference in fillet moisture content in the current study in the absence of other significant alterations such as corresponding significant increases in fillet lipid content likely have minimal physiological impacts. Notably, previous research by Mancini *et al*. (2018) and Huyben *et al.* (2019) reported that the dietary inclusion of BSFL meal had no adverse effects on rainbow trout fillet quality.

Previous research examining the lipid content of BSFL has

Table 5 Growth performance and organosomatic indices of juvenile rainbow trout fed diets containing different inclusions of full-fat (FF) or defatted (DF) BSFL meals and/or BSFL lipid (BSFL Lipid) for 12 weeks

	Final Fish Wt[1]	Weight Gain[2]	FCR[3]	Feed Intake[4]	Viscero-somatic Index[5]	Fillet Ratio[6]	Hepatosomatic Index[7]
	(g)	*%*	*feed g/gain g*	*% bw*	*%*	*%*	*%*
Control	194	1,755	0.86	1.84	10.0	57.8	1.3
BSFL Lipid	184	1,687	0.87	1.81	9.8	58.5	1.3
5% FF	184	1,678	0.86	1.79	10.0	55.3	1.3
10 % FF	189	1,756	0.88	1.83	10.4	58.2	1.2
5% DF	196	1,718	0.84	1.76	8.4	54.0	1.2
10% DF	193	1,729	0.87	1.86	9.7	59.6	1.2
Pooled SEM	13	143	0.04	0.08	1.31	3.78	0.17
Prob > F	0.7321	0.9513	0.8333	0.5885	0.5200	0.3959	0.6808

[1] Final tank weight (g) / number of fish in the tank. [2] Percent increase (%) = (final weight – initial weight) x 100 / initial weight. [3] FCR, feed conversion ratio = g feed consumed / g weight gained. [4] Feed intake (%) = g dry feed consumed/average fish biomass (g) /culture days x 100. [5] Viscerosomatic index (%) = viscera mass x 100 / fish mass. [6] Fillet ratio (%) = fillet with rib mass x 100 / fish mass. [7] Hepatosomatic index (%) = liver mass x 100 / fish mass.
Values are means (n = 4).

Table 6 Whole body proximate composition[1] and protein retention efficiency[2] of juvenile rainbow trout fed diets containing different inclusions of full-fat (FF) or defatted (DF) BSFL meals and/or BSFL lipid (BSFL Lipid) for 12 weeks

	Moisture	Lipid	Protein	Protein Retention Efficiency
Control	69.4	6.8	17.3	42.9
BSFL Lipid	69.4	6.5	17.5	43.3
5% FF	69.8	7.0	16.9	41.7
10% FF	69.9	6.6	17.5	42.4
5% DF	68.8	7.1	17.7	45.3
10% DF	68.9	6.9	17.9	43.4
Pooled SEM	0.78	0.44	0.62	2.25
Prob > F	0.3282	0.4544	0.3280	0.4697

[1] Whole body composition based on three fish composite sample per tank. [2] PRE, protein retention efficiency = g protein gain x 100/g protein fed.
Values are means (n = 4).

Table 7 Fillet moisture, lipid and fatty acid (%) composition of juvenile rainbow trout fed test diets containing different inclusions of full-fat (FF) or defatted (DF) BSFL meals and/or BSFL lipid (BSFL Lipid) for 12 weeks

	Moisture	Lipid	12:0	14:0	16:0	16:1 n-7	18:0	18:1 n-9	18:1 n-5	18:2 n-6	20:4 n-6	20:5 n-3	22:6 n-3
Control	72.1	5.4	0.1	2.5	22.1	8.2	5.1	24.6	5.6	12.7	0.8	2.0	9.2
BSFL Lipid	71.3	5.5	4.2*	3.3*	21.5	7.0*	4.8	22.7	5.3	12.2	0.7	1.3*	8.0*
5% FF	72.6	4.6	2.5*	2.9	21.5	7.1*	5.0	23.1	5.1	13.7*	0.9	1.8	10.0
10% FF	71.1*	6.2	4.8*	3.0*	19.9*	7.0*	5.0	24.3	5.1	14.5*	0.8	1.2*	7.7*
5% DF	71.7	5.1	2.0*	2.9	19.8*	6.9*	5.1	24.3	6.2	14.3*	0.9	1.8	9.0
10% DF	71.3	5.8	3.7*	3.3*	21.0	7.4*	5.0	23.2	5.3	14.1*	0.8	1.7	8.4
Pooled SEM	0.63	0.77	0.71	0.20	0.74	0.47	0.24	1.02	0.97	0.60	0.11	0.33	0.76
Prob > F	0.0298	0.1137	<0.0001	0.0006	0.0374	0.0275	0.3214	0.0847	0.4213	0.0003	0.5208	0.0255	0.0036

Values are means ($n = 4$). Values in the same row with an asterisk are significantly different from the value of Control as determined by Dunnet's test ($P < 0.05$).

Table 8 Fatty acid (%) composition of test diets containing different inclusions of full-fat (FF) or defatted (DF) BSFL meals and/or BSFL lipid (BSFL Lipid) for 12 weeks of growth trial

	12:0	14:0	16:0	16:1 n-7	18:0	18:1 n-9	18:1 n-5	18:2 n-6	20:4 n-6	20:5 n-3	22:6 n-3
Control	0.1	3.4	22.0	7.7	5.7	21.8	2.5	16.9	0.9	5.7	5.5
BSFL Lipid	9.7	4.2	19.7	5.9	4.8	20.0	2.1	19.6	0.6	3.9	3.7
5% FF	5.3	3.9	21.7	7.1	5.5	21.0	2.5	17.6	0.7	4.7	4.6
10% FF	9.4	3.9	20.1	6.1	5.1	20.7	2.1	18.6	0.6	3.7	3.7
5% DF	3.7	3.7	21.0	7.4	5.4	21.5	2.5	17.0	0.8	4.9	4.6
10% DF	7.5	4.3	21.0	7.0	5.2	20.1	2.2	16.9	0.7	4.7	4.4

Means of duplicate analyses on a dry matter basis.

suggested that the lipid component of BSFL meal can pose challenges in feed formulation as it may lead to an imbalanced fat profile in fish (Eide *et al*. 2024) due to the large variation in black soldier fly larvae saturated fatty acids levels (58-72%) and mono and polyunsaturated fatty acids (19-40%) that have been reported (Kroeckel *et al.* 2012). In the current study feeding diets containing BSFL meal or BSFL lipid significantly increased the levels of lauric acid (12:0) in the diets (Table 8) and subsequently, rainbow trout fillets particularly when fish were fed 10% BSFL meal or BSFL lipid diets (Table 8). Conversely, rainbow trout fed the control diet containing no BSFL meal or BSFL lipid had higher fillet levels of palmitic (16:0) and palmitoleic (16:1 n-7) acid. Importantly, rainbow trout fillet polyunsaturated fatty acids levels were altered by BSFL meal inclusion level in that linoleic acid (C18:2 n-6), an omega-6 fatty acid significantly increased with BSFL meal supplementation while docosahexaenoic acid (DHA, 22:6 n-3), and eicosapentaenoic acid (20:5 n-3) significantly decreased in rainbow trout fed the 10% FF BSFL meal or the BSFL lipid diets (Table 7). These results agree with those reported by St-Hilaire *et al*. (2007a, b) and Melenchón *et al*. (2021) who found that feeding high inclusion levels of BSFL meal reduced the omega 3 fatty acid contents in rainbow trout fillets. However, the present results differ from those of Drosdowech *et al*. (2024), who reported that BSFL meal inclusion had no significant effect on DHA levels in rainbow trout fillets. These differing results are likely explained by differences in the total lipid content and fatty acid profiles of the BSFL meals used in the various studies (St-Hilaire *et al.* 2007a, b; Melenchón *et al.* 2021).

Studies have shown that incorporating BSFL products can positively affect immune gene expression and alter inflammatory responses in fish (Elia *et al*. 2018; Koutsos *et al.* 2022; Weththasinghe *et al*. 2022). The current study examined expression of genes that have critical functions in the rainbow trout immune system and provide an indication of overall fish health. Specifically, tumor necrosis factor alpha (TNFα) and interleukin1 β (IL-1β) are pro-inflammatory cytokines whereas interleukin-10 (IL-10) and interleukin-4 like (IL-4 like) are anti-inflammatory cytokines. Defensin β3

is an antimicrobial peptide that helps with wound healing and can inhibit the accumulation of TNFα. Heat shock protein 70 (HSP70) is a molecular chaperone that is expressed in response to stress and UDP-glucose 6-dehydrogenase (UDPG6D) prevents cellular damage from reactive oxygen species. In the current study, no significant effects of BSFL meal on intestinal expression of IL-1β (Fig.1A), TNFα (Fig.1B), IL-10 (Fig.1C), HSP70 (Fig.1D), IL-4 like (Fig.1E), and UDPG6D (Fig.1G) were observed. However, fish fed the BSFL lipid diet exhibited significant upregulation of IL-1β (Fig.1A), TNFα (Fig.1B), IL-4 like gene (Fig.1E), and UDPG6D (Fig.1G) expression and significant down-regulation of IL-10 (Fig.1C) and HSP70 (Fig.1D). Cardinaletti *et al.* (2019) previously reported upregulated expression of IL-10 and TNFα in the

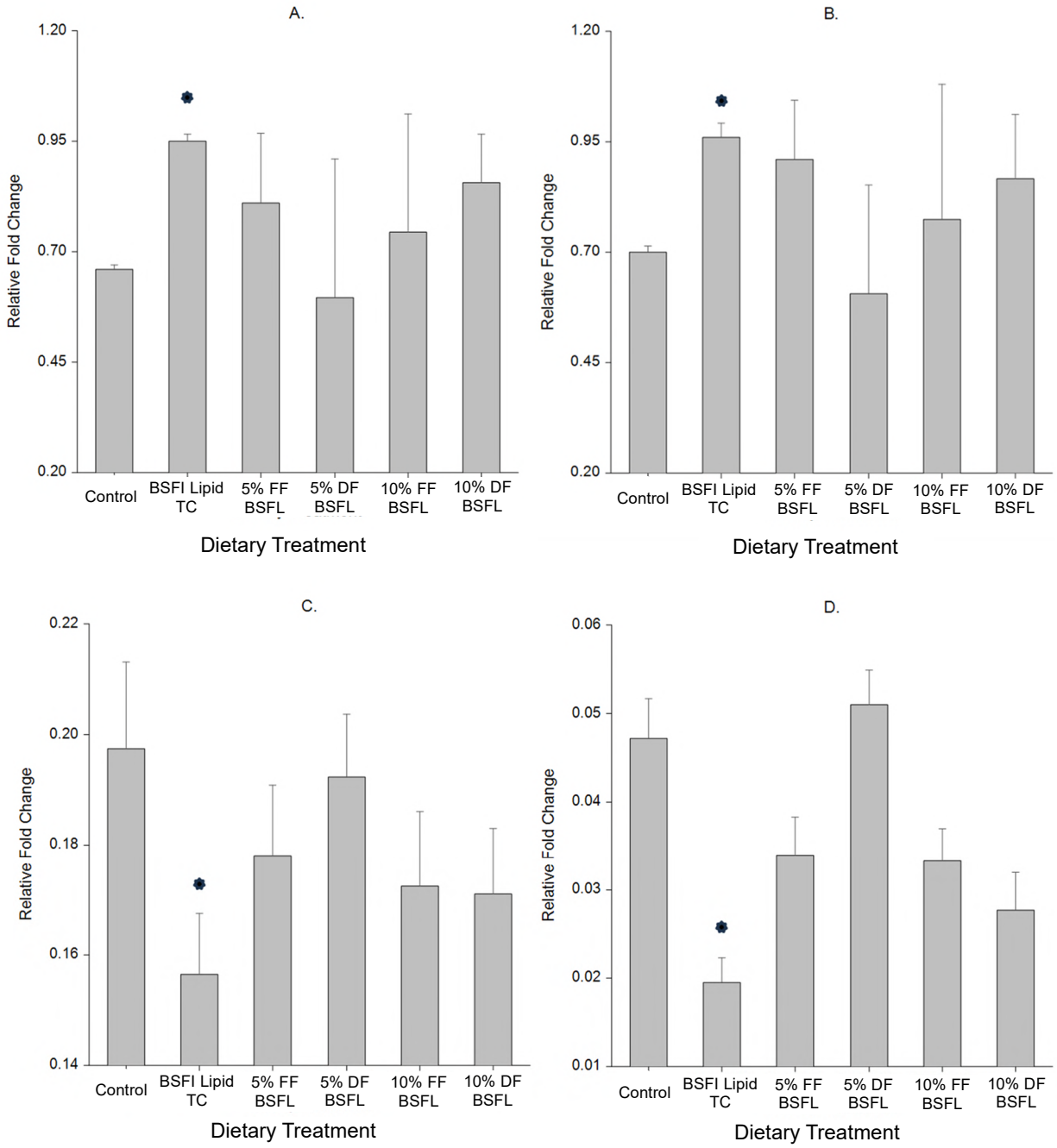

Fig.1 Expression of the intestinal immune related genes IL-1β (A), TNFα (B), IL-10 (C) and HSP70 (D) in rainbow trout fed diets containing different inclusions of full-fat (FF) or defatted (DF) BSFL meals and/or BSFL lipid (BSFL Lipid)

An asterisk indicates that rainbow trout fed the various diets are significantly different from the rainbow trout fed the Control diet as determined by Dunnet's test ($P < 0.05$).

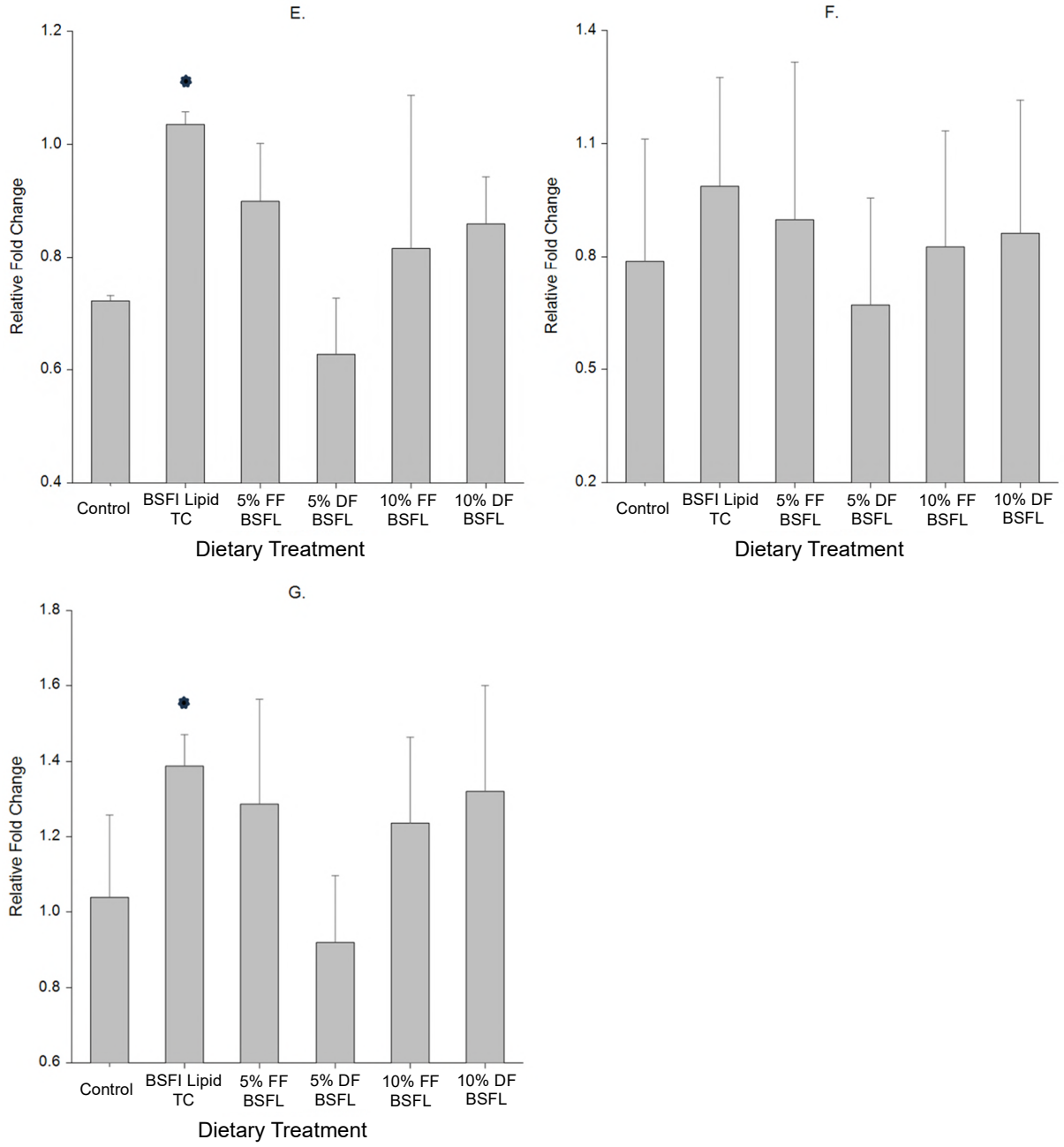

Fig.1 Continued
Expression of the intestinal immune related genes IL-4 like (E), Defensin β3 (F), and UDP glucose 6-dehydrogenase (UDPG6D; G) in rainbow trout fed diets containing different inclusions of full-fat (FF) or defatted (DF) BSFL meals and/or BSFL lipid (BSFL Lipid)

An asterisk indicates that rainbow trout fed the various diets are significantly different from the rainbow trout fed the Control diet as determined by Dunnet's test ($P < 0.05$).

intestine of rainbow trout fed 10.5 or 21% BSFL meal, as well as upregulation of HSP70 in the liver of fish fed 21% BSFL meal. Similarly, Cho *et al.* (2022) reported significantly higher expression of IL-1β in rainbow trout fed 5% BSFL meal. Gaudioso *et al.* (2021) reported different expression patterns of selected markers in the midgut and head kidney of rainbow trout fed BSFL meal. Specifically, significantly higher expression of the pro-inflammatory cytokine IL-1β was observed in head kidney tissues of fish fed BSFL meal, whereas the opposite trend was observed in midgut tissues. The current results suggest that BSFL meals can be used at the tested inclusion rates without negatively affecting immune factors. However, top-coating feed with BSFL lipid resulted in significant modulation of a number of immunoregulatory

factors. This is somewhat similar to the findings of Borland *et al*. (2024) except their diets included supplemental chitin in addition to BSFL meal. Dietary upregulation of immune function is thought to be a positive attribute of some dietary components used as immunostimulants, assuming the animals do not become less responsive to the ingredient over time. The full extent of the effects of BSFL lipid on the rainbow trout immune system have yet to be elucidated, but the effects observed herein are considered modestly immunostimulative suggesting that the ingredient may be potentially useful in that regard.

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Annotated Bibliography of Key Works

(1) English G, Wanger G, Colombo S (2021) A review of advancements in black soldier fly (*Hermetia illucens*) production for dietary inclusion in salmonid feeds. *J. Agric. Food Res*., **5**, 100164. https://doi.org/10.1016/j.jafr.2021.100164

The authors summarize the main findings on the advancement on black soldier fly (BSF) production methods and their use in salmonid aquaculture and highlight the importance of BSF rearing procedures and processing on the suitability of BSF as a nutritious protein source for salmonids. Further, the authors identified areas for future research regarding optimizing rearing and processing procedures for BSF destined for aquafeeds.

(2) Gasco L, Bellezza Oddon S, Vandenberg GW, Veldkamp T, Biasato I (2024) Factors affecting the decision-making process of using insect-based products in animal feed formulations. *J. Insects Food Feed*, **10**, 1707–1718. https://brill.com/view/journals/jiff/10/10/article-p1707_5.xml?srsltid=AfmBOoptqHMf9FusU8AQCMEpGdGIiOCmGduiByrAqwbeSKofIUad7Psk

The authors discuss the various factors to consider when including insect-based products in animal feeds. In particular, the authors identified the importance of insect meal form, insect species, rearing substrates and production processes. The authors highlighted how the increased use of additives during insect processing has created additional sources of variation in commercial insect products. The authors also discussed how feed manufacturing method was an important consideration when using insect-based products in animal feeds.

(3) Koutsos E, Modica B, Freel T (2022) Immunomodulatory potential of black soldier fly larvae: applications beyond nutrition in animal feeding programs. *Transl. Anim. Sci.*, **6**, txac084. https://doi.org/10.1093/tas/txac084

The authors review recent research that demonstrates the potential for the immunomodulatory activity of various components of black soldier fly (BSF)-derived ingredients. The authors focus this review on the actions of three important BSF components that have been shown to alter immune function and disease resistance in companion animals, poultry and livestock including: antimicrobial peptides that are present in BSF hemolymph; lauric acid, a predominant BSF fatty and, the chitin/chitosan components of the insect exoskeleton.

(4) Weththasinghe P, Øvrum Hansen J, Torunn Mydland L, Øverland M (2022) A systematic meta-analysis based review on black soldier fly (*Hermetia illucens*) as a novel protein source for salmonids. *Rev. Aquac*., **14**, 938-956. https://doi.org/10.1111/raq.12635

The authors discuss how black soldier fly (BSF) has gained attention as a sustainable novel protein source in fish feed due to its high nutritional value and low environmental impacts and conducted a meta-analysis to compile and systematically quantify the effect of BSF in diets for salmonids on growth performance and nutrient utilization. The authors present results that demonstrate that overall dietary inclusion of BSF did not compromise the specific growth rate, feed conversion ratio, feed intake, protein digestibility and protein efficiency ratio in salmonids. Importantly, the authors report that when the published literature reviewed was sorted according to the replaced protein source(s), replacing fishmeal by BSF decreased growth rate and feed intake in salmonids but replacing non-fishmeal sources improved growth rate and feed conversion.

水研機構研報, 第 53 号, 31 – 36, 令和 8 年
Bull. Jap. Fish. Edu. Agen. No. 53, 31-36, 2026

Protein assimilation of black soldier fly larvae *Hermetia illucens* in diets for red seabream *Pagrus major*

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Abstract: Fishmeal is a major ingredient for aquaculture feeds. Japanese fish feed manufacturers still heavily depend on imported fishmeal, and include fishmeal into fish feeds at approximately 40% in 2023. Recent price increase in the imported fishmeal makes large impact on the aquaculture practice in Japan, so that the use of alternative protein ingredients has become a crucial interest. Meal of black soldier fly (BSF) larvae is a promising candidate for a fishmeal substitute in aquaculture feeds. Recently, several private companies in Japan have started to produce BSF larvae with food residues. However, a high inclusion of BSF meal (BSFm) in fish feeds has sometimes resulted in growth retardation in several fish species. Thus, in this study, a 42-day feeding experiment was conducted in juvenile red seabream (*Pagrus major*) using diets with various inclusion levels of BSFm. Three isoenergetic diets were prepared with fishmeal to BSFm ratios of 100:0 (Group 1), 50:50 (Group 2), and 22:78 (Group 3). Then, we investigated the protein assimilation from BSFm to fish using conventional growth analysis, stable isotope analysis and metabolomic analysis. Group 1 exhibited a significantly higher specific growth rate (2.92 ± 0.22 %/day) than Group 3 (2.54 ± 0.22 %/day), while Group 2 (2.77 ± 0.21 %/day) did not show a significant difference relative to Group 1. Stable isotope analysis suggested a lower assimilation of BSF protein in Groups 2 and 3, as indicated by their δN15 ratios. *In vitro* digestion evaluation suggested that several essential amino acids (EAAs) in the diet for Group 3 were potentially deficient compared to the diet for Group 1. These results suggest that a diet comprising up to 50% BSFm does not negatively affect the growth of red seabream, but an EAA deficiency may have retarded the growth in Group 3. Therefore, further nutritional improvement is needed for diet with BSFm inclusion level above 50%.

Key words: stable isotope, amino acid balance, *in vitro* digestion, growth, digestive enzymes

Introduction

Future global food crisis that will be exacerbated by global warming and population growth, is a imminent issue. As one of the strategies that could mitigate this crisis, resource circulation in food production sectors is crucial. We should transform unutilized food resources and their residues into higher trophic forms for animal feeds and eventually, for human foods. Some insects which grow on various residues from agriculture, food processing, and livestock, are nutritious and could be used as feed ingredients for fish aquaculture. Notably, larvae of black soldier fly (BSF, *Hermetia illucens*) can grow well on residues of vegetables and livestock, and fish offal (Meneguz *et al*. 2018; Spranghers *et al*. 2017; Joly and Nikiema 2019; Yuan and Hasen 2022).

BSF is a saprophytic insect that can grow even on spoiling matter (Kortsmit *et al*. 2023). The conversion efficiency of food in BSF is higher than that of yellow mealworm (*Tenebrio molitor*) and house cricket (*Acheta domesticus*). BSF has the shortest life cycle among the candidates of insects for animal

Accepted on Feb 26, 2026
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foods (Oonincx *et al*. 2015). BFS larvae contain up to 63% protein or 39 % fat on a dry matter basis (Barragan-Fonseca *et al*. 2017), establishing the potential of BSF larvae that convert unavailable residual nutrients into valuable ones for carnivorous fish. The production of insect proteins, which includes BSF and yellow mealworm, is expected to grow into a $1.14 billion market by 2027 (Gruber and Melton 2023).

BSFm is nutritious feed ingredient with favorable effects on fish physiology. The effectiveness of BSFm has been evaluated in various fish species (Belghit *et al*. 2019; Ido *et al.* 2021; Takakuwa *et al*. 2022). However, diets with high proportions of BSFm resulted in growth retardation in several fish species.

The aims of this study are as follows: 1) evaluation of the performance of red seabream (*Pagrus major*), a marine carnivorous fish fed diets with various substitution levels of fishmeal by BSFm, and 2) investigation of the mechanism causing growth retardation when red seabream are fed with BSFm.

Material and methods

Dried whole BSF larvae, having been raised on brewer's spent grain, were purchased from San-U Fish Farm (Osaka, Japan). They were partially defatted and then powdered using a motor-driven mill. This was designated as BSFm in this study. The formulation and nutrient composition of the diets are summarized in Table 1. Red seabream juveniles (four-month-old) were divided into groups of 15 or 16 individuals each to ensure an identical initial fish density between groups. The groups were as follows: Group 1 was served as the control and fed the 100 fishmeal/0 BSFm diet, Groups 2 and 3 were the experimental groups, and fed the 50 fishmeal/50 BSFm diet and 22 fishmeal/78 BSFm diet, respectively. The water temperature for rearing was maintained at 25.2 ± 0.4°C. The seabreams were hand-fed twice a day until apparent satiety for 42 days. Diets, protein ingredients, and muscle samples of the initial and final fish were subjected to various analyses.

An intestinal digestive enzyme complex was isolated from the intestinal content of three individuals of one-year-old red seabream. The intestinal content was extracted under the ice-cooling condition, and the solid body was removed by centrifugation. The supernatant was then dialyzed overnight, and designated as the digestive enzyme complex (EC). An *in vitro* digestion test was performed according to Andoh *et al*. (2023), with slight modifications. The amount of amino acids produced by the digestion with EC was calculated using the following equation:

$$AAa = TAAa - PAAa$$

In this formula, AAa represents the amount of free amino acids (AA) produced by the digestion by EC. TAAa stands for the total amount of free amino acids in the digesta, and PAAa is the amount of free amino acids in the diet without EC supplementation.

Muscle and diet samples were lyophilized and then defatted. The muscle samples were analyzed for measurement of δ15N. Stable isotope ratios were expressed in the conventional δ notation as parts per-mil (‰) according to the following equation:

$$\delta Xm = ((Rsample/Rstandard) - 1) \times 1000$$

Table 1 Formulation (%) and proximate composition (dry matter basis) of diets

	Group 1	Group 2	Group 3
Fishmeal:BSF powder	100:0	50:50	22:78
Fishmeal	72.6	36.3	16.1
Defatted BSF powder	0.0	36.3	56.5
Wheat gluten	8.1	8.1	8.1
Fish oil	8.9	8.9	8.9
Soybean lecitin	4.0	4.0	4.0
Choline chroride	0.8	0.8	0.8
α-Tocopherol	0.0	0.0	0.0
Others (vitamins and minerals)	5.6	5.6	5.6
Crude protein (%)	56.0	51.2	48.4
Crude fat (%)	15.0	17.4	18.7
Gross energy (kcal/g)	5.03	4.99	4.97

where Xm is δ15N and R is the corresponding δ15N ratio.

The differences in values between diet and muscle were calculated individually according to the following equation:

$$\text{Difference} = \delta Xm - \delta Xd$$

where Xd is δ15N of a diet supplied to the corresponding group.

Results

In the rearing experiment, the specific growth rate (SGR) of Group 1 (2.92 ± 0.22 %/day) was the highest and significantly higher than that of Group 3 (2.54 ± 0.22 %/day) (Table 2). The SGR of Group 2 (2.77 ± 0.21 %/day) was not significantly different from that of Group 1. There were no significant differences among the three groups in terms of condition factor, condition factor without viscera, hepatosomatic index, viscera somatic index, and intraperitoneal fat ratio. The FCR values were similar across groups (1.01 to 1.04). No differences were visually observed in the feeding behavior, external body appearance, hepatopancreas, or viscera among the three groups.

In the analysis of free amino acids in the *in vitro* digesta of diets for Groups 1, 2, and 3, 19 amino acids and taurine (Tau) were detected. Cystine was either not detected or present only in trace amounts in the diets. In the reactant of EC alone without diet, no amino acids were detected by the amino acid analyzer. Three amino acids (Leu, Phe, and Trp) showed no significant differences among three diet Groups (Fig.1). Twelve amino acids (Val, Ile, Thr, His, Ala, Ser, Tyr, Gly, Gln, Asn, Asp, Pro) were higher in Group 3 than in Group 1. The lower amino acids in Group 3 than in Group 1 were Lys, Arg, and Met, and Tau. The percentages of the amounts of amino acid of Group 3 to Group 1 were 82.6 ± 4.4% in Lys, 75.8 ± 4.1 % in Arg, 74.4 ± 3.3 % in Met, and 24.9 ± 1.1 % in Tau. The values for Group 2 were intermediate between

Table 2 Growth performance and fat content of red seabreams in the rearing experiment

	Group 1	Group 2	Group 3
Fishmeal:BSF powder	100:0	50:50	22:78
Initial number of individual	15	16	16
Final number of individual	14	16	15
Initial body weight (g)	26.4 ± 1.6^{a}	26.3 ± 1.6^{a}	26.3 ± 1.5^{a}
Final body weight (g)	90.0 ± 10.1^{a}	84.5 ± 6.1^{ab}	76.7 ± 5.6^{c}
Specific growth rate (%/day)	2.92 ± 0.22^{a}	2.77 ± 0.21^{ab}	2.54 ± 0.22^{c}
Condition factor	2.18 ± 0.18^{a}	2.18 ± 0.09^{a}	2.08 ± 0.10^{a}
Condition factor without viscera	1.96 ± 0.15^{a}	1.97 ± 0.08^{a}	1.87 ± 0.10^{a}
Feed conversion ratio	1.04	1.02	1.01

Values with a different letter indicate a significant difference ($p < 0.05$).

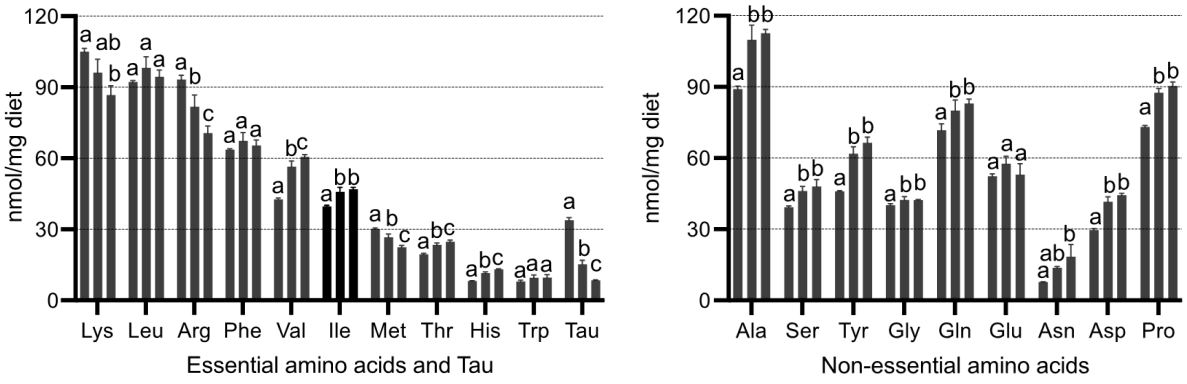

Fig.1 Amino acids produced by *in vitro* digestion with a red seabream digestive enzyme complex (EC) in three diets for Groups 1, 2, and 3

Tau is not an essential amino acid, but its essentiality has already been recognized. The bars in each amino acid indicate that the left is Group 1, the center is Group 2, and the right is Group 3. Different letters indicate a significant difference ($p < 0.05$).

Groups 1 and 3 or not significantly different from Group 3.

The amounts of total amino acid in the digestion reactants in Groups 2 and 3 were significantly higher than in Group 1 (Fig.2). The total amounts of NEAA in Groups 2 and 3 followed a trend similar to the total AA, and the total amounts of EAA were not significantly different among the three groups. Although free amino acids were also detected in the diets, they were at negligible levels except for Tau.

The δ15N ratios of BSFm and wheat gluten were lower than that of fishmeal. The δ15N ratios of the seabream muscle after 42 days of feeding changed significantly from the initial values in all groups. Compared to Group 1, the δ15N ratios of the diets for Groups 2 and 3 decreased in accordance with the dietary BSFm inclusion level (Table 3). The δ15N ratios of the muscles in these groups also decreased with the increase in the dietary BSFm level. In contrast, the difference in the δ15N ratio between the diet and its corresponding muscle in Group 1 was +2.094. For Group 2, it was +4.373, and for Group 3, it was +5.644. These increases was correlated with the increase in the BSFm inclusion level in the diet. The difference in Groups 2 and 3 is biased toward that of fishmeal.

Discussion

There were no significant differences in all parameters of growth performance between Groups 1 and 2, suggesting that substituting fishmeal with BSFm at 50 % does not affect the growth of red seabream. Conversely, a significant growth retardation compared to Group 1 was observed in final BW and SGR in Group 3 that was fed a diet with BSFm at 78 %. This suggests that growth performance becomes lower when an excess amount of BSFm as a replacement of fishmeal is included in red seabream diet. However, this growth retardation does not seem to be due to a physiological disorder, as there were no significant differences in the condition factor, condition factor without viscera, and hepatosomatic index among Groups 1, 2, and 3. Values of feed conversion ratio were also at similar levels. Visual differences in the liver color, body color, feeding behavior, and swimming activity were not observed among all groups throughout the feeding trial. There is a possibility that BSFm, as a feed ingredient, lacks some substances necessary for normal body growth of red seabream.

Three free EAAs produced by in vitro digestion, namely Lys, Arg, and Met, and Tau as well, in Group 3 were significantly lower than Group 1 (Fig.2). This difference in the free amino acid concentrations is one of the possible factors affecting the

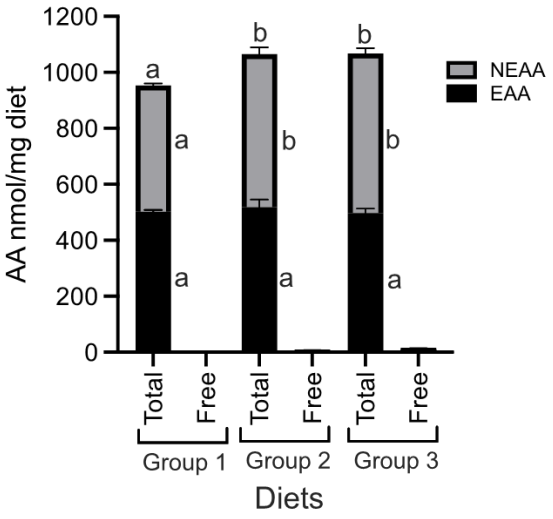

Fig.2 Total essential and non-essential amino acids produced by *in vitro* digestion with a red seabream digestive enzyme complex (EC) in three diets for Groups 1, 2, 3

"Total" indicates the sum of the amount of each amino acid produced by the EC digestion and the amount of free amino acids in each diet. "Free" indicates the amount of free amino acids in each diet. Different letters indicate a significant difference between "Total" amino acids.

Table 3 δ15N (%) of diets used, ingredients and muscle samples of red seabream

Group and ingredient	Diet and ingredient	Muscle	Difference
Initial		+14.24 ± 0.14[a]	
Group 1	+13.4	+15.49 ± 0.11[b]	2.094 ± 0.106[a]
Group 2	+8.7	+13.07 ± 0.13[c]	4.373 ± 0.128[b]
Group 3	+5.9	+11.54 ± 0.21[d]	5.644 ± 0.206[c]
Fishmeal	+15.1		
BSFm	+3.6		
Wheat gluten	+3.1		

Values are means ± SD. Values with a different letter indicate a significant difference ($p < 0.05$).

growth retardation in Group 3. In particular, Lys and Arg along with Leu are the top three EAAs that constitute the body protein in gilthead seabream (*Sparus auratus*, Kaushik 1998), a species phylogenetically close to red seabream. Deficiencies of Lys and Arg could directly affect body mass growth. Taurine deficiency is also a potential factor for the growth retardation, but no symptoms of taurine deficiency were observed in Group 3.

Compared to Group 1 fed the diet without BSFm, difference in the δ15N ratios between the diet and its corresponding muscle of seabreams increased significantly in Groups 2 and 3, which were fed diets containing BSFm,. The δ15N ratio generally increases in fish body compared to its feed, as incorporated 15N in nutrients is concentrated in fish body through metabolism (Gamboa-Delgado 2022). The difference in the δ15N ratio between the diet and muscle in Group 3 was highest (5.644), and more biased toward the δ15N ratio of fishmeal. This suggests that the assimilation of nitrogen from BSFm in the diet was inferior to that from fishmeal in Group 3. This finding is consistent with the hypothesis that the deficiency of three EAAs, namely Lys, Arg, and Met, is a causative factor in the growth retardation of seabreams fed the diet with the highest BSFm level.

In conclusion, it was demonstrated that substituting dietary fishmeal with BSFm at 50% did not affect the growth of red seabream. However, a significant growth retardation was observed when fish were fed a diet with BSFm at 78% due probably to the deficiency in certain essential amino acids. Therefore, further improvements in the nutritional quality are needed for diets with higher inclusion levels of BSFm.

Acknowledgements

Ms. T. Yamawaki, Ms. M. Momosaki and Ms. Kuniyoshi helped us during the sampling and measuring works. This work is a part of Andoh *et al*. (2025) and was supported by the Cabinet Office, Government of Japan, Moonshot Research and Development Program for Agriculture, Forestry and Fisheries (funding agency: Bio-oriented Technology Research Advancement Institution).

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Annotated Bibliography of Key Works

(1) Andoh T, Yasuike M, Ishihara K, Fujiwara A (2023) Effects of heating duration on the digestibility of fish protein powders at 99°C *in vitro* using yellowtail *Seriola quinqueradiata* digestive enzymes. *Fish. Sci.*, **89**, 671–685.

The effects of heating duration on the digestibility of the muscle powder of three fish (pollock [WP], yellowtail [YT], and mackerel [CM]) were assessed *in vitro* using a digestive enzyme complex (EC) extracted from yellowtail pyloric caeca. This research will contribute to the establishment of optimum feed processing conditions for yellowtail. The powder samples were heated at 99°C for 0, 3, 20, 72, and 240 min, followed by hydrolysis with the EC to assess the heating effects. After 3 min of heating WP, YT, and CM, the average production of essential amino acids (EAAs) by the EC digestion decreased to 81.3%, 72.0%, and 66.9%, respectively, compared to the non-heated controls. The production further decreased with the increase of heating duration from 3 to 240 min, although most of the decrease occurred within the first 3 min in WP, YT, and CM.

(2) Takakuwa F, Tanabe R, Nomura S, Inui T, Yamada S, Biswas A, Tanaka H (2022) Availability of black soldier fly meal as an alternative protein source to fishmeal in red sea bream (*Pagrus major*, Temminck & Schlegel) fingerling diets. *Aquacult. Res.*, **53**, 36–49.

The authors replaced fishmeal (FM) in red sea bream (*Pagrus major*) diets with black soldier fly meal (BSFM) to investigate the effects of the diets on growth and feed utilization. Six isonitrogenous and isolipidic experimental diets were prepared by substituting 0%, 20%, 40%, 60%, 80%, and 100% of FM protein with BSFM (control, BSFM20, BSFM40, BSFM60, BSFM80, and BSFM100, respectively). After an eight-week feeding trial, final body weight, weight gain, specific growth rate, and feed efficiency decreased linearly with increasing BM levels ($p < 0.05$). The results suggest that BSFM can replace a maximum of 41.7% of FM in the red sea bream diet without compromising growth performance and feed efficiency for 56 days.

(3) Ido A, Ali MFZ, Takahashi T, Miura C, Miura T (2021) Growth of yellowtail (*Seriola quinqueradiata*) fed on a diet including partially or completely defatted black soldier fly (*Hermetia illucens*) larvae meal. *Insects*, **12**, 722. doi.org/10.3390/insects12080722

Yellowtail, the most popular farmed fish in Japan, is carnivorous; its diet requires a high proportion of fishmeal (FM). This study represents the first example of yellowtail fed on a diet including black solder fly (BSF) meal as an FM replacement. Partially defatted BSF meal (PDBSFM) comprised 49.0% crude protein and 23.2% crude fat, while completely defatted BSF meal (CDBSFM) that was obtained by defatting PDBSFM using hexane, achieved less than 10% crude fat, a level identical to that of FM. In their feeding trials, fish growth performances were reduced with the increase of both PDBSFM and CDBSFM. Therefore, even 10% of BSF meal inclusion, irrespective of the defatting methods, inhibited the juvenile yellowtail growth.

水研機構研報，第 53 号, 37 – 44，令和 8 年
Bull. Jap. Fish. Edu. Agen. No. 53, 37-44, 2026

Omics analysis of red seabream (*Pagrus major*) fed a soybean meal-based diet

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Abstract: In response to the increasing demand for seafood, aquaculture production has been expanding worldwide in recent years. On the other hand, the high demand and unstable supply of fishmeal (FM) has made feed manufacturers difficult to use FM as the main ingredient in aquaculture feeds. Soybean meal (SBM) is one of the most promising substitutes for FM in aquaculture feeds because of its economic advantages and nutritional properties. Although SBM has a relatively high protein content and balanced amino acid composition, it has been reported that feeding of a large amount of SBM has adverse effects on the physiology and growth of fish. To facilitate the development of more effective aquaculture feeds containing SBM, it is required to elucidate the effects of dietary SBM on fish nutritional metabolism in detail. In this study, comprehensive approaches, i.e., transcriptomics and metabolomics analyses, were employed to investigate the effects of SBM on nutritional metabolism in red seabream (*Pagrus major*). Red seabream fingerlings were fed an SBM-based diet (SBMD) and FM-based control diet (FMD) for eight weeks and the hepatopancreas collected at 2, 4, and 8 weeks were used for transcriptome analysis. Growth retardation, and several abnormal physiological conditions such as lower serum cholesterol content and tissue degeneration in the distal intestine, were observed in the SBMD group. Transcriptome analysis (RNA-Seq) identified differentially expressed genes between the SBMD and FMD groups and showed increases in the expression levels of genes involved in the terpenoid and steroid biosynthesis pathways in the former group, suggesting that hepatic cholesterol biosynthesis was up-regulated in the SBMD group. The hepatopancreas of fish fed each diet for 8 weeks was also subjected to metabolome analysis and the results, coupled with the transcriptome analysis, suggest that SBMD affects the glutathione and glycine metabolism. Additionally, transcriptome of the distal intestine at 8 weeks revealed the changes in the expression levels of genes related to the cell cycle control and cholesterol metabolism. This study provides valuable information that enhances the understanding of the effects of dietary SBM on red seabream metabolism, which will lead to the development of a novel technology that relieves the abnormal physiological conditions. Findings obtained from omics analysis could be used to identify the indices of negative effects caused by respective alternative ingredients and thereby lead to the improvement of the quality of future aquaculture feeds.

Key words: *Pagrus major*, transcriptome, metabolome, soybean meal

Accepted on Feb 26, 2026
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Introduction

In recent years, there has been a global trend to reduce the use of fishmeal (FM) in aquaculture feeds. One of the reasons is the increasing demand and price of FM along with the worldwide expansion of aquaculture production (FAO 2020). FM has been used as the main protein source in aquaculture feeds for various fish species (Olsen and Hasan 2012), and aquaculture feeds are still heavily relying on FM. This situation leads to further increase in the demand and price of FM, which has been a serious problem in the aquaculture industry. Additionally, as far as FM production depends on natural fish stock, unstable and limited supply of FM will continue to be inevitable for the aquaculture industry (Olsen and Hasan 2012). Increasing demand of natural fish stock for human foods should also be considered (Naylor *et al*. 2000).

To reduce the use of FM in aquaculture feeds, various ingredients are proposed as FM substitutes (Hussain *et al*. 2024). Protein sources originated from plant, such as corn and soybean, have already been used practically in aquaculture feeds. Animal proteins from livestock by-products are also included in some cases. Poultry by-product meal, meat and bone meal, blood meal and feather meal are representative animal ingredients. However, their use in aquaculture feeds is limited due largely to sanitary and ethical concerns. As novel ingredients from single cell proteins derived from bacteria, yeasts and microalgae, and insect proteins from black soldier fly, meal worms and crickets are still under consideration for aquaculture feeds (Samsing *et al*. 2024; Gao *et al*. 2024; Agboola *et al*. 2021; Alfiko *et al*. 2022). Soybean meal (SBM), a by-product of oil extraction from soybean, is one of the most promising ingredients as a substitute for FM (Gatlin *et al*. 2007). The advantages of SBM are its relatively low price, high availability, and favorable protein content. However, SBM has some issues to be considered for use as a main protein source in aquaculture feeds: deficiency of nutrients such as methionine and taurine, poor palatability, and presence of several anti-nutritional factors. Negative effects of SBM on fish physiology such as growth retardation, tissue degeneration, disfunction of digestive organs, and bile acid depletion in the gallbladder of salmonids have been reported (Romarheim *et al.* 2008; van den Ingh *et al*. 1991; Yamamoto *et al*. 2007). In red seabream *Pagrus major*, Murashita *et al*. (2018) reported that an SBM based diet caused delayed growth, lower gallbladder weight, lower activity and gene expression of digestive enzyme. To facilitate the development of more practical aquaculture feeds comprising of FM substitutes, it is required to investigate the effects of respective ingredients on fish nutritional metabolism in detail.

In fish physiological and nutritional studies, omics analysis could provide various novel insights (Wang *et al*. 2022; Nazari *et al*. 2021; Schock *et al*. 2012). For example, a transcriptome study suggests that dietary eicosapentaenoic acid and docosahexaenoic acid levels influence the expression levels of genes related to lipid and glucose metabolism, redox homeostasis and immune function in Atlantic salmon *Salmo salar* (Xue *et al*. 2020). Another trans-omics study using transcriptome and metabolome analyses in leopard coral grouper *Plectropomus leopardus* suggests that branched-chain amino acids are used for energy supply in fasting, and circadian changes of these metabolic processes were observed (Mekuchi *et al*. 2017). In the present study, to obtain deeper insights into the physiological effects of dietary SBM, we performed the histological and omics analyses in red seabream, an important cultured species in Japan (Yoshinaga *et al*. 2023).

Materials and methods

One hundred and five red seabream fingerlings (initial body weight, 15.7 ± 0.8 g), purchased from a local hatchery, were introduced into each of six 500 L tanks (3 replicates per diet). An FM-based diet (FMD) and SBM-based diet (SBMD) were prepared and fed to the fish twice daily for 8 weeks at 23.4 ± 0.9°C. At start, 1, 2, 3, 4, 6, and 8 weeks, samples for histological and other biological analyses were collected. The hepatopancreas samples were taken for transcriptome analysis (RNA-Seq) at 2, 4 and 8 weeks, and for metabolome analyses at 4 and 8 weeks. The feeding condition, method of histological observation, and procedures of transcriptome and metabolome analyses of the hepatopancreas were described in Yoshinaga *et al*. (2023). The distal intestinal samples (only at 8 weeks) were also collected for transcriptome analysis. Total RNA was extracted from the distal intestinal sample using a commercial kit (RNeasy mini kit, Qiagen) and RNA-Seq was performed at Novogene Co., Ltd. (Beijing, China) for the transcriptome analysis. The RNA-Seq reads were mapped to the red seabream reference genome and analyzed using the same method as described in Yoshinaga *et al*. (2023).

Results and discussion

Effects of SBMD on physiology and histology in red seabream

Compared with the FMD group, the body weight of the

SBMD group had already been significantly lower at 2 weeks and greatly retarded after 6 weeks. Specific growth rate and feed efficiency also significantly decreased after 6 weeks. In the SBMD group, the hepatosomatic index, serum cholesterol concentration, and biliary bile acid concentration became significantly lower at 1 to 3 weeks, suggesting that the fish physiology had already been damaged in early half of the experimental term (Yoshinaga *et al*. 2023). The hepatocytes were atrophied in the SBMD group from 1 week. Matsunari *et al*. (2015) also reported that an SBM based diet caused growth retardation and histological degeneration of the hepatopancreas in red seabream, and the negative effects on growth and hepatopancreas histology were relieved by replacing SBM with soy protein concentrate and ethanol-washed soy protein isolate. These results suggest that the ethanol-soluble fraction of SBM is causative of the physiological disorder in red seabream.

The relationship between SBM intake and histological change of the distal intestine was reported in several fish species, such as Atlantic salmon (Uran *et al*. 2009), rainbow trout *Oncorhynchus mykiss* (Romarheim *et al*. 2008; Iwashita *et al*. 2008) and gilthead seabream *Sparus aurata* (Bonaldo *et al*. 2008). We also confirmed that the distal intestine of red seabream fed SBMD had morphological changes. The distal intestine of fish in the FMD group showed normal features throughout the experimental term: abundant supranuclear vacuoles (SNV), well-developed microvilli, and scattered goblet cells (Fig.1). In contrast, the SBMD group showed a decrease of SNV from 1 week onward and showed severe abnormality at 6 and 8 weeks: disappearance of SNV and microvilli, and decrease of goblet cells. Iwashita *et al*. (2007) reported that saponin and other ANFs contained in SBM caused the liver and distal intestinal histological changes in rainbow trout. Moreover, deterioration caused by a prolonged period of feeding an SBM based diet was reported in Atlantic salmon; the higher the inclusion level of dietary SBM and the longer the experimental period, the severer the histological condition in the distal intestine was damaged (Uran *et al*. 2009).

Analysis of transcriptome and metabolome in the hepatopancreas

Transcriptome analysis of red seabream hepatopancreas was conducted at 2, 4, and 8 weeks after the start of SBMD feeding. The sequence data were used to detect differentially expressed genes (DEGs), which showed significant increase or decrease in the SBMD group compared with the FMD group. Then, 454, 353, and 566 DEGs at 2, 4, and 8 weeks, respectively, were subjected to enrichment analysis to identify the metabolic

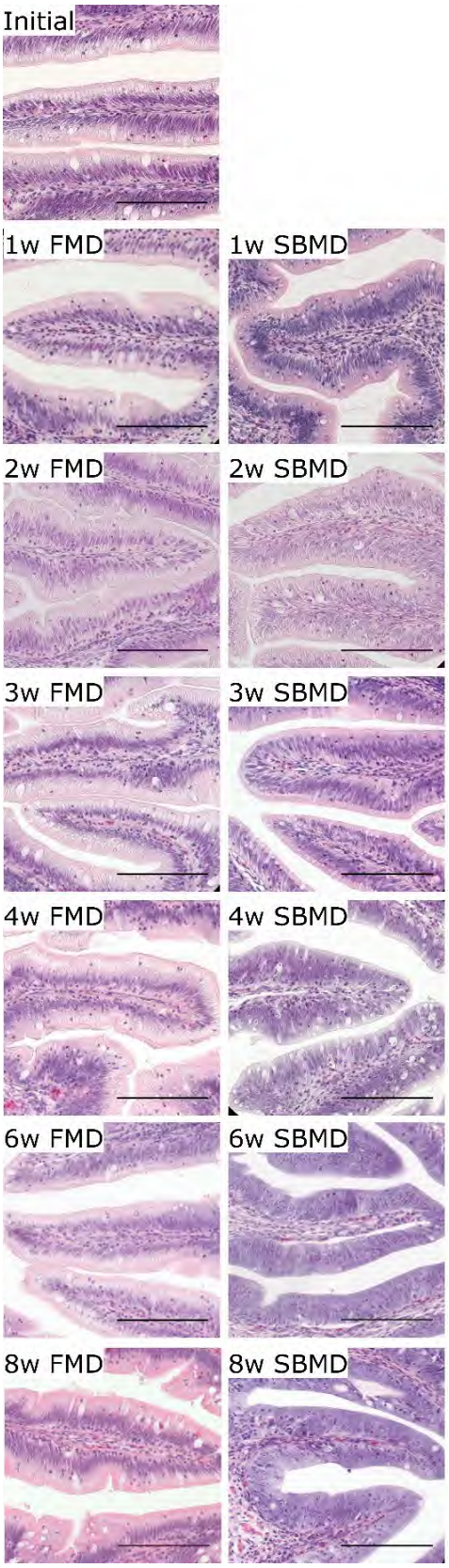

Fig.1 Morphology of the distal intestine of red seabream fed a fish meal diet (FMD) and soybean meal diet (SBMD) for 8 weeks

Scale bars represent 50 μm.

process affected by the diets. The enrichment analysis of DEGs revealed that the genes involved in steroid metabolic process and isoprenoid biosynthetic process were significantly abundant in the SBMD group throughout the feeding trial (Yoshinaga *et al*. 2023). Fig.2 shows the changes of expression levels of genes related to the cholesterol biosynthesis in the hepatopancreas of the SBMD group. The results suggest that biosynthesis of cholesterol is promoted by SBMD. Our result coincides with several previous studies that suggest that SBM intake affects the cholesterol metabolism in fish (Zhu *et al*. 2018: Kortner *et al*. 2013; Kemski *et al*. 2020; Takagi *et al*. 2002). Cholesterol plays a variety of biological roles, such as a constitution in the biological membrane, hormone biosynthesis, and bile acid production (Babin and Vernier 1989; Sheridan 1988). SBM-induced abnormality in fish may result from the changes in the cholesterol metabolic process.

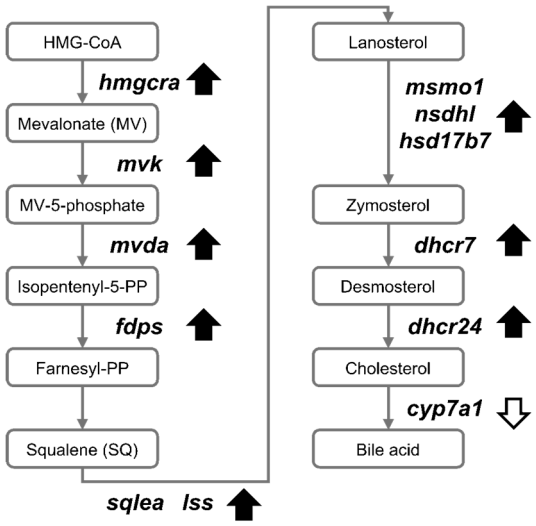

Fig.2 Expression levels of genes related to cholesterol metabolism in the hepatopancreas of red seabream fed a soybean meal diet

Black and white arrows represent the up-regulated and down-regulated genes, respectively. This figure is referenced from Yoshinaga *et al*. (2023).

In the metabolome analysis, the metabolites that showed quantitatively significant changes between diet groups were selected and subjected to the pathway analysis. The results of metabolome analysis, taken together with those of the transcriptome analysis, revealed significant changes in the glycine, serine, and threonine metabolism. Furthermore, metabolism of glutathione, which is a metabolite related to the oxidative stress response in fish (Sukhovskaya *et al*. 2017), was also affected. These results imply that SBMD induced oxidative stress in red seabream and impaired the stress response, which might have resulted in the growth retardation.

Analysis of transcriptome in the distal intestine

The distal intestinal samples of red seabream fed SBMD for 8 weeks were also used for transcriptome analysis. The raw read datasets were deposited in the DDBJ sequence Read Archive under accession nos. DRR656721-DRR656732 and

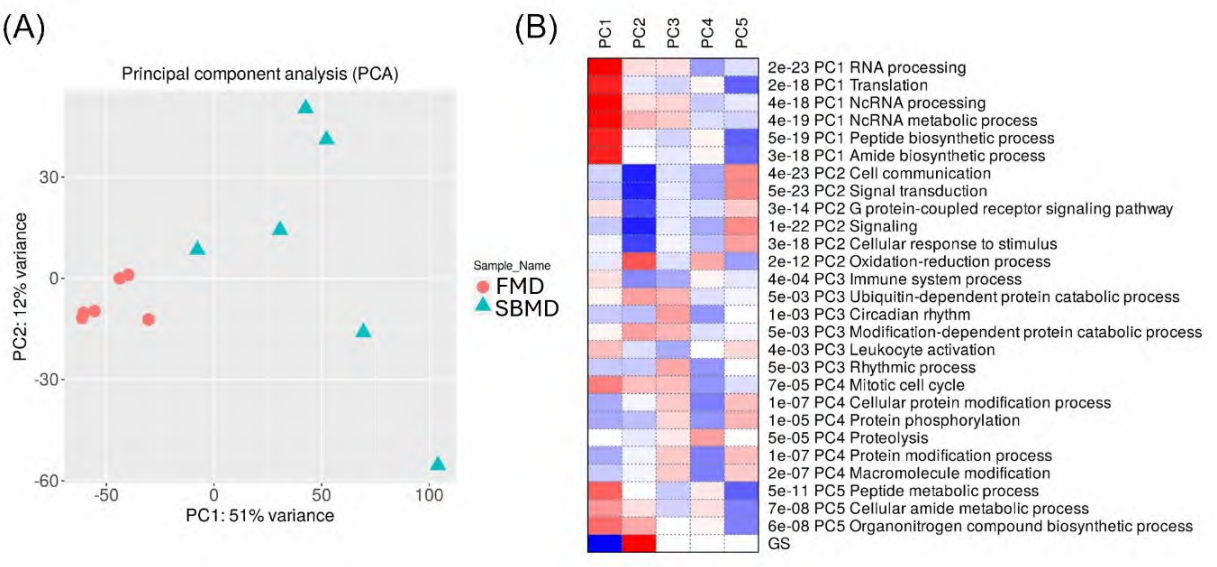

Fig.3 Results of principal component analysis of the transcriptome in the distal intestine of red seabream fed a soybean meal diet

(A), score plot in principal component (PC) 1 and 2; (B), loading factor.

the result of principal component analysis is shown in Fig.3. Compared with the FMD group, 1629 and 1228 genes were identified as up-regulated and down-regulated DEGs at 8 weeks, respectively, and these DEGs were subjected to enrichment analysis (Fig.4). Several genes related to cholesterol metabolism, such as methylsterol monooxygenase 1 (*msmo1*), hydroxysteroid 17-beta dehydrogenase 7 (*hsd17b7*), NAD (P) dependent steroid dehydrogenase like (*nsdhl*), squalene epoxidase a (*sqlea*) and lanosterol synthase (*lss*), were also up-regulated as found in the hepatopancreas. The genes related to cellular lipid metabolism, cellular catabolic process, and lysosomal function were down-regulated. The expression levels of genes involved in DNA replication including MCM complex, ORCs, and cyclin-dependent kinases, increased significantly. At the same time, expressions of genes related to cell cycle regulation, such as DNA-dependent protein kinase, cyclin-dependent kinase inhibitor 1, and 14-3-3 protein, were up-regulated in the SBMD group. In the eukaryotic cells, the cell cycle is regulated by the synthesis and degradation of cyclins and CDKs (Alberts *et al*. 2008), and the progression of cell cycle is controlled by monitoring the cell environment and DNA replication status. Previous studies suggested that intake of SBM-based diets affects the cell cycle and cell stress in the intestinal tract of Atlantic salmon (Sanden *et al*. 2005; Bakke-McKellep *et al*. 2007). These findings imply that SBM promotes the cell division for regeneration of the distal intestinal enterocytes, but meanwhile the cell division is halted due to the DNA damage. The changes observed in the transcriptome in the distal intestine of red seabream fed SBMD could partly be attributed to the growth retardation.

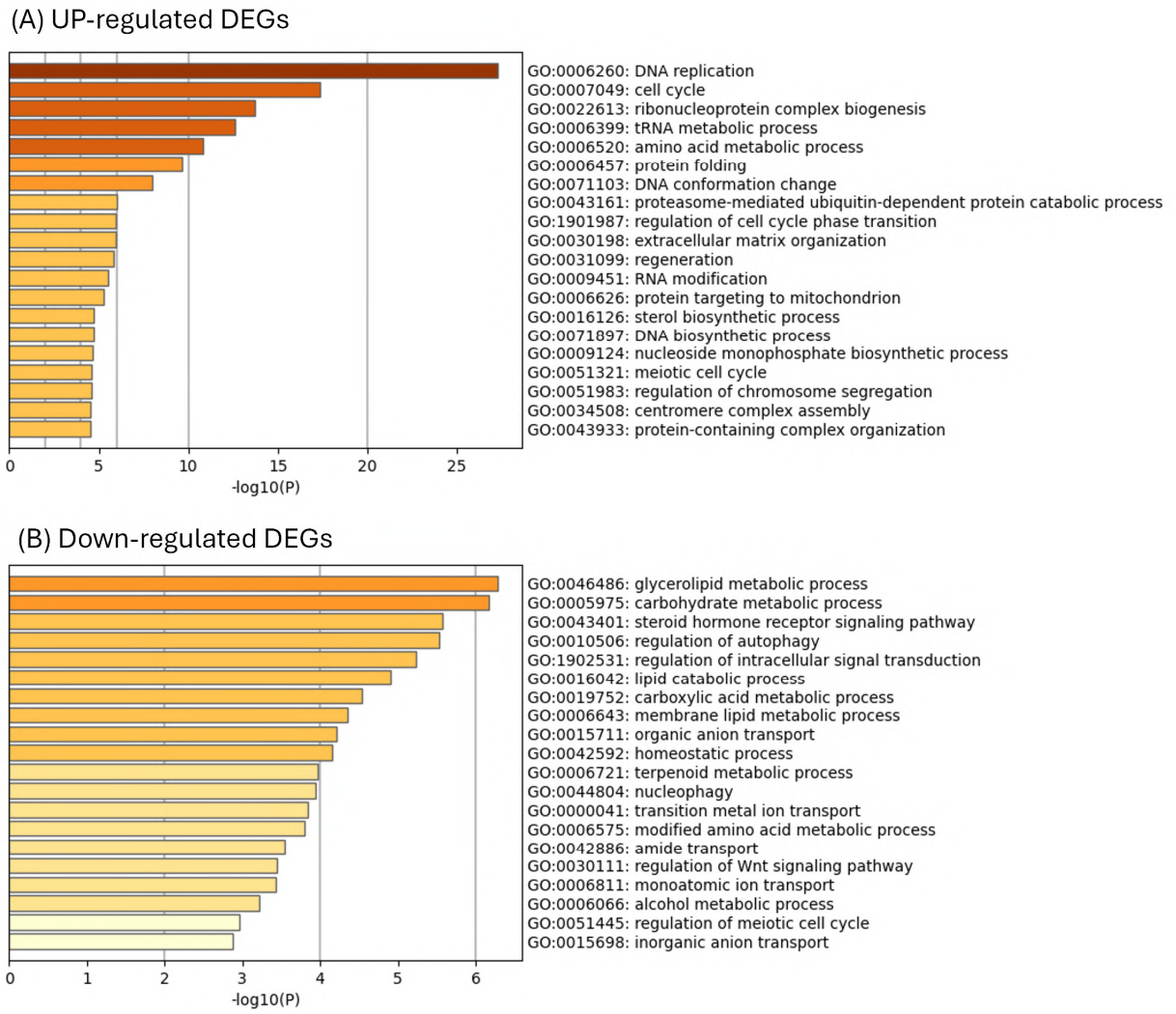

Fig.4 Heatmaps of enriched process and pathway (gene ontology term related to biological process) in the DEGs of the distal intestine of red seabream

DEGs were detected between soybean meal diet group and fish meal diet group. Up-regulated DEGs (A) and down-regulated DEGs (B) in the soybean meal diet group compared with the fishmeal diet group were used for the analysis.

Evaluation of feed ingredients by omics analysis

This study has shown that SBMD gradually induced various physiological changes; hepatocyte atrophy, degeneration of the distal intestine, decreases of serum cholesterol and biliary bile acid concentrations gradually progressed in red seabream fed SBMD. Additionally, transcriptome and metabolome analyses also imply that SBMD affected the metabolic processes, such as enhancement of cholesterol biosynthesis and abnormality of oxidative stress response. These results obtained through the omics analysis revealed the effects of a particular ingredient on the metabolism of a given fish, and will contribute to the development of a new technology that relieves the soybean-induced abnormal physiology. Moreover, as demonstrated in this study, omics analyses will help us find out suitable indices to evaluate the physiological effects of novel feed ingredients.

Acknowledgements

The work was supported by the Fisheries Agency of Japan (research project for transformation of the aquaculture industry into a growth industry).

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Annotated Bibliography of Key Works

(1) Lu F, Haga Y, Satoh S (2015) Effects of replacing fish meal with rendered animal protein and plant protein sources on growth response, biological indices and amino acid availability for rainbow trout *Oncorhynchus mykiss*. *Fish. Sci.*, **81**, 95-105.

In this study, rainbow trout were fed six diets, a fish meal diet and 5 other diets in which fishmeal was replaced by one or in combination of poultry by-product meal, feather meal, blood meal, soybean meal and corn gluten meal. Growth performance was assessed by a feeding trial of 12 weeks. The result of biological indices and amino acid availability showed that a combination of some protein sources could effectively be substituted for fishmeal. This report shows the necessity of using multiple alternative protein sources instead of a single

protein source as a substitute for fishmeal.

(2) Murashita K, Matsunari H, Furuita H, Rønnestad I, Oku H, Yamamoto T (2018) Effects of dietary soybean meal on the digestive physiology of red seabream *Pagrus major*. *Aquaculture*, **493**, 219-228.

The authors reported acute and chronic effects of dietary soybean meal on the digestive physiology of red seabream. Red seabream were fed fishmeal and soybean meal-based diets, and the authors investigated the gastric transit, digestive enzyme activity and expression levels of genes encoding digestive enzymes. In the acute trial, trypsin activity in the intestinal content was lower, suggesting that the soybean meal diet reduces the trypsin secretion. In the chronic trial, in addition to the growth retardation, changes in the gastric transit rate, lower gallbladder weight, and changes in the expression levels of hepatopancreatic enzyme genes were observed in fish fed the soybean meal diet. This paper is one of the reports that clearly shows the SBM effects on fish nutritional physiology in detail.

(3) Roques S, Deborde C, Richard N, Skiba-Cassy S, Moing A, Fauconneau B (2020) Metabolomics and fish nutrition: a review in the context of sustainable feed development. *Rev. Aquacult.*, **12**, 261-282.

The authors reviewed the usefulness of metabolomic approaches for the development of aquaculture feeds. It is crucial for aquaculture sectors to improve the quality of aquaculture feeds using plant resources and other raw materials as substitutes for fish meal and fish oil. In this review, previous studies related to fish nutritional metabolism using metabolomic analysis are summarized. Appropriate sample for metabolome analysis, future applications of metabolomics in fish nutritional study and development of aquaculture feed, evaluation of fish fillet quality, and non-invasive monitoring of fish state were discussed.

(4) Yoshinaga H, Yasuike M, Mekuchi M, Soma S, Yamamoto T, Murashita K, Matsunari H, Oku H, Furuita H (2023) Multi-omics analysis of hepatopancreas of red seabream (*Pagrus major*) fed a soybean meal-based diet. *Aquaculture*, **574**, 739631.

The authors analyzed the hepatopancreatic transcriptome and metabolome in red seabream fed a soybean meal diet for 8 weeks. Fish fed the diet showed delayed growth and physiological abnormalities relative to fish fed a fishmeal-based diet. The results of omics analyses suggest the soybean meal diet affects the metabolism of cholesterol, glutathione, and glycine.

水研機構研報, 第 53 号, 45 – 54, 令和 8 年
Bull. Jap. Fish. Edu. Agen. No. 53, 45 - 54, 2026

Frass from black soldier fly larvae as an aquafeed ingredient: Nutritional value and potential health benefits

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Abstract: Insects have gained global research interest as potential protein sources, increasing insect production to meet the rising demand for protein. The insect meal production produces a considerable amount of frass, which consists of waste produced by larvae, leftover food, and exoskeleton remnants. Two separate trials were conducted to explore the nutritional value of frass generated from black soldier fly larvae, *Hermetia illucens* fed dried distillers' grains for channel catfish, *Ictalurus punctatus*, and hybrid tilapia, Nile x Mozambique (*Oreochromis niloticus* x *O. mossambicus*. Our study aimed to evaluate the impact of adding various levels of frass to fish diets on overall performance and health. Five isocaloric practical-type diets containing frass at 0% to 30% were formulated. Dietary frass served as a partial replacement for a combination of soybean meal, wheat shorts, and corn meal, all on an equal protein basis. The diets were fed to catfish (average weight 5.24 ± 0.04 g) and tilapia (average weight 2.6 ± 0.035 g) over 10 weeks. Each treatment group included four replicate tanks, with 50 fish per tank. After that, fish were evaluated for growth, feed utilization, body proximate, and liver mineral composition. Representative fish were bled for hematological and serological assays and challenged to determine the effects of dietary frass on disease resistance. Final weight gain significantly increased in catfish fed diets containing frass at levels from 10% to 30%. Feed intake of catfish increased with increasing dietary levels of frass. Feed and protein efficiencies, however, were significantly lower in catfish fed frass at levels of 20% and higher compared to the control diet. On the other hand, final weight gain significantly increased in tilapia fed the diet containing the highest level of frass (30%). Tilapia fed diets containing frass (5% to 30%) had significantly higher protein efficiency ratio than the control group. Dietary treatments did not significantly affect feed intake and utilization efficiency of tilapia. Survival, whole-body composition, and mineral content of both fish species were not affected by frass. In catfish, hematological parameters (red blood cell count, hemoglobin, and hematocrit) were improved with the inclusion of frass. Hematological parameters and serum biochemistry of tilapia were unaffected by dietary treatment. In both species, dietary frass enhanced serum alternative complement activity and improved disease resistance from fish pathogenic bacteria. Diets containing frass at levels 20% or more showed significantly higher survival rates against *Flavobacterium covae* than that of control catfish or fish on diets with lower levels of frass. Tilapia fed the diets containing frass showed significant dose-dependent trends in survival against both *F. covae* and *Streptococcus iniae* challenges. Insect frass can be utilized as a feed ingredient to enhance feed palatability, promote the growth of channel catfish and hybrid tilapia, and improve fish's overall resistance to pathogenic bacteria.

Key words: insect larvae frass, alternative feed ingredient, aquaculture nutrition

Accepted on Feb 26, 2026
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Introduction

The growing global population is increasing the demand for animal protein while also generating more waste (Henchion *et al*. 2017). Animal production plays a significant role in this waste generation and resource exploitation. In the United States, farm-raised channel catfish, *Ictalurus punctatus*, represents the largest aquaculture industry, contributing about 75 % of the finfish aquaculture (FAO 2022; Hegde *et al*. 2021). Tilapia is also one of the most widely cultured species globally, with a global annual production of 6,100,719 t in 2020 (FAO 2022). To keep up with the growth of the aquaculture sector, a well-balanced prepared feed is crucial for achieving high yields and promoting rapid growth at the lowest possible cost (Manam 2023).

To promote sustainable and efficient animal nutrition, insect farming is gaining recognition as a method to mitigate the environmental impacts associated with animal feed production. Fly larvae can be produced economically from low-quality substrates, such as various sources of organic waste, and can be incorporated as a primary protein source in animal feed (Gasco *et al*. 2023; Sogari *et al*. 2019). These characteristics make insects an ideal novel protein source for animal feed formulations while reducing the burden of waste management, promoting a more circular economy, and improving global food security (Diener *et al*. 2009; van Huis *et al*. 2013; Makkar *et al*. 2014). Recent studies indicate that insect-based protein meals can serve as a more sustainable alternative to conventional protein sources, such as fish and plant protein meals, in aquaculture (Prakoso *et al*. 2022; Nunes *et al*. 2023; Islam *et al*. 2024). However, the production of insects results in significant amounts of processing residue, known as “frass” (Gasco *et al*. 2023). Frass, which consists of larval feces, residual larval excrement, and undigested remnants of the substrates, is rich in nutrients (Ravi *et al*. 2020).

Frass is regarded as a biofertilizer that can be used as an alternative to conventional fertilizers for various purposes. It can enhance plant growth and resistance, stimulate seed germination, increase drought and stress tolerance in plants, and support soil microbial communities (Schmitt and de Vries 2020; Barragán-Fonseca *et al*. 2022). Due to its protein, lipid, and bioactive components, along with beneficial microbes (Lopes *et al*. 2022; Mudalungu *et al*. 2021), frass has the potential to serve as an immunomodulatory functional feed ingredient. Thus, the objective of this study was to evaluate the effects of incorporating frass from black soldier fly larvae, *Hermetia illucens*, into the diet at varying levels on the growth, feed utilization, body composition, hematology, serum biochemistry, immune response, and disease resistance of channel catfish and hybrid tilapia.

Material and Methods

Experimental diets, fish, feeding, and sampling

A nutritionally complete practical basal diet was formulated to contain approximately 31.5 % crude protein and 6.2% lipid, based on feedstuff values reported in the NRC (1993) (Table 1). Five experimental diets were prepared, incorporating frass (0 %, 5 %, 10 %, 20 %, and 30 %) as partial replacements for a combination of soybean meal, wheat shorts, and corn meal, all on an equal protein basis. The frass used in the study was sourced from black soldier flies (*H. illucens*) larvae fed dried distiller's grains and provided by EnviroFlight LLC (Yellow Springs, OH, USA).

Channel catfish (*I. punctatus*) with an average weight of 5.24 ± 0.04 g and hybrid tilapia (Nile x Mozambique, *Oreochromis niloticus* x *O. mossambicus*) fingerlings, averaging 2.6 ± 0.035 g, were randomly stocked into 20 aquaria, each with a volume of 110 L, at a density of 50 fish per aquarium. The aquaria were supplied with flow-through dechlorinated city water, heated to 28°C, and maintained a flow rate of about 0.7 L/min. Each diet was randomly assigned to four aquaria and fed for 10 weeks. Daily feed consumption was recorded. Fish in each aquarium were group-weighed and counted at two-week intervals.

Tissue and blood sampling

At the conclusion of the growth trial, livers from four fish per tank were collected, pooled, and stored at -80°C for subsequent mineral content analysis. The mineral content of the experimental feed and liver samples was measured using a mineral panel analyzed by inductively coupled plasma mass spectrometry (ICP-MS).

At the end of the feeding period, three fish from each tank were anesthetized using tricaine methanesulfonate (MS-222) at 150 mg/L. Blood samples were collected using dried heparinized tuberculin syringes (100 IU) for hematological assays, which included red and white blood cell counts (RBC and WBC), hemoglobin (Hb), and hematocrit (Ht), as described by Yildirim-Aksoy *et al*. (2007). An additional set of fish (4 fish/tank) were bled using non-heparinized tuberculin syringes and allowed to clot and serum samples were collected following centrifugation for the determination of innate immune responses. Lysozyme activity and serum

Table 1 Percentage composition and determined nutrient content of experimental diets

	Experimental diets (%) [1]				
	1	2	3	4	5
Menhaden fish meal	8	8	8	8	8
Soybean meal	45	44	43	41	39
Frass	--	5	10	20	30
Wheat short	24	20.4	16.9	9.8	2.5
Corn meal	14	13.8	13.5	13	12.8
Corn oil	4	3.8	3.6	3.2	2.8
Dicalcium phosphate	1	1	1	1	1
CMC	3	3	3	3	3
Vitamin premix [2]	0.5	0.5	0.5	0.5	0.5
Mineral premix [3]	0.5	0.5	0.5	0.5	0.5
Proximate composition (%) -catfish					
Dry matter	91.68	91.68	91.85	91.78	91.56
Protein	31.80	31.28	31.77	31.83	31.51
Lipid	6.25	6.15	6.33	6.11	6.01
Ash	7.20	7.32	7.74	8.26	8.81
Proximate composition (%) -tilapia					
Dry matter	91.68	91.68	91.85	91.78	91.56
Protein	31.60	31.00	31.20	31.10	31.00
Lipid	5.28	5.52	5.71	5.56	5.49
Ash	6.80	6.97	7.16	7.39	7.68

CMC, carboxymethyl cellulose; Frass is by-product of black soldier fly (*H. illucens*) larva meal industry.
[1] Diets 1, 2, 3, 4, and 5 contained 0, 5, 10, 20, and 30% frass, respectively.
[2] Vitamin premix, diluted in cellulose, provided by following vitamins (mg/kg diet): vitamin A (520,400 IU/g), 7.7; vitamin D3 (108,300 IU/g), 18.5; vitamin E (250 IU/g), 200; vitamin K, 10; thiamin, 10; riboflavin, 12; pyridoxine, 10; calcium pantothenate, 32; nicotinic acid, 80; folic acid, 2; vitamin B12, 0.01; biotin, 0.2; choline chloride, 400; and L-ascorbyl-2-polyphosphate (35% vitamin C activity), 60.
[3] Trace mineral premix provided by following minerals (mg/kg diet): zinc (as $ZnSO_4\_7H_2O$), 150; iron (as $FeSO_4\_7H_2O$), 40; manganese (as $MnSO_4\_7H_2O$), 25; copper (as $CuCl_2$), 3; iodine (as Kl), 5; cobalt (as $CoCl_2\_6H_2O$), 0.05; and selenium (as Na_2SeO_3), 0.09.

natural hemolytic (alternative pathway) complement activity were performed as described in Yildirim-Aksoy *et al*. (2007). Lysozyme activity in serum involved measuring the lytic activity of catfish serum against the bacterium *Micrococcus lysodeikticus* (Sigma, St. Louis, MO, USA). The complement assay relied on the hemolysis of sheep erythrocytes (Remel Inc., Lenexa, KS, USA) by complement present in serum.

Bacterial Challenge

To utilize a common garden approach, at the end of the feeding period, 30 (tilapia) and 15 (catfish) of the remaining fish in each aquarium were tagged with Visible Implant Elastomer (VIE) tags (Northwest Marine Technology, Inc., Shaw Island, WA, USA). The tagged fish were distributed into new aquaria, each housing a total of 30 fish (six fish each from all five dietary treatments, resulting in 30 fish per aquarium for challenge tests).

Then, the tagged catfish or tilapia in the 10 aquaria (totaling 60 fish per dietary treatment) were exposed to predetermined concentrations of *Flavobacterium covae* through immersion at 1 x 10^9 cells/mL and 2 x 10^5 cells/mL, respectively. Another set of tagged tilapia in the 10 aquaria (60 fish/dietary treatment) were injected with a predetermined concentration of *Streptococcus iniae* at 1 x 10^4 cells/ml concentration. During this period, the fish were fed the control diet and monitored twice daily, recording and removing any moribund or dead fish for seven days.

Statistical analysis

Data were analyzed using one-way ANOVA with the general linear model. Comparisons of treatment means were conducted using Tukey's multiple comparison test in GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA). The trends for mean responses across different levels of dietary larval frass, as well as weight gain and feed utilization parameters, were analyzed through orthogonal polynomial contrasts using SAS software version 9.4 (SAS Institute, Cary, NC, USA). A significance level of 0.05 or below was considered statistically significant.

Results

Growth performance, feed utilization, and survival are given in Table 2. Dietary frass at 10% significantly ($p < 0.0026$) increased the weight gain of catfish without affecting feed and protein efficiencies. Weight gain and feed intake were linearly increased, whereas feed efficiency (FER), protein efficiency ratio (PER), and apparent protein utilization (APU) were linearly decreased with increasing levels of dietary frass. Fish fed dietary frass at a level of 20% had the highest weight gain but significantly reduced the FER, PER, and APU. Including frass at a level of 30% significantly enhanced the weight of tilapia. Fish fed diets containing frass (5 % to 30 %) had significantly higher PER than the group fed a diet without frass (control diet). Fish fed diets with frass also showed improved APU, but it was only significantly higher in fish fed the diet with 30% frass than that of the control fish. Weight gain ($p < 0.0048$), PER ($p < 0.0091$), and APU ($p < 0.0395$) linearly increased with increasing levels of dietary larval frass (Table 2).

The liver concentration of iron increased incrementally with increasing concentrations of dietary frass, but the values were significantly higher only at the highest dietary frass level (30 %) (Table 3). The inclusion of frass resulted in the improvement of hematological (red blood cell (RBC) count, hemoglobin, and hematocrit) parameters of catfish (Fig.1). The RBC count of fish fed with a 10 % or higher frass diet was significantly ($p < 0.01$) higher than that of the control fish. Hemoglobin concentration and hematocrit increased at each incremental level of dietary frass and significantly ($p < 0.05$) higher in catfish fed a 30 % frass diet. Dietary levels of frass did not influence the hematological values of tilapia. Serum complement activities increased in both catfish and tilapia fed frass diets (data not shown).

In the challenge tests, catfish that had been fed diets containing 20 % and 30 % frass and tilapia that had received 10 % and 30 % frass demonstrated significantly better survival rates ($p < 0.05$) than those on lower frass levels and the control diet. Additionally, tilapia that had been fed diets with 30 % frass showed improved survival rates when challenged with *S. iniae* (Fig.2). The post-challenge survival rates from both *F. covae* ($R^2 = 0.699$) and *S. iniae* ($R^2 = 0.881$) showed a linear response to the dietary frass levels.

Table 2 Weight gain, feed intake, FER, PER, APU, and survival of channel catfish and hybrid tilapia (Nile x Mozambique) fed diets containing various level of frass for 10 weeks [1]

	Dietary levels of frass (%)					*P*-value	Linear (Pr > F)
	0	5	10	20	30		
Channel catfish							
Weight gain (g)	21.72	22.15	24.34*	25.49**	25.01**	0.0026	0.0002
Feed intake (g)	28.03	30.39*	32.92***	34.86****	35.35****	<0.0001	<0.0000
FER [2]	0.78	0.74	0.74	0.73*	0.71***	0.0017	0.0005
PER [3]	2.44	2.37	2.33	2.30	2.25**	0.0039	0.0002
APU (%) [4]	39.70	37.80	37.20	36.60*	35.60**	0.0039	0.0002
Survival (%)	98.5	97.3	98	99	100	0.3391	0.2105
Hybrid tilapia							
Weight gain (g)	48.97	51.26	51.81	51.88	55.26*	1.265	0.0048
Feed intake (g)	59.81	60.29	60.85	60.58	64.94	1.517	0.0483
FER [2]	0.82	0.85	0.85	0.85	0.85	0.011	0.0579
PER [3]	2.58	2.72*	2.73*	2.73*	2.74*	0.034	0.0091
APU (%) [4]	39.24	41.4	41.23	41.21	41.94*	0.695	0.0395
Survival (%)	92.35	88.78	87.24	93.88	84.69	3.666	0.3978

FER, feed efficiency ratio; PER, protein efficiency ratio; APU, apparent protein utilization.
[1] Values are means of four replicates per treatment. Asterisks indicate significant difference between the control and frass fed groups. Number of asterisks represent degree of statistically significant difference from control: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.
[2] FER = weight gain (g)/dry feed fed (g).
[3] PER = wet weight gain (g)/crude protein fed (g).
[4] APU = 100 x [body protein gain (g)/crude protein fed (g)].

Table 3 Liver mineral analysis (dry matter basis) of channel catfish fed diets containing various levels of frass for 10 weeks [1]

	Dietary levels of frass (%)					*P*-value
	0	5	10	20	30	
Macro minerals (mg g^{-1} dry liver)						
Calcium	0.27	0.28	0.26	0.27	0.27	0.7265
Potassium	8.6	7.98	8.45	8.29	8.53	0.6579
Magnesium	0.58	0.57	0.58	0.54	0.57	0.2036
Phosphorus	7.38	7.52	7.12	7.23	7.35	0.731
Sulfur	0.11	0.11	0.1	0.1	0.11	0.6453
Trace minerals (μg g^{-1} dry liver)						
Manganese	2.9	2.8	3.43	2.98	3.14	0.0826
Copper	18.32	17.35	18.25	16.16	15.75	0.3415
Iron	64.28	69.65	69.71	80.46	96.8*	0.0103
Aluminum	2.73	2.8	2.58	2.19	2.74	0.8243
Zinc	99.05	99.15	98.47	91.73	93.99	0.5754

[1] Values are means of two determinations of pooled liver samples of four fish per tank and four tanks per treatment. Asterisks denote statistically significant differences at $p < 0.05$.

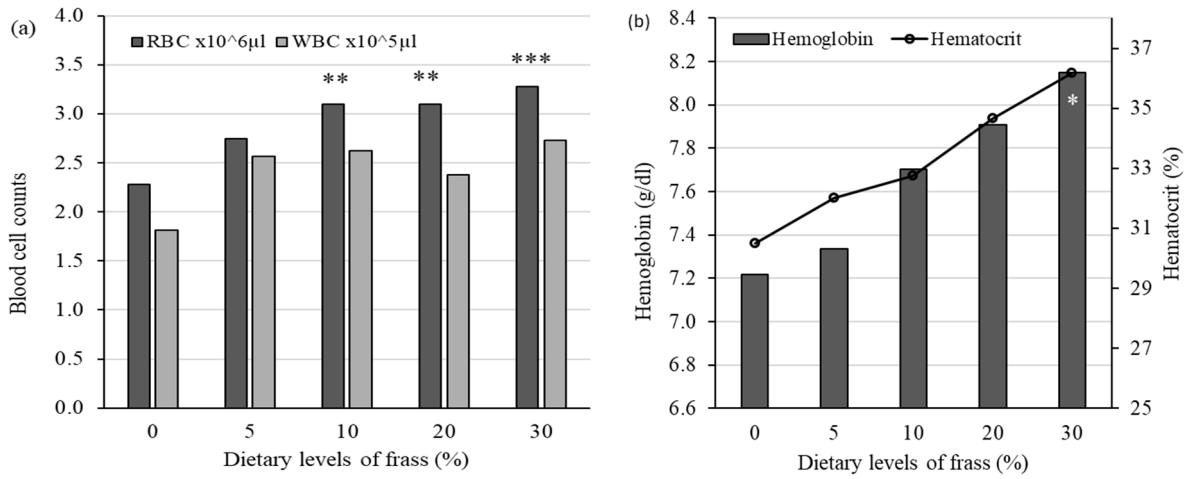

Fig.1 Hematological values of channel catfish fed diets containing various level of frass for 10 weeks

RBC, red blood cell count; WBC, white blood cell count; Hb, hemoglobin; Ht, hematocrit. Values are means of one determination per fish, four fish per tank and four tanks per treatment. Asterisks indicate significant difference between the control and frass fed groups. Number of asterisks represent degree of statistically significant difference from control: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Discussion

Our research indicates that while larval frass was efficiently utilized by both juvenile channel catfish and hybrid tilapia, its effectiveness on catfish performance is limited. After 10 weeks on a diet containing 10% or more frass, the catfish's weight increased significantly. This growth improvement was associated with a significant increase in feed intake. However, increasing the frass content from 20% to 30% did not lead to further growth benefits; it reduced feed and protein efficiency. This indicates that a careful balance is necessary when incorporating frass into fish diets. Similarly, improvements in both catfish and plant growth have been observed in aquaponic systems that used dietary frass at a ratio of 10% (Romano *et al.* 2023). The authors suggested that the increased growth in catfish may be linked to the upregulation of genes associated with growth, a reduction in intestinal inflammation, and a significant enhancement in feed intake.

For tilapia, including frass at a 30% level significantly boosted their weight. Although no significant differences were noted in feed intake or feed efficiency ratio (FER) among fish fed diets with varying frass levels, there was a

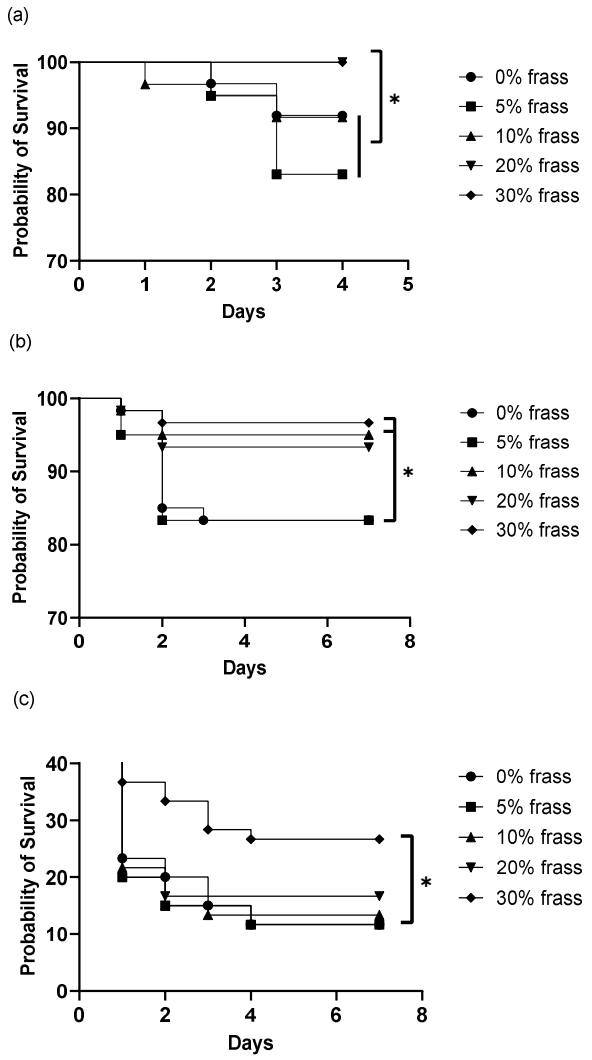

Fig.2 Percent survival of channel catfish challenged with *Flavobacterium covae*, ALG-00-053 (a) and hybrid tilapia (Nile x Mozambique) challenged with *F. covae*, ALG-00-053 (b) and *Streptococcus iniae* #60 (c) previously fed various level of frass for 10 weeks

Values are means of 60 fish per treatment. Asterisks indicate significant difference in the survival curves between the control and frass fed groups at $p < 0.05$.

noticeable trend of improvement with the inclusion of frass. Fish consuming diets with frass (ranging from 5 % to 30 %) displayed significantly higher protein efficiency ratios (PER) compared to the no-frass group. Furthermore, the fish that consumed frass-rich diets showed improved apparent protein utilization (APU), with a significant increase observed in fish fed the 30% frass diet compared to the control group. There were no significant differences in the whole-body proximate composition of catfish or tilapia across the different frass diet levels (5 % to 30 %) (Yildirim-Aksoy *et al*. 2020a, 2020b). Similarly, Romano *et al*. (2024) fed Mozambique tilapia (*O. mossambicus*) a diet consisting of 10% frass from larvae that were fed an expired fish diet (45 % crude protein) over 8 weeks, which significantly enhanced tilapia growth compared to those fed the control diet.

These findings suggest hybrid tilapia seem more efficient than channel catfish in converting ingested frass feed into fish body mass. This increased efficiency may be due to hybrid tilapia's higher chitinase activity, which allows them to better degrade chitin compared to catfish. Removing chitin from insect meal has been shown to improve the digestibility of insect protein (Bonomini *et al*. 2024). This improvement occurs because chitin is a complex matrix of proteins, lipids, and other components. The hydrolysis of chitin enhances the accessibility of digestive enzymes to proteins and lipids, thereby increasing protein digestibility (Bonomini *et al*. 2024). Additionally, the poorer FER observed with higher levels of frass in the catfish diets may be due to the higher ash content of the frass used in catfish diets compared to the tilapia study (Table 1).

Including frass has shown promise as a beneficial dietary supplement, enhancing fish's hematological and innate immune parameters. A significant increase in complement activity was observed in both catfish and tilapia fed diets with frass (Yildirim-Aksoy *et al*. 2020a, 2020b). Additionally, Sankappa *et al*. (2024) reported changes in global gene expression and activation of innate and adaptive immunity in channel catfish that consumed diets containing frass. Furthermore, a diet including 20% frass in Pacific white shrimp (*Litopenaeus vannamei*) significantly increased the serum's ability to inhibit the growth of *Vibrio parahaemolyticus* (Yildirim-Aksoy *et al*. 2022). In yellow catfish (*Pelteobagrus fulvidraco*), enhanced immune responses were also noted when fish meal protein in the diet was substituted with 13-48 % black soldier fly larvae meal protein (Xiao *et al*. 2018). Including insect meal in the Nile tilapia diet improved skin, mucus lysozyme, and peroxidase activities (Tippayadara *et al*. 2021). A related study on red seabream (*Pagrus major*) found that incorporating low doses (0.75 % and 7.5 %) of housefly (*Musca domestica*) pupae into their diet for ten days resulted in a significant enhancement of the phagocytic activity of peritoneal macrophages (Ido *et al*. 2015). Notably, fish that received 5 % dietary housefly pupae for two months experienced 100 % survival against the bacterial pathogen *Edwardsiella tarda*, while all control fish perished within 12 days of the bacterial challenge (Ido *et al*. 2015). In the present study, catfish fed diets containing 20 % and 30 % frass

exhibited better survival rates following challenges with *F. covae* than those on the control diet or diets with lower levels of frass. Similarly, tilapia that received a diet with 30% frass showed a significant increase in survival against challenges from both *F. covae* and *S. iniae*. In another study, small European sea bass (*Dicentrarchus labrax*) fed *Tenebrio molitor* larvae meal for six weeks showed anti-inflammatory responses, including increases in ceruloplasmin, myeloperoxidase, and nitric oxide levels (Henry *et al*. 2018).

The positive responses observed could be linked to the bioactive components found in insect frass. Various components derived from insects, such as antimicrobial peptides (AMPs), lauric acid, beneficial microbes, and chitin, can serve as immunomodulatory functional feed ingredients (Koutsos *et al*. 2022). Insects are among the richest sources of AMPs (Yi *et al*. 2014; Vogel *et al*. 2018; Silveira *et al*. 2021). Research has demonstrated that AMPs are effective in killing bacteria and controlling pathogen infections in animals including fish (Rodrigues *et al*., 2021; Silveira *et al*., 2021; Wang *et al*., 2022). Insect meal and frass are rich sources of chitin, which can activate the immune system in mammals (O'Neil *et al*. 2006). The benefits of dietary chitin and/or chitosan have been well-documented in fish and shellfish (Harikrishnan *et al*. 2012; Gopalakannan and Arul 2006). Furthermore, chitin may act as a prebiotic, being non-digestible by the host, while selectively promoting the growth of beneficial intestinal microbiota that could help prevent the growth and colonization of pathogenic bacteria (Mousavi *et al*. 2020; Sankian *et al*. 2018).

In conclusion, frass generated from the production of black soldier fly larvae has the potential to be used as a feed ingredient that can increase feed intake and improve fish growth. Although frass may not be an ideal dietary component for carnivorous fish (Kagata and Ohgushi 2012; Banavar *et al*. 2022), incorporating it into the diets of omnivorous fish, especially at higher levels, could provide benefits such as boosting innate immune components and enhancing fish resistance to bacterial infections. In addition to its environmental advantages, converting organic waste into a high-value feed ingredient could offer significant economic benefits.

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Yildirim-Aksoy M, Eljack R, Beck BH, Peatman E (2022) Nutritional evaluation of frass from black soldier fly larvae as potential feed ingredient for Pacific white shrimp, *Litopenaeus vannamei*. *Aquac. Rep.*, **27**, 101353. https://doi.org/10.1016/j.aqrep.2022.101353

Annotated Bibliography of Key Works

(1) Romano N, Datta SN, Pande GSJ, Sinha AK, Yamamoto FY, Beck BH, Webster CD (2023) Dietary inclusions of black soldier fly (*Hermetia illucens*) larvae frass enhanced production of channel catfish (*Ictalurus punctatus*) juveniles, stevia (*Stevia rebaudiana*), and lavender (*Lavaridula angustifolia*) in an aquaponic system. *Aquaculture*, **575**, 739742. https://doi.org/10.1016/j.aquaculture.2023.739742

For the first time, the authors explored the potential of frass to enhance fish and plant production using a dietary approach in an aquaponic system. Channel catfish (*Ictalurus punctatus*) juveniles were fed with or without 10% black soldier fly (*Hermetia illucens*) larvae frass for 8 weeks. The authors used a 2 x 2 factorial design, with the main effects of dietary frass inclusion and media type at two different plant bed types. Overall, the authors reported improved fish and plant growth in an aquaponic system with dietary frass. The authors suggested that increased catfish growth was likely due to the upregulation of genes responsible for growth, reduction in intestinal inflammation, and a significant enhancement in feed intake. Additionally, it was noted that dietary frass contributed more water-borne nutrients for the plants, resulting in better plant growth.

(2) Romano N, Yamamoto F, Rawles SD, Webster CD (2024) Type of black soldier fly (*Hermetia illucens*) larvae frass influences the nutritional value when included in a prepared diet for Mozambique tilapia (*Oreochromis mossambicus*). *Aquaculture*, **589**, 740946. https://doi.org/10.1016/j.aquaculture.2024.740946

The authors, for the first time, present the nutritive value of frass from black soldier larvae fed on different substrates for Mozambique tilapia (*Oreochromis mozambicus*). They hypothesized that the initial substrate would influence the composition of frass and, thus, the nutritive values for fish. The study examined frass from larvae fed either an expired fish diet (45% crude protein) or a combination of fruits/vegetable

peels (9.3 % crude protein) at 5 % and 10 % dietary inclusion levels over 8 weeks. They showed that expired fish diet-based frass at 10 % significantly enhanced tilapia growth compared to fish fed the control diet. Additionally, the authors provided histological scoring for the liver and intestine, noting mild liver inflammation but improved intestinal histomorphology. The findings suggest that the initial substrate fed to black soldier fly larvae has important implications for the nutritional value of their frass and the associated liver and intestinal health of the fish consuming it.

(3) Yildirim-Aksoy M, Eljack R, Beck BH, Peatman E (2022) Nutritional evaluation of frass from black soldier fly larvae as potential feed ingredient for Pacific white shrimp, *Litopenaeus vannamei*. *Aquac. Rep*., **27**, 101353. https://doi.org/10.1016/j.aqrep.2022.101353

For the first time, the authors present the nutritional value and health benefits of frass derived from the larvae of black soldier flies fed dried distillers' grains in shrimp. They examined the inclusion of frass at levels up to 30 % in the diets of Pacific white shrimp, *Litopenaeus vannamei*. Overall, the authors showed a quadratic growth trend with increasing dietary levels of frass, with 5 % frass at the highest and 30 % frass at the lowest weight gain. Feed efficiency and body composition were also provided. Additionally, the authors assessed the health benefits of incorporating dietary frass by analyzing serum samples for immune parameters and antibacterial activities. The findings highlight the implications of feeding shrimp different levels of frass, which are discussed in detail in this paper.

(4) Banavar A, Amirkolaei SK, Duscher L, Khairunisa BH, Mukhopadhyay B, Schwarz M, Urick S, Ovissipour R (2022) Nutritional evaluation of black soldier fly frass as an ingredient in Florida pompano (*Trachinotus carolinus* L.) diets. *Animals* (*Basel*), **12**, 2407. https://doi.org/10.3390/ani12182407

The authors examined the potential of using frass as a dietary ingredient for a carnivorous fish species for the first time. They evaluated the impact of three different levels of dietary frass -8 %, 12 %, and 18 % -on the growth performance and feed utilization of Florida pompano (*Trachinotus carolinus* L.). The findings suggest that pompano cannot fully utilize frass, resulting in decreased growth performance as the level of frass increases. Additionally, the authors analyzed the intestinal microbiome, which revealed the highest diversity of gut flora in the control diet, while diets containing frass displayed signs of community imbalance. The authors concluded that frass is not an ideal dietary component for carnivorous fish.

(5) Sankappa NM, Lange MD, Yildirim-Aksoy M, Eljack R, Kucuktas H, Beck BH, Abernathy JW (2024) Transcriptome analysis and immune gene expression of channel catfish (*Ictalurus punctatus*) fed diets with inclusion of frass from black soldier fly larvae. *Front. Physiol.*, **14**, 1330368. doi: 10.3389/fphys.2023.1330368

The authors reported systemic and mucosal tissue gene expression, especially regarding the growth and immune-related genes of channel catfish (*Ictalurus punctatus*) fed various levels of frass for 10 weeks. They examined liver, head, kidney, gill, and intestine samples for gene expression analyses. Further, they identified differential expression of genes using targeted quantitative PCR panels for both innate and adaptive immune genes from channel catfish. Overall, the authors showed alteration of global gene expression and activation of innate and adaptive immunity of channel catfish fed diet with frass.

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Effect of feeding black soldier fly larvae diets on growth and culture condition of kuruma prawn (*Penaeus japonicus*)

Katsutoshi ITO*[1, †], Mana ITO*[1], and Ryuhei NAKAMURA*[2]

Abstract: Insect-based feeds are attracting considerable attention in aquaculture industries as alternatives to traditional fish-based feeds. Insect-based ingredients could be potential replacements for fishmeal in aquaculture feeds due to their implication in enhancing fish growth and disease resistance. However, their environmental impact should be fully assessed before insect-based feeds are used more frequently and on a larger scale. Although several attempts have been made to examine the utilization of insect-based diets in aquaculture species, the experimental conditions are limited. As a result, the comprehensive understanding of their impacts on environments remain elusive. To fill the knowledge gap, this research introduces the utilization of black soldier fly *Hermetia illucens* larvae (BSFL) as an alternative to fishmeal in diet for Kuruma prawn, *Penaeus japonicus,* focusing on the effects on growth and culture condition against a traditional fishmeal-based diet. A feeding experiments was conducted using juvenile Kuruma prawns (body weight, 106 ± 9.5 mg) and experimental diets with 0% BSFL (100% fishmeal), 50% BSFL (50% of fishmeal was substituted with BSFL), and 80% BSFL (80% of fishmeal was substituted with BSFL). The prawn was individually kept in a glass container and fed respective diets once every two days. The feeding trial was conducted at 20°C. The body weight of prawn in the 50% BSFL treatment was significantly higher than that in the 0 % BSFL treatment after one week, while the body weights were not significantly different between the 0% BSFL and 80% BSFL treatments. The concentrations of dissolved inorganic nutrients in the rearing water were measured periodically during the one-week feeding period. The concentration of dissolved ammonium nitrogen became lower with the increase of BSFL content ($p < 0.05$). On the other hand, phosphate concentrations tended to be higher in the BSFL-supplemented treatments. However, no significant difference was found between the 0% (100% fish meal) and 50% BSFL treatments, the latter of which noted a higher prawn growth. Oxidative conditions of the bottom sediment are important for monitoring the health of benthic organisms such as Kuruma prawn. Therefore, we monitored the redox potential of the bottom sediment in real time. The redox potential was found to be highest in the 80% BSFL treatment, lowest in the 0% BSFL treatment, and at an intermediate level in the 50% BSFL treatment (80% BSFL > 50% BSFL > 0% BSFL). Overall, our results indicate that for prawn culture, 50% of dietary fishmeal could potentially be replaced by BSFL with growth promotion and a lower environmental impact.

Key words: aquaculture, insect-based diets, dissolved inorganic nutrients, redox potential

Introduction

With the increasing demands, technological advancements, and advanced efforts, the global production of shrimp has been increasing rapidly. Aquaculture has been contributing substantially to maintaining the sustainable use of marine resources and is essential to ensure the food security (Costello *et al*. 2020). Most of fish and crustacean aquaculture practices are sometimes criticized for the disruption of the food web, resulting in an ecological imbalance. As a result of using wild pelagic fish (e.g., sardines and anchovies) for the production of fishmeal that have been used as a feed ingredient,

Accepted on Feb 26, 2026
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particularly for farming marine carnivorous fish at higher inclusion levels (Naylor *et al*. 2000), the disruption tends to be more serious. Despite not being commonly used, insect-based ingredients are attracting significant attention as alternative sources relative to traditional fishmeal in aquaculture industries (Henry *et al*. 2015). The insect-based ingredients could be potential replacements for fishmeal in aquaculture feeds due to their implication in enhancing fish growth and disease resistance (Ido *et al*. 2019). The effects of different feeds used in aquaculture, including insect-based feeds, on the rearing environment should be assessed because the knowledge is limited.

To further improve the aquaculture production in terms of growth and profitability, the improvements in feed formulation and quality are greatly needed. Black soldier fly *Hermetia illucens*, the larvae of which (BSFL) could grow on low-value organic matters and are able to supply a protein-rich and high-value biomass (Diener *et al*. 2009), is a promising species and commercially exploited in various countries. Thus, the use of BSFL as an alternative protein source in aquaculture industries has been rising because BSFL is considered to supply required nutrients needed in aquaculture production (Bruni *et al*. 2018; Foysal *et al*. 2019). Kuruma prawn (*Penaeus japonicus*) is distributed in the areas of Indo-Pacific regions including Japan except for Hokkaido Island (Hayashi 1992), and has been an important commercial aquaculture species. Diet quality is always a vital factor affecting the shrimp aquaculture practices and BSFL could provide nutrients required for maintaining and improving the health of aquaculture species. However, utilization of BSFL in Kuruma prawn diet has been unknown in terms of the effects on their growth, survival, and aquaculture environment. Especially, the environmental impact assessment should be addressed in advance to practical use of insect-based feeds at a larger scale. To fill the knowledge gap, this research introduces the results of our study examining the effects of dietary inclusion of BSFL as an insect-based ingredient on the growth and environmental condition of Kuruma prawn against a traditional fishmeal-based diet. Since Kuruma prawn are sensitive to the condition of the bottom sediment, maintaining the bottom sediment environment in a healthy condition is extremely important in their aquaculture. In this study, we also report the results of measurements of the growth of Kuruma prawn fed BSFL supplemented diets and a fish meal-based diet, and the impact on the bottom sediment environment using a newly developed bottom sediment monitoring system (Shono *et al*. 2022).

Short Materials and Methods

Feeding performance of kuruma prawn using BSFL-containing diets

A feeding experiment was conducted using juvenile Kuruma prawn (body weight, 106 ± 9.5 mg) and experimental diets with 0 % BSFL (100 % fishmeal), 50 % BSFL (50 % of fishmeal was substituted with BSFL), and 80 % BSFL (80 % of fishmeal was substituted with BSFL). Individual prawn was kept in a glass container and fed one of the diets once every two days. The feeding trial was conducted at 20°C for one week. The feed conversion ratio (FCR) was calculated for individuals using the following formula.

$$\mathrm{FCR} = \frac{\text{Total feed consumed (g)}}{\text{Weight gain (g)}}$$

Evaluation of rearing environment using BSFL-containing diets

For dissolved inorganic nutrient analysis, seawater samples were collected from each experimental breeding container, frozen and stored until analysis. Concentrations of dissolved inorganic nitrogen (NH_4-N, NO_2-N, and NO_3-N), dissolved inorganic phosphate (PO_4-P) and dissolved silicate (DSi) were measured by an autoanalyzer QuAAtro 39 (BL-TEC, Osaka, Japan), following the standard methods detailed in Strickland and Parsons (1972).

We also measured the redox potential of the bottom sediment. A two-electrode electrochemical cell (EC, 160 ml capacity) was assembled using an Ag/AgCl/saturated KCl as reference electrodes. A fluorine-doped tin oxide (FTO)-coated glass electrode (surface area of 28.26 cm^2, SPD Laboratory, Shizuoka, Japan) was used as a working electrode and was placed on the bottom of the EC reactor (Fig.1). Twenty grams of wet sediments and 145 ml of sea water were added to the EC reactor. One reactor was placed in each experimental container by inserting the receptor end inside the shallow benthic sedimentary layer (Fig.2) and was maintained at 20°C. Redox potential measurements were continuously conducted using open circuit potential measurements performed with an automatic polarization system HAL-3001A (Meiden Hokuto, Tokyo, Japan).

Statistical analysis

All data were expressed as mean ± standard deviation. Statistical analysis was performed using Microsoft Excel Touki 2010 for Windows version 1.03 (Esumi, Tokyo, Japan). The

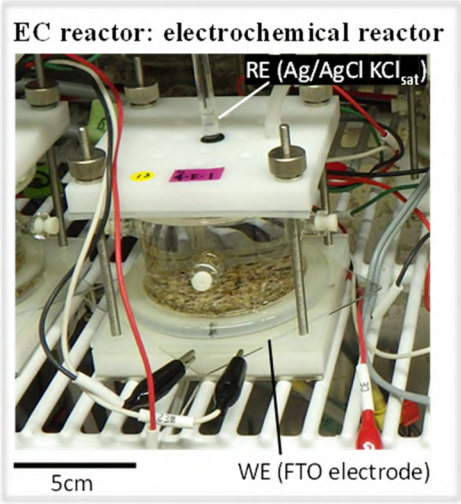

Fig.1 (A) the model benthic ecosystem constructed in an electrochemical (EC) reactor

RE, and WE denote the reference and working electrodes, respectively.

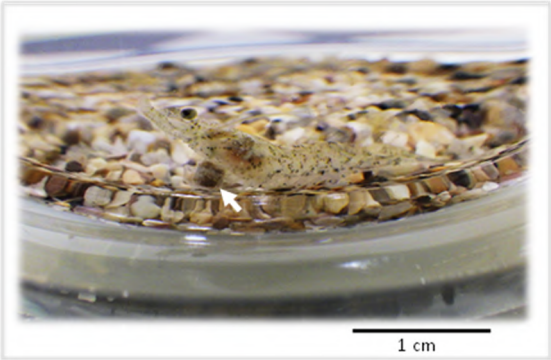

Fig.2 Feeding behavior of Kuruma prawn for a BSFL diet (white arrow) in the EC reactor

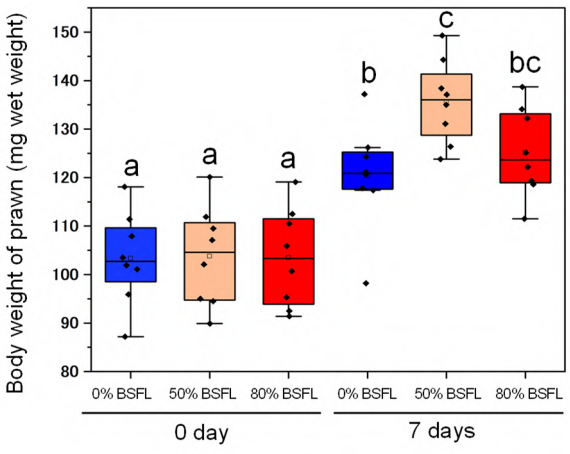

Fig.3 The body weights of Kuruma prawns before and after one week of feeding experiment

Blue, orange, and red indicate the results of 0%, 50%, and 80% BSFL treatments, respectively. Values with different letters are significantly different from each other ($p < 0.05$).

differences between treatment means were tested using Tukey's multiple comparison test and were considered significant at $p < 0.05$.

Results and Discussion

Growth performance

Survival rate was more than 90% across the experimental treatments and no significant difference was observed during the experimental period. The Kuruma prawn actively foraged within each test container and actively consumed the diets during the experimental period (Fig.2). The feeding behavior of Kuruma prawn for a BSFL-based diet is shown in Fig.2. An improved growth performance was observed in prawn fed the BSFL-containing diets compared to that of the control diet (Fig. 3). The final body weight of prawn in the 50% BSFL treatment was significantly higher than that in the 0% BSFL treatment while the weight in the 80% BSFL treatment was intermediate between the values of the other two treatments (Fig.3). The utilization of insect larvae has been extensively studied in aquaculture fish while that in crustaceans has rarely been studied. Different metamorphic stages (i.e., larvae or pupae) of a given insect could bias the growth performances of aquaculture shrimp, due to the variabilities in their nutritional compositions (Huyben *et al*. 2019). The FCR in the 50% BSFL treatment was the lowest value of 2.1, indicating that nutrients in the BSFL diet were efficiently converted into prawn body (Table 1). Kuruma prawn is omnivorous and feeds on certain invertebrates such as bivalves, polychaetes and crustaceans, and other fauna in the wild. Therefore, the prawn could be able to efficiently assimilate BSFL.

Previous studies reported the growth promotion due to the substitution of fishmeal with BSFL (Renna *et al*. 2017; Wang *et al*. 2019), which fully agrees with the finding of the present study. The composition of the fatty acids in BSFL could trigger the growth performance compared to that of fishmeal. Wang *et al*. (2021) demonstrated that dietary inclusion of BSFL initiated the hepatocyte interruption and lowered the unsaturated fatty acids, leading to the increase in growth

Table 1 The feed conversion ratio of Kuruma prawn fed each diet

	Feed conversion ratio
0% BSFL	4.0 ± 1.8
50% BSFL	2.1 ± 0.7
80% BSFL	3.0 ± 0.7

performance in Pacific white shrimp *Litopenaeus vannamei*. The excessive substitution of fishmeal with alternative ingredients does not always improve the growth performance as it might affect the metabolism, intestinal histology, and digestion of shrimps (Rahimnejad *et al*. 2019; Shao *et al*. 2020). These observations support the finding of our study that 80 % substitution negatively affected the growth performance relative to that of the 50 % BSFL treatment (Fig.3). Ling *et al*. (2025) comprehensively investigated the multifaceted impacts of BSFL on shrimp aquaculture and recommended its potential as a suitable and sustainable alternative protein source relative to traditional fishmeal. They concluded that BSFL is a promising ingredient that enhances the growth performance and feed efficiency in certain conditions, contributing to the economic viability of shrimp aquaculture practices. On the other hand, it has been stated that substitution of 6-8 % fishmeal with BSFL is an appropriate level in a diet for *L. vannamei*, a species closely related to Kuruma prawn, and that 10 % substitution with BSFL may induce pathological abnormality in the intestine and decrease the shrimp's immunity (Chen *et al*. 2023). Although the species are different, our study suggests that substitution of fishmeal up to 50 % with BSFL might enhance the immunity of Kuruma prawn. In a future study, the effects of long-term culture of Kuruma prawn with a BSFL diet not only on their growth but on their gut flora that is known to be involved in the immune system, should be addressed.

Rearing environment for Kuruma prawn due to dietary BSFL inclusion

The concentrations of dissolved inorganic nutrients in the rearing water were measured periodically in each experimental container for one week (Fig.4). The concentration of dissolved ammonium nitrogen became lower with the increase of BSFL content ($p < 0.05$). On the other hand, phosphate concentrations tended to be higher in the BSFL-supplemented treatments, but no significant difference was found between the 0 % BSFL (100 % fish meal) and 50% BSFL treatments, the latter of which noted a higher prawn growth. The redox potential in the sediment during the prawn culture was generally higher in the order of 80 % BSFL > 50 % BSFL > 0 % BSFL. These results indicate that 50 % substitution of fishmeal with BSFL could be favorable for prawn culture, increasing the growth with a lower environmental impact.

Traditionally, the environmental assessment of aquaculture fields uses various indicators, such as chemical and biological ones (e.g., benthic fauna; Holmer *et al*. 2008). However, these approaches are not suitable for assessing short-term environmental changes, such as those that fluctuate on hourly and daily basis. Although chemical indicators, such as oxygen, nitrogen, phosphorus, and sulfur compounds, could instantly respond to the changes caused by feeding, enormous efforts are required for on-site water and sediment samplings and comprehensive analysis of complex chemical compounds.

In the present experiment, the sediment of the 0 % BSFL treatment with a higher dissolved ammonium nitrogen concentration (Fig.4A, blue bar) had lower redox potential levels and was in a reduced environment (Fig.5, blue line). On the other hand, the sediment of the 80 % BSFL treatment with a lower dissolved ammonium nitrogen concentration (Fig.4, red bar) had higher redox potential levels, indicating the sediment was in an oxidizing environment (Fig.5, red line). The dissolved inorganic phosphate (PO4-P) in the 50 % BSFL treatment was not statistically different from that of the control treatment in the current study (Fig.4D). The water quality is known to be negatively influenced by the feeding activities, leading to growth retardation of aquatic animals. He *et al*. (2022) reported the negative correlation between the concentrations of NH_4-N and NO_3-N and the inclusion levels of BSFL, which partially supports the finding of the current study (consistent decrease in NH_4-N with BSFL; see Fig.4A). Generally, 20-30 % of dietary nutrients are efficiently utilized by the animals in shrimp aquaculture, while the others are accumulated in the bottom environment as leftovers that are often responsible for the deterioration of sediment conditions in aquaculture farms (Paez-Osuna 2001). Our results indicate that the BSFL diets were possibly utilized more efficiently by Kuruma prawns compared to the fishmeal-based diet. By the measurement of redox potential which is a physicochemical value determined by the integration of various factors such as biological, chemical, and physical parameters, we have recently successfully implemented a real-time monitoring system at an aquaculture site (Ito *et al.*, in preparation). In the future, it is expected that the sediment environment which has been adversely impacted by aquaculture could be monitored and evaluated by the real-time redox potential monitoring of the sedimentary environment. In conclusion, 50 % replacement of fishmeal by BSFL was found to be ideal in terms of limited feed loss, higher growth performance, survival, and quality of the environmental characteristics. Especially, the introduction of unique redox potential monitoring system that integrates various physicochemical measurements in the future aquaculture designs could lead to the improvement in monitoring the aquaculture environment.

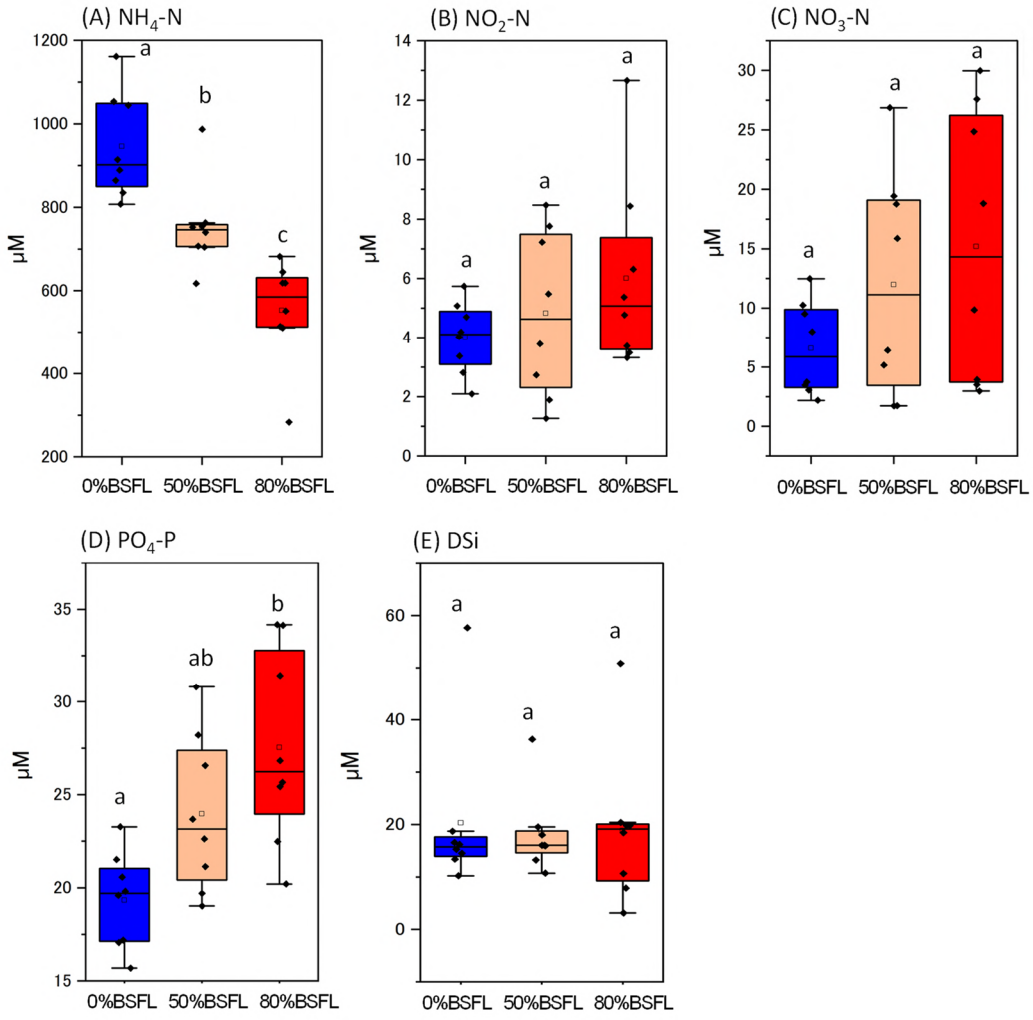

Fig.4 The concentrations of dissolved nutrients in rearing seawater after one week of feeding experiment in Kuruma prawn fed BSFL diets

The concentrations of dissolved inorganic nitrogen, NH_4-N (A), NO_2-N (B), and NO_3-N (C), dissolved inorganic phosphorus (PO_4-P) (D), and dissolved silicate DSi (E) are presented. Values with different letters are significantly different from each other ($p < 0.05$).

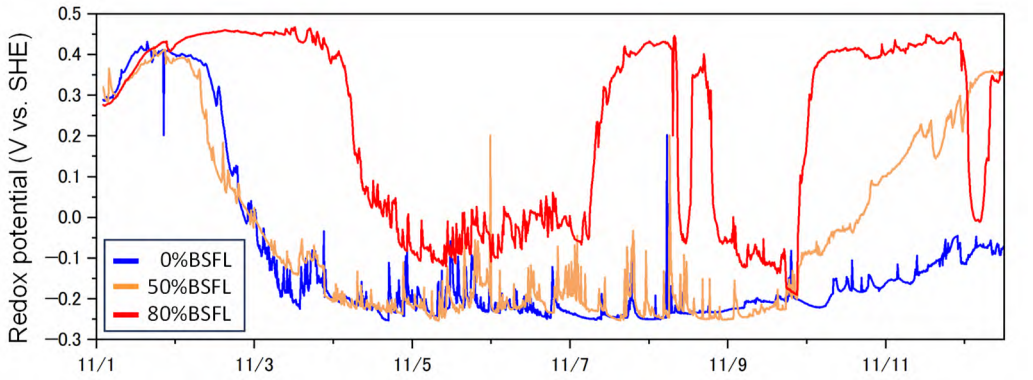

Fig.5 The fluctuations in the redox potential of the bottom sediments in each rearing container during one week feeding experimet in Kuruma prawn

The blue, orange, and red lines represent 0%, 50%, and 80% BSFL treatments, respectively.

Acknowledgement

The authors express their sincere gratitude to Dr. T. Andoh, Dr. K. Abe, Dr. A.M. Khorshed, and Ms. M. Ishimoto of Fisheries Technology Institute, Japan Fisheries Research and Education Agency, for their generous assistance with the experiments.

This work was supported by Cabinet Office, Government of Japan, Moonshot Research and Development Program for Agriculture, Forestry and Fisheries (funding agency: Bio-oriented Technology Research Advancement Institution) (JPJ009237).

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Annotated Bibliography of Key Works

(1) Shono N, Ito M, Umezawa A, Sakata K, Li A, Kikuchi J, Ito K, Nakamura R (2022) Tracing and regulating redox homeostasis of model benthic ecosystems for sustainable aquaculture in coastal environments. *Front. Microbiol*., **13**, 907703. https://doi.org/10.3389/fmicb.2022.907703

Aquaculture practiced in coastal environments has an increasingly important role in the world's food supply; however, the accumulation of organic compounds on the seafloors due to overfeeding harms benthic ecosystems. To assess the ecological resilience of aquafarms to nutrient influx, they investigated the redox homeostasis of benthic ecosystems using a marine oligochaete as a model benthic organism in aquaculture fields. Real-time monitoring of the redox potential of a model benthic ecosystem constructed in an electrochemical reactor allowed evaluation of the homeostatic response of the system to nutrient addition. Although the detrimental effects of overfeeding were confirmed by irreversible potential changes in the sediment, redox homeostasis was reinforced through a cooperative relationship between oligochaetes and sediment microorganisms. Specifically, the oligochaetes exhibited reversible changes in the metabolism and body position in response to dynamic changes in the sediment potential between -300 and 500 mV, thereby promoting the decomposition of organic compounds. The potential-dependent changes in the metabolism and body position were reproduced by artificially manipulating the sediment potential in the electrochemical reactors. Given the importance of benthic animals in sustaining coastal ecosystems, the electrochemical monitoring and physiologic regulation of marine oligochaetes could offer an intriguing approach toward sustainable aquaculture.

水研機構研報, 第 53 号, 63 – 69, 令和 8 年
Bull. Jap. Fish. Edu. Agen. No. 53, 63-69, 2026

Current status of artificial seed production and selective breeding in Japanese yellowtail *Seriola quinqueradiata*: The progress achieved by Japan Fisheries Research and Education Agency

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Abstract: Japanese yellowtail *Seriola quinqueradiata* is one of the most important commercial fish species in Japan. The aquaculture of yellowtail has been practiced since the 1920s, using wild juveniles caught in the Pacific coast of southern Japan. However, the catch of juveniles is occasionally unstable, likely owing to various environmental factors. Additionally, the peak period of the harvest of yellowtail, which have been cultured for nearly two years from wild juveniles that could be obtained only in early spring, is from late fall to winter. Consequently, the quantity of yellowtail available in the market significantly decreases from May to September. Therefore, artificially produced seeds have begun to be utilized to overcome these problems. In this situation, a family lineage or strain with fast growth and disease resistance traits would be valuable. Therefore, the development of such a strain via selective breeding and production of artificial seed having beneficial traits have drawn significant interest in Japan. In the selective breeding program for yellowtail at Japan Fisheries Research and Education Agency (FRA), the seeds obtained via artificial spawning were raised to approximately 5 cm and then cultured until they reach the harvest sizes in commercial aquaculture farms. Subsequently, 2,000 adults (approximately 60 cm) were selected, and their body length and body weight data, and samples of fin chip were obtained for pedigree reconstruction from the individuals. Finally, based on the estimated breeding values using pedigree-based best linear unbiased prediction and inbreeding coefficients, 200 individuals were selected as broodstock candidates. Thus far, good correlations of breeding values between the first and second generations have been observed in both fish length and body weight, indicating that our breeding program is promising.

Key words: *Seriola quinqueradiata*, artificial seedling, selective breeding, BLUP

Accepted on Feb 26, 2026

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Introduction

Japanese yellowtail *Seriola quinqueradiata* is one of the most important commercial fish species in Japan. Hence, aquaculture of this species is prevalent in southwest Japan, particularly in Kagoshima, Oita, Ehime, Miyazaki, Kochi, and Nagasaki Prefectures. The Pacific sub-population of this species spawns in the Satsunan area, southern Kyushu, Japan, and the juveniles appear off the coast of Kagoshima Prefecture from March to April, following floating seaweeds. The wild juveniles of approximately 50-100 mm length are caught from April to May, cultured in cages until they reach 3-5 kg, and then shipped from late autumn of the following year.

Aquaculture of this species in Japan began in 1927 in the Adoike cove, which is separated from the Seto Inland Sea by the bank, in Kagawa Prefecture (Miyashita 2008). This practice is considered the first successful case of modern aquaculture of marine fish in the world. After the 1950s, the "cage culture method" had been developed in Japan, and promoted new entrants to aquaculture of this species. Therefore, the production of this species rapidly increased after 1964. Recently, the gross domestic production of this species, both through capture fishery and aquaculture, has been over two hundred thousand tons per year (Fishery and aquaculture statistics of Reiwa 6; Ministry of agriculture, Forestry and fisheries 2025). In 2023, the export value of fishery products showed that Japanese yellowtail positioned in the 3rd place (41.8 billion yen) after Japanese scallops and pearls, marking a steady increase from the previous year's value (36.3 billion yen) (FY2023 White Paper on Fisheries; Ministry of agriculture, Forestry and fisheries 2024).

However, in Japanese yellowtail aquaculture, the practice of using wild-caught juveniles as seedling is still common. Thus, the aquaculture production largely depends on the amount of wild-caught juveniles, which is occasionally unstable likely owing to various environmental factors. The poor harvest of wild juveniles in 2021 led to a drastic decrease in the aquaculture production in 2022, resulting in the short supply and price surge in the market. Additionally, the peak period of the harvest of yellowtail, which have been cultured for nearly two years from the wild juveniles that could be obtained only in early spring, is from late fall to winter. Thus, the quantity of yellowtail available in the market significantly decreases from May to September. Currently, although the aquaculture industry relies on approximately 20 million wild-caught juveniles each year, artificially reared seedlings account for only 5 % of the demand. Hence, promoting the use of artificial seedlings is a valid way to overcome the problems mentioned above, and is expected to further increase the productivity through selective breeding.

Application of conventional selective breeding methods has been attempted in common carp *Cyprinus carpio* (Moav and Wohlfarth 1976), coho salmon *Oncorhynchus kisutch* (Hershberger *et al*. 1990), rainbow trout *O. mykiss* (Kincaid *et al*. 1977; Hörstgen-Schwark 1993), and red seabream *Pagrus major* (Murata *et al*. 1996). In addition, parentage analyses using DNA markers, such as microsatellite DNA and single nucleotide polymorphisms (SNPs), have been advanced, and the breeding values now could be determined using the pedigree-based best linear unbiased prediction (BLUP) method, which allows the selection of individuals with the most beneficial phenotype. In particular, the parentage analysis using SNP markers results in higher parental assignment rates than those using microsatellite DNA markers (Hauser *et al*. 2011; Trọng *et al*. 2013). The former method has been applied to various aquatic species, such as yellowtail kingfish *S. lalandi* (Premachandra *et al*. 2019), Atlantic salmon *Salmo salar* (Holman *et al*. 2017), rainbow trout (Liu *et al*. 2016), European abalone *Haliotis tuberculate* (Harney *et al*. 2018), and Australian greenlip abalone *H. laevigata* (Arbon *et al*. 2021). In the case of Japanese yellowtail, the SNP markers have recently been developed for a parentage analysis (Uchino *et al*. 2020), in addition to the conventional microsatellite DNA markers (Shimada *et al*. 2019).

In this study, we raised offspring (first generation) produced by artificial mating of wild individuals of yellowtail. The first broodstock candidates were then selected based on the breeding values using pedigree-based BLUP for adult size. Subsequently, offspring (second generation) were obtained from the first broodstock and raised to adult size. Finally, we assessed the effects of our selective breeding program by comparing the growth differences between the first and second generations in terms of adult size.

Materials and Methods

Samples

For the first generation (G_1), 26 family lineages were obtained from 26 (13 sires and 13 dams) wild fish (G_0) via insemination in April 2019 by a 1 × 2 factorial mating in Goto Field Station, Fisheries Technology Institute (FTI) (Nagasaki, Japan). The fertilized eggs were divided into two subsamples, and were grow in Goto Field Station and Kamiura Field Station (Oita, Japan), with 26 and 14 family lineages allocated to each

station, respectively. After they grew to adult sizes, we selected 20 individuals, taking the breeding values and inbreeding coefficient into account, for the parents of the next generation (see the next sub-section for details).

For the second generation (G_2), 48 and 84 family lineages were obtained from the 18 (14 sires and 4 dams) and 20 (14 sires and 6 dams) adult G_1 fish, by performing a 1 × 5 factorial mating in Goto Field Station and a full factorial mating in Kamiura Field Station, respectively, via insemination in April 2022. The first (48 family lineages) and second (84 family lineages) fertilized eggs resulting from these mating plans were allocated to Goto and Kamiura Field Station, respectively.

The obtained seedlings were raised to approximately 5 cm in indoor rearing tanks for approximately 70 days during which fish were sorted twice to uniform sizes to avoid cannibalism (Fig.1A). These seedlings were then transferred to two commercial aquaculture farms, designated here as A and B in the Kyushu region (Fig.1B), and cultured to harvest sizes. The G_1 seedlings were transported from Kamiura Field Station and G_2 seedlings from Goto Field Station, to both aquaculture farms. After approximately 18 months, ca 1,000 adult fish were randomly selected from each aquaculture farm, and fish length (total length or fork length) and body weight were measured, and samples of fin chip were obtained from the individuals for pedigree reconstruction (Fig.2A, 2B).

Finally, 100 individuals were selected (see the next sub-section) from approximately 1,000 individuals in the cages. The individuals were identified based on PIT tags (Biomark,

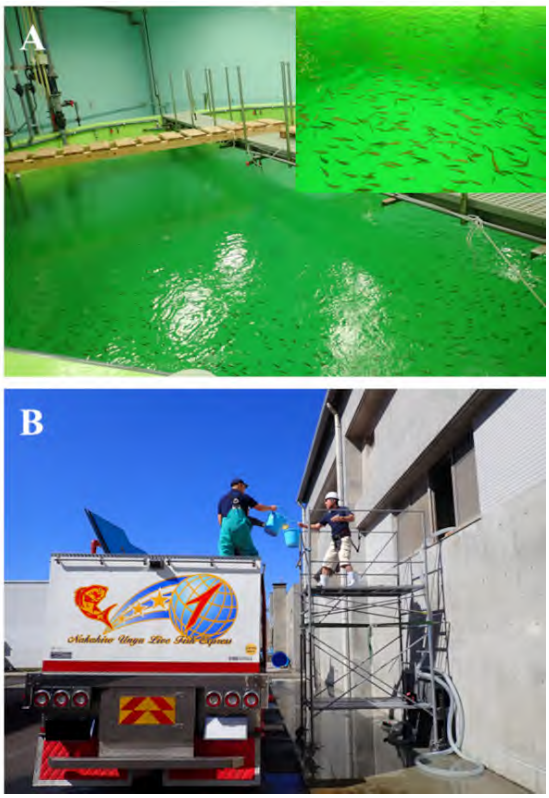

Fig.1 Mass production of artificial seedlings of Japanese yellowtail in Kamiura Field Station

A, artificial seedlings that are raised to approximately 5 cm in 120 kL indoor rearing tanks for approximately 60 days. B, transportation of artificial seedlings from Kamiura Field Station to aquaculture farms after approximately 70 days post hatching.

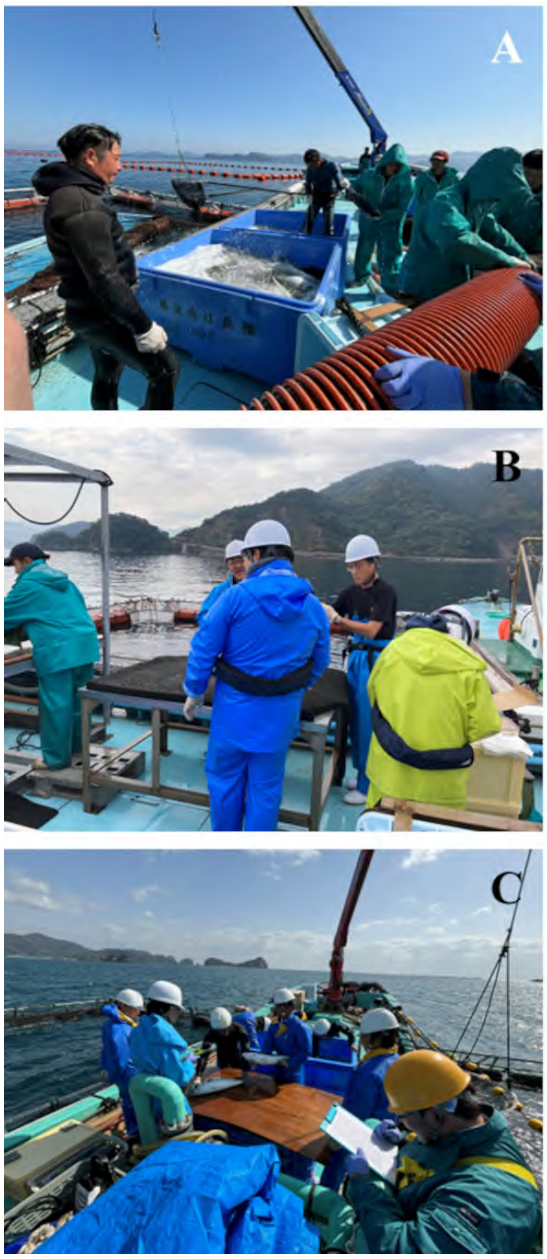

Fig.2 Phenotyping and selection of broodstock candidates of yellowtail in aquaculture farms

A, anesthetization of fish; B, measurement of body weight and fish length; C, identification of individuals for selection using PIT Tag data.

ID, USA) that had been implanted in their bodies (Fig.2C), and used as the broodstock.

Parentage analysis

We extracted total DNA from all fin chip samples using the InnuPure C96 and Smart DNA prep (a) kits (Analytik Jena, Jena, Germany). Uchino *et al*. (2020) designed and synthesized the SNP probes for 96 loci using SNP Type Assay (Fluidigm, CA, USA). We amplified these SNP markers using a 2-step PCR protocol and genotyped using the Biomark HD system and BioMark Genotyping Analysis Software v4.5.1 (Fluidigm). The obtained genotype data allowed us to perform parental assignments using an exclusion method (Sekino and Kakehi 2012), and then we reconstructed the pedigree of the samples. We estimated heritability and breeding values for the growth traits of the reconstructed pedigree. We applied the BLUP method to the data under the animal model using the breedR package of R (Muñoz and Sanchez 2019). Based on the breeding values and inbreeding coefficients, we selected 100 individuals mentioned above as the candidates of the parents for the next generation.

Results

The phenotypic data of our Japanese yellowtail breeding population are summarized in Fig.3. The mean body weight and total length (minimum-maximum value) of G_1 fish in aquaculture farms A and B were 4.5 ± 0.2 kg (4.0-5.7 kg) and 69.6 ± 1.5 cm (65.0-75.5 cm) (n = 1,000), and 3.7 ± 0.2 kg (3.1-4.8 kg) and 65.8 ± 1.5 cm (60.5-71.3 cm) (n = 1,000), respectively. The average values of total length and body weight in aquaculture farm A were larger than those in farm B. The body weight and fork length of G_2 fish in these aquaculture farms were 3.2 ± 0.3 kg (2.4-4.1 kg) and 57.6 ± 1.6 cm (50.2-61.8 cm) (n=1,012), and 3.1±0.3 kg (2.2-4.0 kg) and 61.1 ± 1.6 cm (53.5-65.5 cm) (n=1,040), respectively.

All 14 family lineages of G_1 were detected in both aquaculture farms by the parentage analysis. The parents of 968 individuals from farm A and those of 1,039 individuals from farm B were assigned, and 42 family lineages of G_2 were found in both aquaculture farms.

We then estimated the heritability and breeding values for growth traits. The heritability estimates in G_1 and G_2 for body

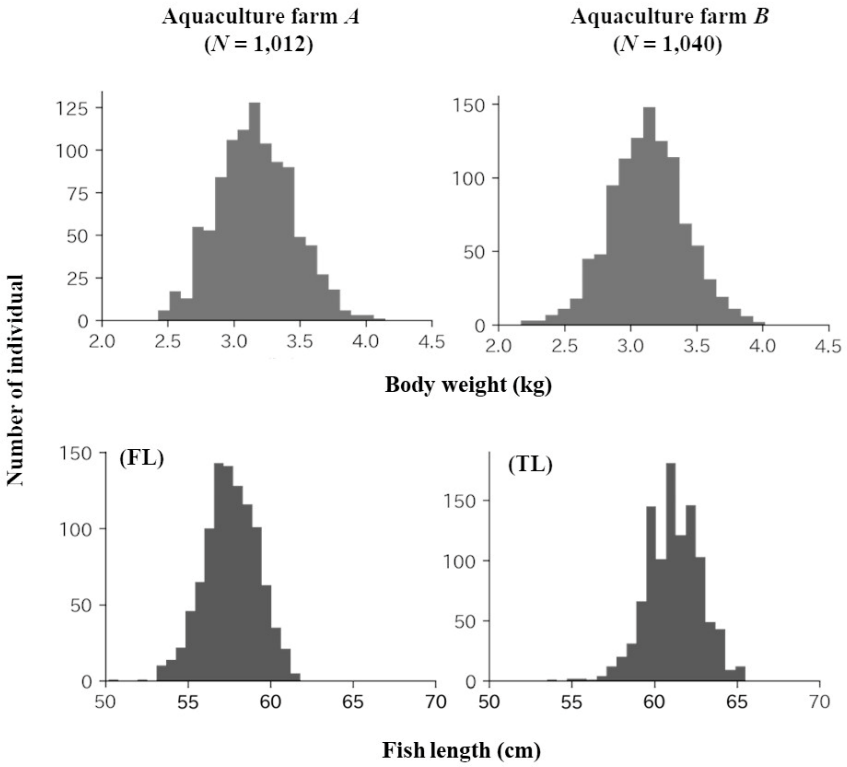

Fig.3 Distributions of body weight (kg) and fish length (cm) in randomly selected 2,000 adults (G_2) in aquaculture farm A and B

N, individual number; TL, total length; FL, fork length.

weight and fish length are shown in Table 1. Despite that we analyzed the data obtained from two independent aquaculture farms, the average breeding values of each family lineage exhibited strong correlations between the two aquaculture farms both in the G_1 and G_2 generations. This suggests that the environmental effects were similar between the two farms.

We further examined if our breeding program is progressing well. Unexpectedly, the average values of the body weight and fish length of G_2 were smaller than those of G_1 in both aquaculture farms though we had selected G_1 individuals with high breeding values as the parents of G_2 generation. This is probably because the individuals of G_2 were raised in much lower water temperatures in both farms than those for G_1. Nevertheless, we observed strong correlations between the average breeding values of a sire and a dam in G_1 and the average ones of their offsprings in G_2 for fish length (farm A, $r = 0.89$; farm B, $r = 0.74$) and body weight (farm A, $r = 0.77$; farm B, $r = 0.82$) (Fig.4). These observations are consistent with what are expected under the animal model; the high breeding values of our selected parents were inherited to their offsprings.

Table 1 The heritability estimates for body weight and fish length in both aquaculture farms

Generation		Aquaculture farm A	Aquaculture farm B
G_1	BW	0.320	0.402
	TL	0.606	0.684
G_2	BW	0.504	0.323
	FL	0.680	0.384

BW, body weight; TL, total length; FL, fork length

Discussion

In this study, we evaluated the efficiency of our selective breeding program for Japanese yellowtail by comparing the differences in growth between two generations, G_1 and G_2 in terms of adult size. Although the mean body weights of G_2 in the two aquaculture farms were lower than those of G_1, positive

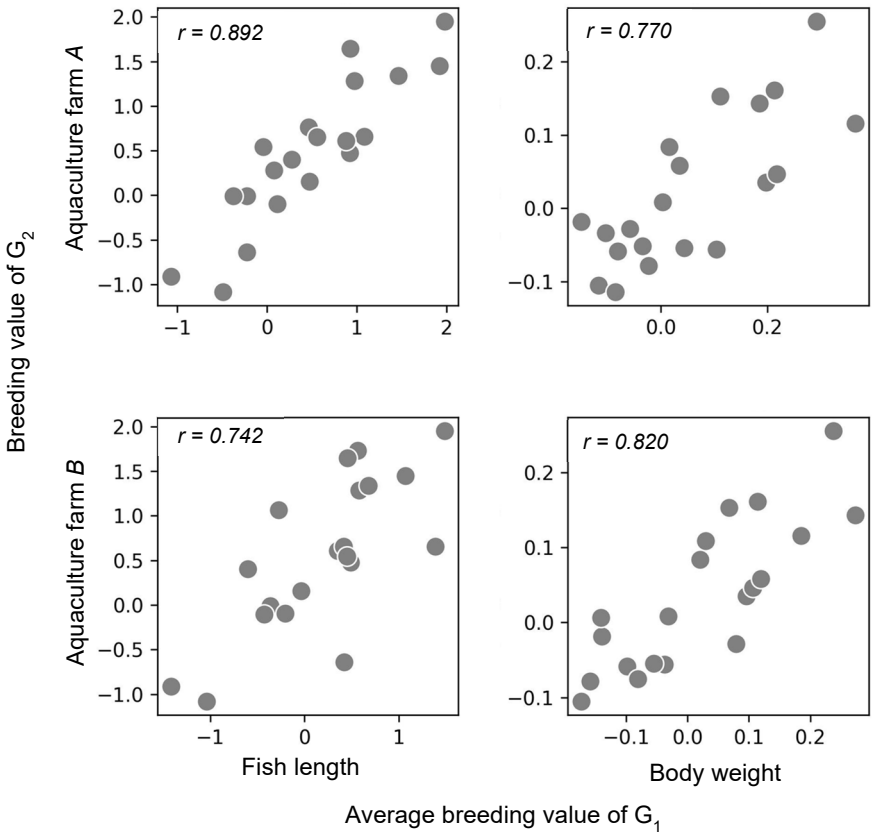

Fig.4 Testing the fit to an additive genetic model

The x-axis represents the average breeding values of sires and dams in the G_1 generation. The y-axis represents the average breeding values of their offspring in the G_2 generation. Each dot represents a sibling. The four panels correspond to combinations of aquaculture farms (A or B) and phenotypes.

correlations were observed between the breeding values of the two generations, indicating that family lineages with high breeding values in G_2 were originated from the family lineages of G_1 with high breeding values. This also implies that the fast-growing family lineage had been appropriately selected using pedigree-based BLUP, thereby making our breeding program promising. Similarly, Morishima (2019) reported that the mean body weight (5 kg) of the group selected by pedigree-based BLUP was higher than the maximum body weight (4 kg) of the group consisting of wild seedlings at the same period. Additionally, selective breeding of this species based on pedigree-based BLUP has also been promoted by other private enterprises in Japan. The developing family lineage at our institute will reach the third generation by 2025 through two selections. In contrast, Shimada *et al*. (2019) reported that the bias in family composition in the G_1 artificial seedlings increased after size selection. As the bias of family composition in the artificial seedlings provided to each aquaculture farm might result in reduced genetic diversity and survival rate in the broodstock in the future, it is necessary to closely monitor the status of the selectively bred next generation. Recently, genome sequencing of various species has been conducted using next-generation sequencing, allowing breeding practices based on genomic selection. Genomic selection is a breeding method introduced by Meuwissen *et al*. (2001), which utilizes genome-wide markers to predict the breeding value (GEBV) of individuals within a breeding population. A modified implementation of the BLUP approach (GBLUP) has already been used in various practical genomic selection applications. In dairy cattle breeding, genomic selection has largely replaced the traditional methods based on progeny testing, which often yielded unparalleled results. Maize and wheat are at the forefront of crop genomic selection (de Koning 2016). In aquatic species such as *S. salar*, *O. mykiss*, yellow croaker *Larimichthys crocea*, Pacific abalone *H. discus hannai*, and Japanese scallop *Patinopecten yessoensis*, the possibility of genomic selection for body size, parasite and disease resistance, fatty acid composition and nutritional quality-related traits has been reported (Ødegård *et al*. 2014; Tsai *et al*. 2015, 2016; Dong *et al*. 2016; Vallejo *et al*. 2016; Hosoya *et al*. 2017; Liu *et al*. 2024). To perform GBLUP in yellowtail, we have been accumulating genome-wide SNP data based on GRAS-Di, which will be beneficial for improving selection strategies and preventing anticipated risks in the future. In summary, this study revealed that our breeding program is promising for selection of yellowtail that grow faster relative to conventional use of wild seedlings. The development of strains via selective breeding and the stable supply of their artificial seedlings will greatly contribute to the expansion of aquaculture production and export to the global market.

Acknowledgments

We thank Dr. A. Ozaki, Mr. K. Yoshida, Dr. K. Okuzawa, Dr. Y. Shimada, Ms. J. Tabata, Mr. T. Noda, Mr. R. Shinoda, and Mr. Y. Nakagawa of Japan Fisheries Research and Education Agency for their valuable suggestions and supports. We thank all members of the HYOSHOKU Co., Ltd. and KEITEN Co., Ltd. for cultivating to harvest size to obtain the main results of this paper.

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水研機構研報, 第 53 号, 71 – 77, 令和 8 年
Bull. Jap. Fish. Edu. Agen. No. 53, 71 - 77, 2026

Evaluation of growth performance and genetic analysis of rainbow trout (*Oncorhynchus mykiss*) reared in freshwater and seawater in Japan

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Abstract: Rainbow trout is a globally important aquaculture species and has traditionally been cultured in freshwater. Recently, production of rainbow trout in seawater has been increasing due to its potential for faster growth relative to rearing in freshwater. However, a high mortality during adaptation from freshwater to seawater and growth retardation during subsequent rearing period often occur. This study aimed to evaluate the genetic potential for growth and survival of rainbow trout in seawater rearing and to validate the effectiveness of genomic selection (GS) for these traits. Two populations, designated as A and B, were subjected to both freshwater and seawater growth trials. Using GRAS-Di analysis, 11,105 biallelic SNPs were identified in these fish. Survival rate in the seawater rearing was 43.8% for population A and 49.8% for population B, respectively, with the mortality being observed among the sexually matured individuals. Body weight and standard length showed positive correlations between the values in freshwater and seawater (Pearson's r = 0.55). Narrow-sense heritability for growth traits was estimated using the GBLUP method, which revealed moderate values of heritability in seawater (0.57 for standard length, 0.50 for body weight). Genomic estimated breeding values (GEBV) were calculated and a correlation of 0.25 was obtained between GEBV for body weight in freshwater and seawater. Cross-validation demonstrated predictive accuracies of 0.80 and 0.54 for the body weights in freshwater and seawater, respectively. These results indicate a potential for genetic improvements through GS in the growth and survival of rainbow trout in seawater, providing valuable insights for the sustainable aquaculture practices.

Key words: rainbow trout, selective breeding, growth, SNP, GBLUP

Introduction

Rainbow trout (*Oncorhynchus mykiss*) is a globally significant aquaculture species due to its rapid growth, adaptability, and high market value (FAO 2022). This species, native to North America, has been successfully introduced and cultured worldwide. In Japan, it was first introduced in 1877, and since then has been continuously bred in various aquaculture facilities and research institutions (Hasegawa 2020; Uchino *et al*. 2021). Traditionally, rainbow trout aquaculture has primarily been practiced under freshwater environments. Recently, there is a growing trend towards seawater aquaculture of rainbow trout, as evidenced by the

Accepted on Feb 26, 2026
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successful practices in Nordic countries like Norway (Aas *et al*. 2022). Similarly, seawater aquaculture of this species in Japan has also been developing. Seawater rearing of rainbow trout offers various potential such as growth acceleration and larger-scale production. However, this challenge often brings a high mortality during seawater acclimation as well as growth retardation (Morro *et al*. 2021). Despite these problems and increasing importance of seawater aquaculture for rainbow trout, studies on the genetic potential to improve the growth and survival in seawater are still limited.

As for freshwater aquaculture, selective breeding of this species has been undertaken, focusing on selected key production traits such as growth and disease resistance, and contributed significantly to the sustainability and profitability of rainbow trout aquaculture (Yáñez *et al*. 2014; Yáñez *et al*. 2023). In recent years, the advance in the selection technologies such as genomic selection (GS) has begun to replace the traditional pedigree-based selection, as GS is a powerful tool for accelerating genetic improvement in aquaculture species (Song *et al*. 2023). For rainbow trout, developments of the technologies for constructing high-quality reference genome and obtaining genome-wide single nucleotide polymorphism (SNP) markers, including SNP arrays, genotyping-by-sequencing (GBS) and low-coverage whole-genome sequencing (lcWGS), have enabled the implementation of GS (Palti *et al*. 2015; Bernard *et al*. 2022; Liu *et al*. 2024). The effectiveness of GS in rainbow trout with the use of these genomic resources, has been validated for numerous traits such as high growth rate, disease resistance, thermal tolerance, and hypoxia tolerance (Yoshida and Yáñez 2022; Prchal *et al*. 2023; Yáñez *et al*. 2023). Thus, the applicability of GS to improve the growth and survival of rainbow trout in seawater culture should be evaluated. As a first step, this requires the acquisition of growth and survival data from seawater-cultured rainbow trout, coupled with the validation of trait heritability.

This study aims at evaluating the genetic potential for growth and survival of rainbow trout in seawater using GS. Specifically, we conducted growth trials under both freshwater and seawater rearing conditions using two rainbow trout populations, designated here as A and B. We collected growth data from both freshwater and seawater rearing conditions and subsequently compared them. Furthermore, genome-wide SNP data were obtained using the GRAS-Di method (Hosoya *et al*. 2019). The efficacy of genomic selection for growth in the seawater culture was then assessed by evaluating the genetic parameters and estimating genomic breeding values (GEBV) via the GBLUP approach. This research will provide valuable insights into the genetic architecture of key traits and contribute to the development of improved rainbow trout strains for sustainable and efficient aquaculture in Japan.

Materials and Methods

Experimental fish and growth trials in freshwater and seawater

The production of experimental fish and the freshwater growth trials were conducted at Fuji Trout Hatchery, Shizuoka Fisheries and Marine Technology Institute (Shizuoka, Japan). Population A was established on November 13, 2019, through a full diallel mating of seven parental fish from four strains: Shizuoka Donaldson, Shizuoka steelhead, Shizuoka 4-year-matured, and Shizuoka farmed broodstock. These four strains had been maintained at Fuji Trout Hatchery. Population B was established on December 2, 2020, through a full diallel mating of eleven parental fish from four strains: Shizuoka farmed broodstock, Shizuoka spring spawning, Tochigi farmed broodstock and Tochigi Donaldson. Of these four strains, the former two had been maintained at Fuji Trout Hatchery, and the latter two at Nikko Field Station, Fisheries Technology Institute, Japan Fisheries Research and Education Agency (Tochigi Japan). Fertilized eggs from populations A and B were disinfected with povidone-iodine solution (50 ppm) for 15 min before water hardening to prevent vertical transmission of *Flavobacterium psychrophilum* (Kumagai and Nawata 2010) and then placed in incubation trays at 10 °C until hatching. The hatched fish from both population A and B were reared in 10 °C freshwater at a flow rate of 1.8 kL/h. Fish were fed twice daily with commercial feeds (Twin Power, Nippon Nosan Kogyo, Kanagawa, Japan). The daily amount of feed supplied was determined based on the body weight and water temperature according to the Leitritz feeding chart. At approximately one year post-hatching, fish were injected with PIT tags for individual identification. Standard length and body weight were measured for population A on October 11, 2021 (698 days post-fertilization), and for population B on November 28, 2022 (726 days post-fertilization), to evaluate the growth performances during the freshwater rearing period.

Growth experiments of population A and B in seawater were performed at Miyazu Field Station, Fisheries Technology Institute, Japan Fisheries Research and Education Agency (Kyoto, Japan). Fish used in the seawater growth experiments were 751 days post-fertilization for population A and 737 days post-fertilization for population B. The fish were acclimated

to seawater in a stepwise manner. First, they were held for 24 h in 50% seawater (16‰ salinity), followed by 24 h in 75% seawater (25‰ salinity), and then they were transferred to UV-sterilized seawater (33-34‰ salinity) at a flow rate of 15 kL/h. Seawater rearing was initiated for population A on December 6, 2021, and for population B on December 11, 2022. The fish were fed five times daily with commercial feeds (Kurenai, Nippon Nosan Kogyo, Kanagawa, Japan). To prevent sexual maturation, a long-day photoperiod was provided from 05:00 to 21:00 using LED lights. The seawater rearing temperature for population A ranged from 10°C to 17°C, while that for population B ranged from 11°C to 18°C. The growth experiments ended for population A on May 19, 2022 (165 days after the start of seawater rearing), and for population B on May 17, 2023 (157 days after the start of seawater rearing). Standard length and body weight were measured at the conclusion of each experiment.

Genetic analysis

Tissue samples from the ventral fin were collected from 195 individuals in population A and 273 individuals in population B, and preserved in 99.5% ethanol. Genomic DNA was extracted using InnuPure C96 and smart DNA prep (a) (Analytik Jena, Jena, Germany), following the manufacturer's instructions. A GRAS-Di library was constructed (Hosoya *et al*. 2019), and sequence data were obtained by 150 bp paired-end sequencing using NovaSeq 6000 system (Illumina, San Diego, CA, USA). Library construction and sequencing were performed by Genebay Co., Ltd. (Kanagawa, Japan). Illumina Nextera adapter sequences and low-quality reads were removed using Trimmomatic v.0.39 (Bolger *et al*., 2014) with the following parameters: LEADING, 30; TRAILING, 20; SLIDINGWINDOW, 4:25; ILLUMINACLIP, 2:30:10; MINLEN, 50. The obtained reads were mapped to the rainbow trout reference genome sequence (Omyk_2.0, 2.3 Gb in genome size) using snap-aligner (Zaharia *et al*. 2011; Ali *et al*. 2025) with the following parameters: -so -F s -F b. Variant calling was performed using elPrep 5 version 5.1.3 (Herzeel *et al*. 2021), and Genome Analysis Toolkit (GATK) version 4.4 with Best Practices Workflow (Van der Auwera *et al*. 2014). SNP filtering using vcftools criteria was set as a maximum read depth of 200 and a minimum genotype quality of 20 (Danecek *et al*., 2011). Missing genotypes in the SNP data were imputed using LinkImputeR software version 1.2.4 (Money *et al*. 2017), with a minimum read depth of 6, a call rate of 0.6, and a minor allele frequency of 1%. Heritability and GEBV were estimated using the GBLUP method with the rrBLUP package version 4.6.1 in R (Endelman 2011), using the SNP data and standard length or body weight under the freshwater or seawater rearing conditions. The prediction accuracy of GEBV for each trait was validated using a 5-fold cross-validation with 10 repetitions.

Results and Discussion

Phenotypes

The phenotypic values of growth-related traits in each population are summarized in Table 1. In population A, phenotype measurements were performed of 445 individuals after freshwater rearing for 698 days post fertilization. The average body weight was 0.92 kg, ranging from 0.41 kg to 1.32 kg. The average standard length was 36.8 cm, ranging from 27.5 cm to 41.8 cm. Subsequently, seawater rearing was conducted for 165 days. The number of individuals surviving at the end of seawater rearing was 195, with a survival rate of 43.8% (Fig.1A). Among the 250 individuals that had died during the seawater rearing period, 44 individuals had developed gonads (ovary or testis). All these individuals died in an early stage of seawater rearing (within 1 month). In the seawater rearing of Atlantic salmon (*Salmo salar*), sexually matured individuals, particularly precocious males, often present a high mortality due to maturation having a negative impact on seawater adaptation (Stien *et al*. 2013). Our results also suggest that the progression of sexual maturity reduces their seawater adaptability. At the end of the seawater rearing period, the average body weight was 2.14 kg, ranging from

Table 1 Growth performance of populations A and B in freshwater and seawater rearing trials

Population		Number of fishes	Date	Standard length (cm)			Body weight (kg)		
				ave.	min.	max.	ave.	min.	max.
A	freshwater	445	211011	36.8	27.5	41.8	0.92	0.41	1.32
	seawater	195	220519	46.1	31.5	58.6	2.14	0.40	4.56
B	freshwater	558	221128	32.4	24.5	39.6	0.63	0.20	1.11
	seawater	273	230517	40.3	28.5	52.7	1.45	0.26	3.49

0.40 kg to 4.56 kg. The average standard length was 46.1 cm, ranging from 31.5 cm to 58.6 cm. Among the 195 individuals that survived at the end of seawater rearing, 26 individuals decreased their body weights compared to the initial weights just before the seawater rearing. These individuals were unlikely to have adapted well to seawater, resulting in almost fasting and subsequent weight loss.

In population B, phenotype measurements were performed of 548 individuals after freshwater rearing for 726 days post fertilization. The average body weight was 0.63 kg, ranging from 0.20 kg to 1.11 kg. The average standard length was 32.4 cm, ranging from 24.5 cm to 39.6 cm. Subsequently, seawater rearing was conducted for 157 days. The number of individuals surviving at the end of seawater rearing was 273, with a survival rate of 49.8 % (Fig.1B). Of the 275 dead individuals examined during the experiment, testis was developed in 31 males and ovary was developed in 1 female. Among these matured individuals, one male died on December 17, 2022, at the beginning of seawater rearing, suggesting a lack of seawater adaptability. The other 31 matured individuals died between February 7th, 2023 and May 16th, 2023. The mortality of fish occurred in March and April could be attributable to the spring maturation rather than the winter maturation. At the end of the seawater rearing period, the average body weight was 1.45 kg, ranging from 0.26 kg to 3.49 kg. The average standard length was 40.3 cm, ranging from 28.5 cm to 52.7 cm. Among the 273 individuals that survived at the end of seawater rearing, 45 individuals decreased their body weights compared to the initial weights just before the seawater rearing. Similar to population A, these individuals were unlikely to have adapted well to seawater, resulting in almost fasting and subsequent weight loss.

The body weights of populations A and B were compared at the end of freshwater rearing and at the end of seawater rearing (Fig.2). A positive correlation was noted between the body weights in the freshwater rearing and seawater rearing. The Pearson's correlation coefficient was 0.55. The seawater rearing seemed to result in a larger dispersion of body weight. This suggests that while individuals with larger body weights after the freshwater rearing tended to be still larger even after the seawater rearing, certain factors such as seawater adaptability may have prevented them from growing consistently in the seawater environment.

Genetic analysis

Following the library preparation for GRAS-Di analysis, 150 bp paired-end sequencing was performed using the

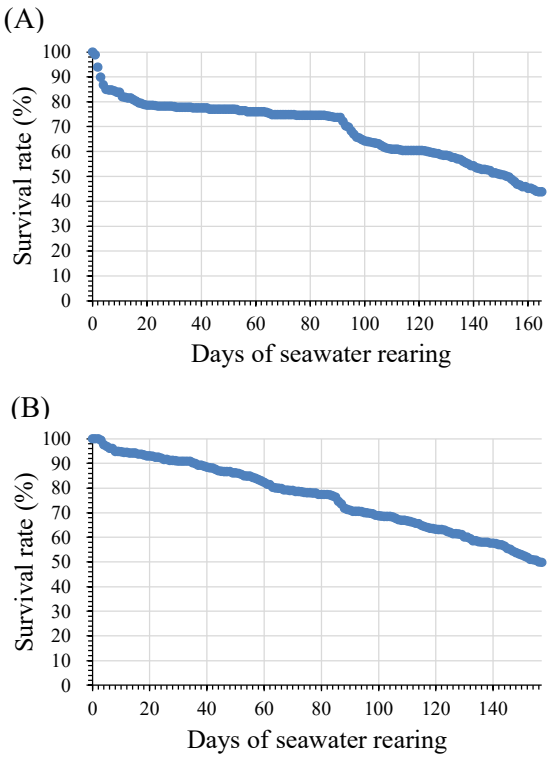

Fig.1 Survival rate after seawater exposure

X-axis represents the number of days after the start of seawater rearing, and Y-axis represents the survival rate (%). (A), population A; (B), population B.

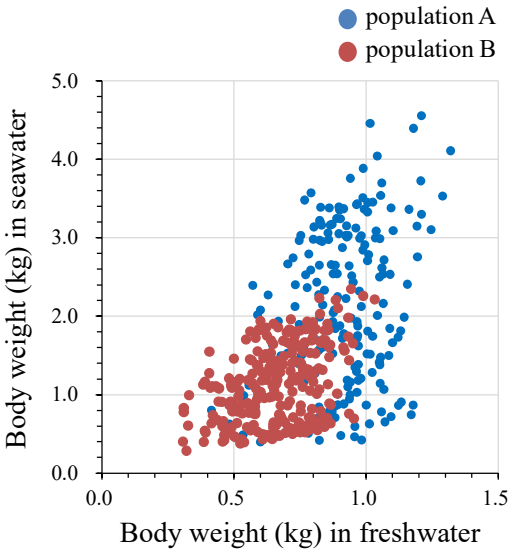

Fig.2 Comparison of body weight in freshwater and seawater

X-axis represents the body weight at the end of freshwater rearing, and Y-axis represents the body weight at the end of seawater rearing. Blue dots indicate the values of population A, and red dots indicate the values of population B.

Table 2 Heritability and prediction accuracy of genomic estimated breeding values for growth-related traits of populations A and B in freshwater and seawater

	heritability		prediction accuracy	
	standard length	body weight	standard length	body weight
freshwater	0.75	0.64	0.76	0.80
seawater	0.57	0.50	0.55	0.54

NovaSeq 6000 system. This sequencing was conducted on the 195 individuals from population A and the 273 individuals from population B, all of which survived at the end of seawater rearing. The sequencing yielded an average of 3.97 million reads and an average of 0.54 Gb per individual. Variant calling yielded 11,105 biallelic SNPs. The narrow-sense heritability for growth traits, specifically body weight and standard body length, was estimated by the GBLUP method using SNP data and is shown in Table 2. The heritability of standard length in the freshwater rearing was 0.75, while that of body weight was 0.64. The heritability of standard length and body weight during the seawater rearing was 0.57 and 0.50, respectively. In the previous report in salmonids, the heritability estimates for body weight measures fell within the range of 0.19-0.50 (Gonzalez-Pena *et al*. 2016). It is important to note that these heritability estimates were obtained in freshwater rearing condition, not in seawater rearing condition (Gonzalez-Pena *et al*., 2016). In the current study, the heritability values of standard body length and body weight in the seawater rearing condition were considered moderate, suggesting a potential for genetic improvement. GEBV for body weight during the freshwater rearing and seawater rearing were estimated using the GBLUP method and then compared (Fig.3). The Pearson's correlation coefficient between GEBV for body weight in freshwater and those in seawater was 0.25. This suggests that the genetic architecture that influence the body weights in the freshwater and seawater environments have some differences. Therefore, selection for body weight in seawater is crucial for the effective selective breeding of rainbow trout that grow rapidly in seawater. The predictive accuracy of GEBV was evaluated by applying cross-validation. In the freshwater rearing period, the predictive accuracy of GEBV for the standard length was 0.76, and that for the body weight was 0.80 (Table 2). In the seawater rearing period, the predictive accuracy of GEBV for the standard length was 0.55, and that for the body weight was 0.54. Gonzalez-Pena *et al*. (2016) reported a prediction accuracy of GEBV as 0.7 for body weight during freshwater

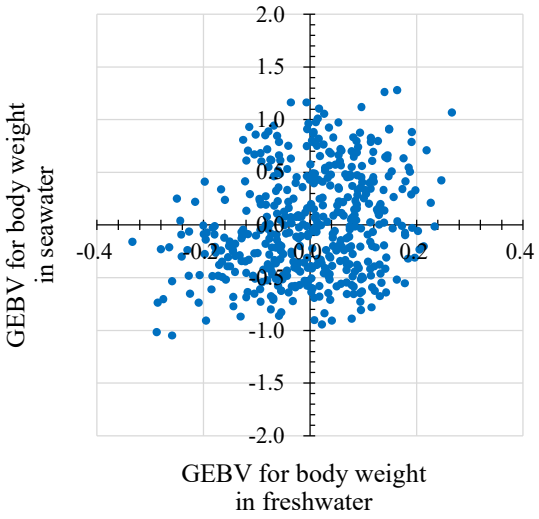

Fig.3 Comparison of genomic estimated breeding values for body weight reared in freshwater and seawater

X-axis represents genomic estimated breeding values (GEBV) for body weight at the end of freshwater rearing, and Y-axis represents GEBV for body weight at the end of seawater rearing.

rearing. In the present study, we obtained a slightly higher prediction accuracy for body weight during the freshwater rearing. The moderate prediction accuracy of GEBV for growth in seawater indicates a benefit potential for the improvement in selection efficiency. Selection of candidate parents for broodstock from individuals rearing in seawater is considered the most effective approach for selective breeding for higher growth in this species. However, returning potential broodstock to freshwater from seawater before use in spawning is often difficult for many rainbow trout farms due to concerns about introducing diseases from external sources. Meanwhile, genomic selection offers a viable alternative approach when availability of future broodstock rearing in seawater is limited. This method suggests that if there are groups of the same origin reared in both seawater and freshwater, potential broodstock can be indirectly selected from the freshwater group based on their GEBV for seawater growth.

Acknowledgements

This study was supported by a grant from "Salmon Aquaculture Promotion Technology Development" project under "Aquaculture Growth Industrialization Technology Development Program" in Fisheries Agency, Government of Japan. We would like to express our sincere gratitude to Dr. T. Yada and the laboratory members of Nikko Field Station of Fisheries Technology Institute, Japan Fisheries Research and Education Agency, for their cooperation in providing the gametes obtained from their rainbow trout strains.

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水研機構研報, 第 53 号, 79 – 88, 令和 8 年
Bull. Jap. Fish. Edu. Agen. No. 53, 79-88, 2026

Monosex breeding technology for sablefish aquaculture: a mini-review

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Abstract: The process of sexual differentiation (i.e. development of ovaries or testes) in fishes is much more plastic than for humans or other mammals. Even in fish species for which genetic sex determination systems have been well described, exogenous factors such as high temperature or steroid exposure may override those genetic controls and induce sex reversal—a mismatch between genetic and phenotypic sex. Sablefish *Anoplopoma fimbria*, also known as black cod (or "gindara" in Japan), is an important United States fishery and emerging marine aquaculture species, and a focal species for intensive aquaculture research and development at the Northwest Fisheries Science Center (NWFSC, Seattle, Washington, USA).

Upon initiating our work with sablefish, we recognized that it exhibits sexually dimorphic growth, with females growing significantly faster and attaining a larger body size than males, a phenomenon seen in many other species as well. This led to a major goal to develop a non-GMO method to produce monosex female (100% female) sablefish stocks for aquaculture to capitalize on their faster growth. A series of studies was conducted to gain an understanding of the basic processes of sex determination and differentiation (e.g., When do the gonads differentiate? What molecular and morphological changes signal the occurrence of gonadal sex differentiation?) and to develop an effective method for feminization of sablefish populations. The labile period of sex differentiation was successfully characterized and indirect feminization was achieved. First, sex differentiation of XX-genotype fish was redirected toward testicular development instead of ovarian development using dietary 17 alpha-methyltestosterone (MT) treatment during the labile period. Second, putative neomale (i.e., XX-genotype male) broodstock were obtained and ultimately bred with normal female broodstock. Offspring resulting from neomale × female crosses were 100% female, while those from control male × female crosses were ~50% female. Sperm from neomale sablefish broodstock could then be routinely utilized to generate all-female populations without the use of hormones, allowing for semi-commercial evaluations of their aquaculture performance.

Economic analyses were conducted using data obtained from the wild fishery, industry, and our own grow-out trials. This highlighted the importance of this monosex breeding technology on the economics of sablefish aquaculture production, including the reduction of time to harvest. Current research seeks to address common reproductive problems observed in neomale sablefish broodstocks through the use of alternatives to MT treatment (aromatase inhibitors) that allow for more 'natural' testicular differentiation to occur in neomales.

In summary, the advancement of monosex breeding technology for sablefish not only enhances the economic viability of sablefish aquaculture but also sets a precedent for sustainable practices in the industry. The successful development of hormone-free methods to produce all-female populations represents a significant step

Accepted on Feb 26, 2026
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toward optimizing growth performance while minimizing potential environmental impacts. This research contributes valuable insights that can be applied to other aquaculture species, supporting the industry's shift toward more sustainable and efficient production practices.

Key words: aquaculture; reproductive physiology; sex differentiation; economics

Background on Sablefish Aquaculture Development

Over half the world's seafood now comes from aquaculture (FAO 2024). The United States is a leading consumer of seafood; however, it recently ranked 17th in aquaculture production worldwide and continues to import much of its farmed faish. To ameliorate this issue, the United States Department of Agriculture (USDA), National Oceanic and Atmospheric Administration (NOAA), and other agencies and research institutes have begun to identify focal species for aquaculture development in the USA to bolster its aquaculture production (e.g., the "Status of Marine Finfish for USA Aquaculture" project and associated species-status publications in Journal of the World Aquaculture Society in 2021).

One such focal species is sablefish (*Anoplopoma fimbria*), often marketed in the USA as "black cod" and in Japan as "gindara." Sablefish is a long-lived, deepwater fish native to the North Pacific Ocean. It is a relatively hardy species that exhibits fast growth in the aquaculture environment (Goetz *et al*. 2021). In addition, there are strong, established markets for sablefish in Japan and other parts of Asia, as well as growing markets in the USA, Europe, and Canada (Hartley *et al*. 2020). At NOAA's Northwest Fisheries Science Center (NWFSC; Seattle, Washington, USA), a multidisciplinary approach to the development of sablefish aquaculture was taken over the past decade. It was seen as critical to first gain a basic understanding of aspects of sablefish biology that are important to aquaculture, such as reproduction, growth, nutrition, and immunology. This involved NWFSC researchers with expertise in various scientific disciplines working together to study sablefish in captivity. Specific research projects focused on improving or refining methods for reproduction of captive broodstocks, larval rearing, and grow-out. One focal area for our own laboratory has been the development of methods for all-female (monosex) production of sablefish, which is the focus of this mini-review.

Capitalizing on Sexually Dimorphic Growth

When we began working with sablefish in captivity, one aspect of its biology we recognized was a distinct sex-associated growth pattern. Our observations of captive fish supported earlier reports in the fisheries literature demonstrating that female sablefish achieve larger sizes than males (Mason *et al*. 1983; Echave *et al*. 2012), a phenomenon known as sexual growth dimorphism or sexually dimorphic growth, which is also observed in many other fishes (Martínez *et al*. 2014). Studies were conducted in our laboratory with juvenile sablefish to determine whether sexually dimorphic growth occurs in cultured fish prior to them reaching the typical harvest size of ~2.5 kg. If so, its impacts could be significant, whereas, if it occurs after harvest size it might have little or no impact on sablefish aquaculture. We found that sexually dimorphic growth indeed occurs in the aquaculture setting and females and males diverge in body weight and length prior to reaching the target harvest size (Luckenbach *et al*. 2017; Hartley *et al*. 2020).

This finding led to a major objective to develop a non-GMO approach to produce all-female (monosex) sablefish populations for aquaculture to capitalize on their faster growth relative to males. Monosex female stocks were anticipated to grow more rapidly than mixed-sex (i.e., mixed male and female) stocks, thereby reducing the duration of grow-out prior to harvest and labor costs for industry operations. If such stocks could be obtained, they would provide significant economic benefits to growers and bolster marine aquaculture production, potentially offsetting the domestic trade imbalance for seafood noted above.

Understanding Sex Determination and Differentiation in Sablefish

Fish exhibit a high degree of sexual plasticity relative to mammals (Nagahama *et al*. 2021; Yamamoto and Luckenbach 2024). Species may exhibit environmental sex determination, whereby abiotic factors (e.g., water temperature) or social/ behavioral cues influence their sex; genetic sex determination, whereby underlying genetically driven signaling determines their sex; or a combination of genetic and environmental sex determination. Regardless of what mechanisms are at play,

and even in species with strictly genetically controlled systems, the sexual phenotype of fish may be readily influenced by exposure to hormones or other factors during the labile or critical sensitive period of sex differentiation (Luckenbach *et al*. 2023; Yamamoto and Luckenbach 2024). This period typically corresponds with fish embryonic or larval development when the gonads are sexually undifferentiated and thus sensitive to exogenous perturbations.

To identify the labile period of sexual differentiation and develop research 'tools' to understand and monitor this process in sablefish, we conducted a series of studies in our laboratory. One study sought to characterize the morphological changes sablefish gonads undergo during sex differentiation using histology. An ontogenetic series of gonad tissue samples was collected to track the development of sexually undifferentiated gonads into either testes or ovaries ("morphological sex differentiation;" Luckenbach and Fairgrieve 2016). We found that morphological sex differentiation was well correlated with fish length, first apparent in fish ~80 mm long, and was completed in all individuals by 150 mm. Thus, we concluded that ~70-150 mm is the period of morphological sex differentiation for sablefish (Fig.1).

Other studies focused on characterizing earlier events associated with sex determination and differentiation (Rondeau *et al*. 2013; Smith *et al*. 2013; Hayman *et al*. 2021; Herpin *et al*. 2021). Advanced molecular biology approaches such as next-generation sequencing and quantitative RT-PCR were used to identify sexually dimorphic genes expressed in the gonads that may regulate the development of either testes or ovaries (Smith *et al*. 2013; Hayman *et al*. 2021). This research provided insights into the sequence of molecular events associated with gonadal sex differentiation (i.e., "molecular sex differentiation;" Fig.1), which precedes morphological sex differentiation, and established robust molecular markers to distinguish each gonadal phenotype (Smith *et al*. 2013; Hayman *et al*. 2021). Molecular markers of sablefish sex differentiation include genes encoding transcription factors, steroidogenic enzymes, gonadotropin and steroid receptors, and transforming growth factors.

Complementary work conducted in collaboration with researchers at the National Research Institute for Agriculture, Food and Environment (INRAE; Rennes, France) and academic partners led to the identification of the sablefish male sex-determining gene, *gonadal soma-derived factor on the Y chromosome* (*gsdfY*), and revealed the ontogeny of its expression and potential mechanisms of action (Herpin *et al*. 2021). The *gsdfY* gene was the ontogenetically earliest sexually dimorphic gene that we identified and showed a male-specific expression pattern that began shortly after embryos had hatched (Herpin *et al*. 2021). Results of this research revealed that sablefish *gsdfY* had evolved through gene duplication and subfunctionalization to become the master sex-determining gene.

Altogether, these basic studies of early reproductive development provided a blueprint of the normal process of gonadal sex differentiation in sablefish (Fig.1) that, as described below, informed our targeted manipulation of the process to create all-female populations via indirect feminization. This approach did not involve the use of genetic modification techniques and instead relied on the inherent plasticity of gonadal sex differentiation in fishes.

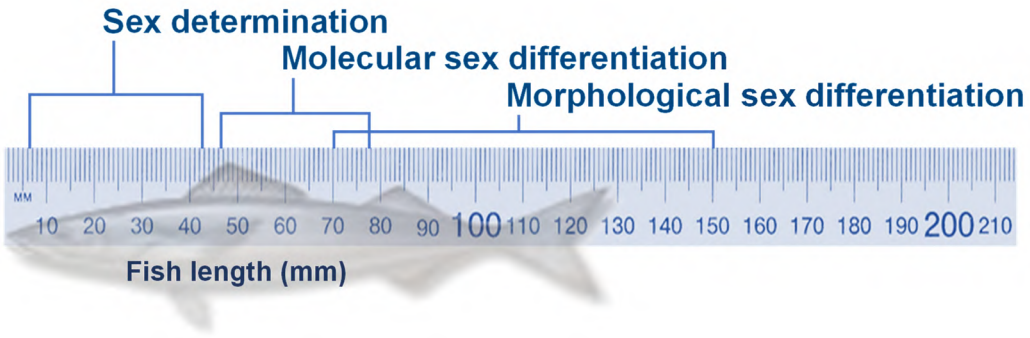


Fig.1 Schematic depicting the developmental timing of sablefish (*Anoplopoma fimbria*) sex determination and gonadal sex differentiation, which were well correlated with and predictable based on fish body length

Data to inform each period were generated through a series of experiments tracking ontogenetic development of sablefish produced and reared in captivity (Smith *et al*. 2013; Luckenbach and Fairgrieve 2016; Hayman *et al*. 2021; Herpin *et al*. 2021).

An Indirect Feminization Strategy

As we set out to devise a non-GMO strategy for monosex female production of sablefish, several potential approaches were considered. One such approach was 'direct feminization' whereby young, sexually undifferentiated fish can be exposed via the diet to low levels of estrogen to promote ovarian differentiation in 100 % of the treated fish (Piferrer 2001; Luckenbach *et al*. 2017). The caveat with this approach, however, is that the food fish (i.e. those intended for human consumption) would be directly treated with exogenous hormones. An alternative and preferred approach was 'indirect feminization,' which often entails treating sexually undifferentiated fish with an androgen, most commonly 17 alpha-methyltestosterone (MT), to induce masculinization of the genetically female proportion of the fish (e.g. the XX proportion in species with an XX/XY sex determination system) (Fig.2). If successful, these will develop as "neomales"—genotypic female individuals with functional testes. The neomales can be grown over several years post-treatment until they reach sexual maturity; they are then crossed with female broodstock to potentially generate all-female offspring in the filial-one (F1) generation (Fig.2). Conceptually, the resulting progeny will be 100% genotypically female and should develop normal ovaries if no unintended environmental effects on sex determination occur. Given the indirect nature of this approach, the food fish are never treated with exogenous hormones, which is preferred for safety and environmental sustainability.

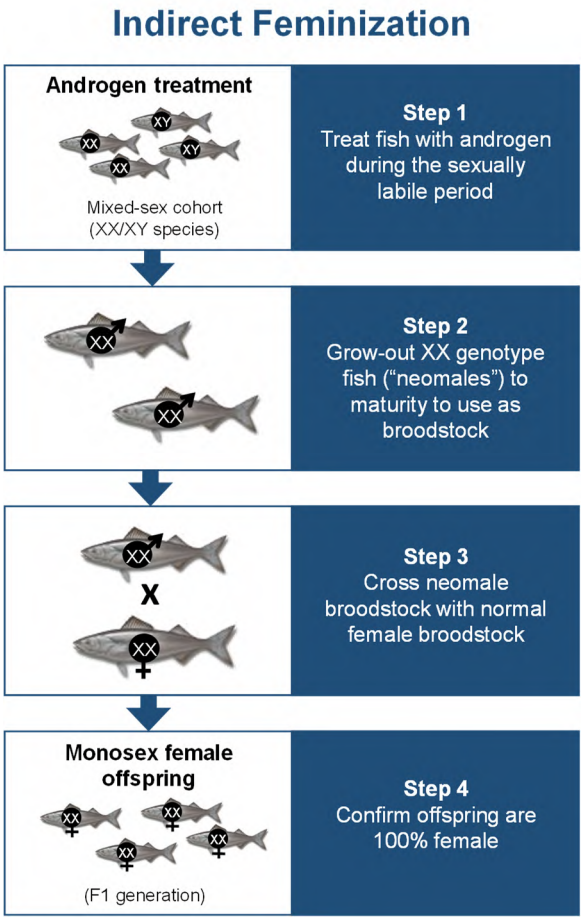


Fig.2 Schematic depicting the indirect feminization approach used to produce monosex female stocks of sablefish via dietary 17 alpha-methyltestosterone (MT) treatment

Modified from Luckenbach *et al*. (2017).

As a first step in the process of indirect feminization (Fig.2), dietary treatment of juveniles with MT was applied so that the treatment spanned the entire period of sex differentiation in an attempt to generate neomale sablefish. The intention was that female genotype fish in the treated population would be redirected toward testicular instead of ovarian development (see Luckenbach *et al*. 2017 for details). After a treatment duration of 2-4 months the fish were provided a standard, untreated diet. After approximately two years of grow-out, the fish were subsampled to evaluate their genotypic and phenotypic sex and maturity status. Gonadal histology and genotypic sexing using the sex marker *gsdf* demonstrated that some genotypic females treated with MT had testes with tubules full of spermatozoa, a promising result (Luckenbach *et al*. 2017).

The remaining fish were reared for an additional year (until reaching 3 years old) and checked for maturation in winter. At that time, some of the neomales and normal males (controls) were producing milt and were used in targeted breeding crosses with normal female broodstock (Fig.2). The results of those crosses clearly demonstrated the success of indirect feminization. As shown in Table 1, eight control male × female crosses yielded an average of 55 % female offspring (not significantly different from a 1 female:1 male sex ratio), while 10 neomale × female crosses yielded 100 % female offspring (Luckenbach *et al*. 2017). Results were consistent between genotypic sex ratios for sampled embryos (determined by the *gsdf* sex marker; Rondeau *et al*. 2013), and later evaluations of the same populations for phenotypic sex (i.e., genotypic females all developed normal ovaries). This study also established that sablefish exhibit an XX/XY-type sex determination whereby the presence or absence of a 'Y chromosome' determines the sex of resulting offspring (Luckenbach *et al*. 2017). In the years that have followed this breakthrough, no phenotypic males have ever been observed

Table 1 Genotypic sex ratios for F1 embryos resulting from control male × female crosses and neomale × female crosses. Summarized from Luckenbach *et al*. (2017)

Control Crosses	Genotypic Sex		Female (%)	Neomale Crosses	Genotypic Sex		Female (%)
	Male (n)	Female (n)			Male (n)	Female (n)	
1	15	26	63.4	1	0	42	100
2	16	15	48.4	2	0	30	100
3	12	23	65.7	3	0	31	100
4	12	19	61.3	4	0	31	100
5	15	16	51.6	5	0	35	100
6	14	18	56.3	6	0	33	100
7	19	12	38.7	7	0	31	100
8	15	16	51.6	8	0	31	100
				9	0	30	100
				10	0	31	100
		Average % female	54.6 ± 8.9			Average % female	100 ± 0.0

in the populations resulting from neomale × female crosses. Milt from neomale broodstock can now be routinely collected from existing broodstock and used to generate all-female populations without the use of hormones.

Of note, follow-up work in our lab has demonstrated that low proportions of neomales can also be generated by rearing genotypic female sablefish at high water temperatures throughout the period of sex differentiation (Huynh *et al*. 2019). However, this approach requires further investigation to evaluate its potential for aquaculture.

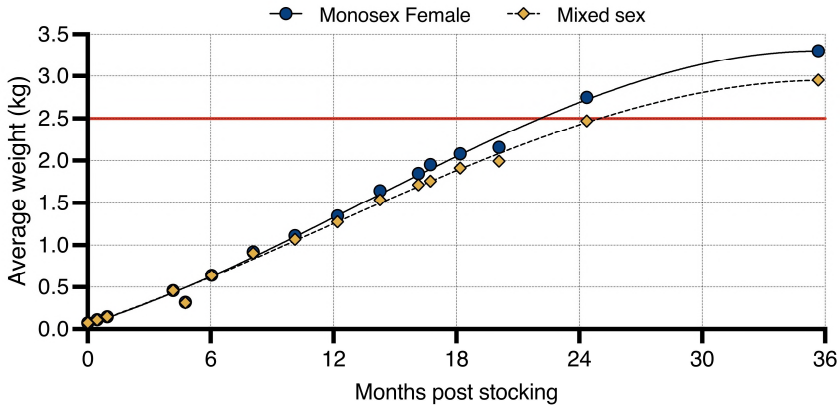
Economic Benefits of Monosex Female Production

After successfully producing monosex female stocks of sablefish, there was interest in knowing whether the growth rate advantages of using monosex stocks in place of mixed-sex would be a game changer for commercial-scale aquaculture of sablefish. The performance of monosex and mixed-sex stocks was evaluated under research conditions in trials conducted at the NWFSC Manchester Research Station (Port Orchard, Washington, USA). We found that mixed-sex and monosex fish grew equally well through the first winter and spring post-stocking, but that thereafter, the weight gain of male fish slowed dramatically compared with females. Net productivity of the mixed-sex stock was reduced by an increased time to harvest, greater losses due to higher total mortality, and a higher proportion of undersized, mostly male fish in the mixed-sex population. Based on this information, a semi-commercial grow-out trial was conducted with monosex female sablefish in marine net pens. The monosex sablefish grew rapidly and were ready for harvest at an average weight of 2.48 kg, almost 3 months earlier than a typical net pen stocked with mixed-sex sablefish (Hartley *et al*. 2020; Goetz *et al*. 2021).

A comprehensive econometric analysis was conducted using data obtained from the wild fishery, the small Canadian sablefish aquaculture industry, and our own grow-out trials (Hartley *et al*. 2020; Goetz *et al*. 2021). Cash-flow simulation models (Monte Carlo simulations) were developed to estimate the bottom-line impact of using monosex females instead of mixed-sex sablefish in net-pen aquaculture were developed. Two different simulations were run using actual weight-at-age data from our tank- and net-pen-based studies to make predictions (Fig.3A). In the first simulation, the grower harvests all of their fish at once, while in the second simulation, the grower selectively size-grades and harvests fish as they reach a market size of ~2.5 kg (Hartley *et al*. 2020). The scenario shown in Fig.3A reflects the former, where all fish are harvested after a fixed 24-month production cycle. In the simulation, both monosex and mixed-sex stocks were grown in commercial pens and harvested after 24 months, allowing for five complete production cycles to take place over a 10-year simulation period. We assumed fixed operational costs equivalent to commercial salmon net-pen farms that were operating in Puget Sound, Washington (USA), at that time. We also assigned size-based premium prices to the farmed fish that were equivalent to those for wild-caught sablefish as larger sablefish command a higher price, particularly in the Japanese market.

Results of the cash-flow simulations clearly demonstrated the advantages of farming monosex female stocks relative to mixed-sex stocks (Fig.3B; Hartley *et al*. 2020). Monosex

A



B

	Mixed sex	Monosex female	Difference
Average fish weight (kg)	2.4 kg	2.7 kg	+12.50%
Sized-based price ($/kg)	$9.42	$9.96	+5.70%
Operational costs ($/kg)	$5.82	$5.51	-5.30%
Gross margin ($/kg)	$3.60	$4.41	+22.50%

Fig.3 Growth rate data used for cash-flow simulations of monosex female versus mixed-sablefish stocks during the grow-out phase of aquaculture (A) and key information derived from the cash-flow simulation of a 2-year production cycle (B)

See Hartley *et al*. (2020) for details.

female sablefish had a higher average weight and received a better price. Because of the greater biomass produced, operational expenses consisting of feed, labor, purchase of fingerlings, and harvesting costs were reduced, leaving a higher gross margin. Overall, the cash-flow simulations predicted that using monosex female stocks would add 6-15% to the Internal Rate of Return (IRR) for growers compared to mixed-sex stocks (Hartley *et al*. 2020). In practical terms, a higher IRR translates to higher profits for growers who switch from farming mixed-sex to monosex stocks.

Conclusions, Challenges, and Future Directions

In summary, a multidisciplinary effort within NOAA has led to several key advances in sablefish aquaculture. Research on sexually dimorphic growth in this species paved the way for targeted research to capitalize on the superior growth of females relative to males through the development of methods for monosex female production. Basic studies were conducted using captively reared sablefish to first develop an understanding of sex determination and differentiation toward ultimately controlling those processes to generate monosex stocks to enhance sablefish aquaculture. Through the use of dietary MT treatment, indirect feminization of sablefish was achieved whereby monosex female offspring could be produced *en masse* via neomale broodstock without the use of genetic modification or exogenous hormone treatment.

Econometric analyses showed clear benefits of this monosex female breeding technology, including higher profits to growers when compared to previous mixed-sex farming practices. Most importantly, this technology has been adopted by industry: monosex sablefish are now being farmed in the United States and Canada. This line of research also generated immense biological and genetic information about sablefish, one of the highest-value fishery species in the United States on a per-weight basis (Hartley *et al*. 2020). Hence, information obtained should continue to be of value to sablefish aquaculture and fishery management well into the future.

The advancement of monosex breeding technology for sablefish not only enhances the economic viability of sablefish aquaculture but also sets a precedent for sustainable practices in industry. The successful development of indirect methods to produce all-female populations represents a significant step toward optimizing growth performance while minimizing potential environmental impacts. This research contributes valuable insights that can be applied to other aquaculture species, supporting the industry's shift toward more sustainable and efficient production practices.

Despite all of these successes, several challenges have been encountered with neomale sablefish broodstock that affect their utility for commercial-scale production of monosex offspring. The problems observed that may negatively affect

neomale broodstock performance or having sufficient gametes for spawning include anomalous testis morphology, inability to manually express and collect milt for spawning, and/or low sperm motility. For example, a high proportion of neomales possess testes that have anomalous morphology and intersex characteristics (Fig.4). Intersex gonads are not encountered in wild-sourced or untreated, captively reared sablefish, and therefore likely reflect incomplete masculinization by MT. It is therefore critical to refine the current MT treatment protocol to generate fully masculinized neomale broodstock for commercial-scale aquaculture.

Current research in our lab seeks to address the reproductive problems observed in neomale broodstock through the use of an alternative to MT treatment. Our approach was informed by examining the natural process of ovarian versus testicular differentiation in sablefish and emerging evidence in the field suggesting a lack of estrogens, and not necessarily the presence of androgens, is critical to testicular differentiation in fishes (Fig.5; Zhou *et al*. 2021). Since MT is an androgen, and neomales (genotypic females) likely have competing endogenous estrogen signaling, MT treatment does not mimic the natural process of testicular

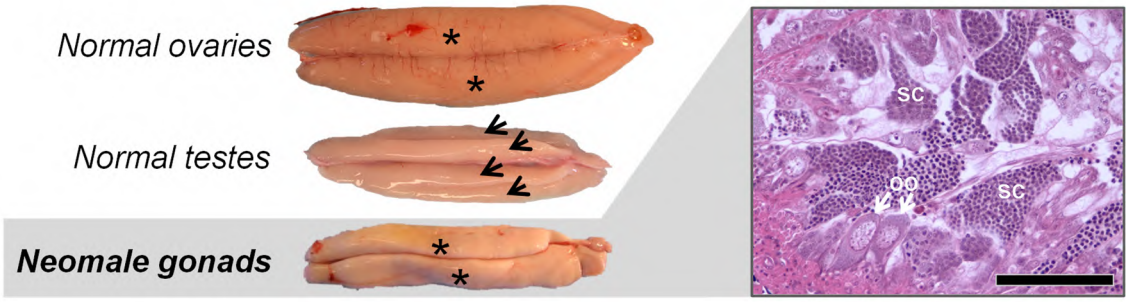


Fig.4 Typical gonadal morphology of female and male sablefish compared to that of a neomale sablefish generated by methyltestosterone treatment

Normal ovaries are cylindrical in shape and single-lobed (i.e., lacking folds) as indicated by asterisks, whereas normal testes are dual-lobed as indicated by arrows. Neomale gonads are single-lobed like ovaries and may contain both male and female germ cells (sc, spermatocytes; oo, oocytes), which are indicative of an intersex phenotype due to incomplete sex reversal. Scale bar = 100 µm. Images of normal gonads from Guzmán *et al*. (2017).

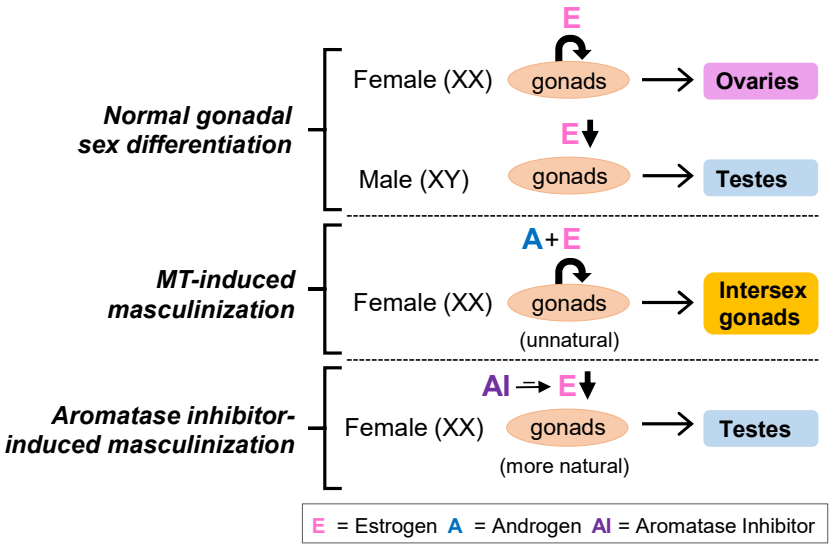


Fig.5 Conceptual diagram of the normal gonadal sex differentiation process; methyltestosterone (MT)-induced testicular differentiation, which overrides endogenous E2 effects; and aromatase inhibitor (AI)-induced testicular differentiation, which blocks E2 production and is viewed as a more 'natural' induction method

Curved arrows indicate the positive feedback of estrogen (E) production on the gonads.

differentiation. We hypothesized that a better approach would be to block estrogen production via treatment with an aromatase inhibitor (AI). AIs are widely used in human medicine and have also been vetted in fish to block estrogen synthesis by inhibiting aromatase/Cyp19a1, the rate-limiting enzyme responsible for the conversion of testosterone to estrogen (Piferrer 2001; Guiguen *et al*. 2010; Luckenbach *et al*. 2023). Thus, AI treatment may allow more 'natural' testicular differentiation to occur in neomales as opposed to the unnatural actions of MT (Fig.5). We are excited about this ongoing research using AIs to overcome prevalent issues with sablefish neomale broodstock—as well as those of other aquaculture species—and the future expansion of monosex breeding technologies to improve the economic and environmental sustainability of aquaculture.

Acknowledgments

This research was supported by a variety of funding sources including the NOAA Office of Aquaculture via Internal Competitive Aquaculture Funds (ICAF) and the NWFSC Internal Grants Program. The authors are grateful to S. Kim and A. Brown (NWFSC NOAA) for their helpful suggestions on an earlier version of the manuscript.

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This article reviews the state-of-the-art for fish sex determination and approaches to control sex ratios in aquaculture in order to capitalize on enhanced performance characteristics of either females or males. The primary characteristic of interest in aquaculture is growth, which may differ between sexes. This phenomenon, known as sexually dimorphic growth or sexual growth dimorphism, is common in fishes and may greatly influence the time to harvest, uniformity of harvested fish, and other aspects of aquaculture. The authors begin with an introduction on the main features of early gonadal development, including the processes of sex determination and gonadal sex differentiation (i.e., differentiation of the gonads into either ovaries or testes), as well as gonadal gene expression and morphological changes observed in most fish species. They also highlight the complex interplay between environmental and genetic factors that influence sex determination, with particular focus on how manipulating environmental conditions (e.g., temperature, hormones) or employing genetic breeding schemes can be used to control sex ratios. These approaches are of particular interest for improving productivity in aquaculture settings, where the selective production of a single sex can lead to faster growth rates or other desirable traits. A number of examples of sex-control strategies for various species, such as turbot, European sea bass, and tilapia are provided. Finally, the authors discuss the potential ethical and ecological implications of these methods, offering a comprehensive view of both the benefits and challenges of sex ratio manipulations in aquaculture.

(2) Luckenbach JA, Kikuchi K, Iwamatsu T, Nagahama Y, Devlin RH (2023) Chapter 11-The lasting impact of Toki-o Yamamoto's pioneering chapter on fish sex determination and differentiation: A retrospective analysis of its contributions to reproductive biology and influences on aquaculture and fisheries sciences. *Fish Physiol*., **40**, 401-419. https://doi.org/10.1016/bs.fp.2023.08.003

This chapter, developed in collaboration with Japanese and Canadian colleagues, provides a retrospective analysis of Dr. Toki-o Yamamoto's foundational work on fish sex determination and its continuing influence on modern aquaculture practices. While honoring Yamamoto's 1969 chapter in the famous *Fish Physiology* book series edited by Hoar and Randall, the authors place significant emphasis on how Yamamoto's research has informed current strategies for sex control and genetic selection in aquaculture. The text outlines key advancements in understanding the genetic and environmental mechanisms governing fish sex differentiation, offering insights into the development of techniques such as hormonal sex reversal and breeding of monosex populations. These methods, derived from Yamamoto's early findings, are

critical for enhancing fish production in aquaculture by enabling the manipulation of sex ratios to produce all-male or all-female stocks, depending on the desired traits (e.g., faster growth rates in one sex). The chapter also highlights the application of these approaches in various species and the potential for integrating genetic selection techniques to improve growth performance and disease resistance. Overall, the analysis underscores the enduring relevance of Dr. Yamamoto's work for optimizing genetic outcomes in aquaculture, making it a good resource for researchers and practitioners focused on sustainable fish production.

(3) Luckenbach JA, Fairgrieve WT, Hayman ES (2017) Establishment of monosex female production of sablefish (*Anoplopoma fimbria*) through direct and indirect sex control. *Aquaculture*, **479**, 285-296. https://www.sciencedirect.com/science/article/abs/pii/S0044848617306361

This paper focuses on the development of methods for both direct and indirect feminization of sablefish populations for aquaculture and establishes for the first time that sablefish have an XX/XY system of sex determination. For direct feminization, the authors administered estradiol-17 beta (E2) via the diet starting just after weaning for two or four months. They found that E2 treatment successfully feminized genetic males, as fish sampled at both time points all developed ovaries. For indirect feminization, 17 alpha-methyltestosterone (MT) was used, with the intention to masculinize genetic females. After four months of MT treatment, fish had all developed testes, while a two-month treatment resulted in intersex gonads, indicating incomplete sex reversal. Once sexually mature, the "neomales" (genetic females with masculinized traits) were crossed with females and their offspring were all female, confirming that sablefish have an XX/XY sex determination system. The study demonstrates that indirect feminization using neomales can yield all-female populations of sablefish for aquaculture without the need for hormone treatment of food fish. This approach, which avoids direct hormone treatment of market fish, offers significant advantages to the aquaculture industry by improving production and ensuring consumer safety.

(4) Hartley ML, Schug DM, Wellman KF, Lane B, Fairgrieve WT, Luckenbach JA (2020) Sablefish aquaculture: An assessment of recent developments and their potential for enhancing profitability. NOAA Technical Memorandum NMFS-NWFSC-159, Seattle, Washington, 89 p. https://doi.org/10.25923/cb0y-n468

This article provides a comprehensive assessment of the history of sablefish aquaculture and recent advancements in research, and evaluates their potential to improve the profitability of the industry. The authors, some of whom are economists, summarize technological innovations and biological research aimed at overcoming key production challenges, such as feed efficiency, broodstock management, and disease resistance. In particular, they emphasize developments in monosex female sablefish production and the use of novel feeds, both of which have the potential to reduce production costs and enhance growth rates. The authors report findings from the first economic simulation of monosex (all female) compared to mixed-sex (both females and males) grow-out for sablefish, which demonstrated a significant increase in the internal rate of return to growers. Additionally, the article explores market factors, such as consumer demand and pricing trends, offering insights into how improved production practices could meet market needs and increase profitability. This resource is important to stakeholders seeking to enhance the sustainability and economic viability of sablefish farming. Many of the principles therein may apply broadly to aquaculture of other fish species.

水研機構研報, 第 53 号, 89 – 98, 令和 8 年
Bull. Jap. Fish. Edu. Agen. No. 53, 89-98, 2026

Developing vaccination strategies for the prevention of atypical furunculosis in sablefish *Anoplopoma fimbria*

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Abstract: Sablefish (also known as black cod) represent a promising and high-value species for marine aquaculture. Research efforts to optimize culture strategies and methods have been ongoing for more than two decades. This species is commercially produced using a combination of land-based tank and marine net-pen aquaculture in British Columbia, Canada, and Washington, USA. To set the stage for expanded production of farmed sablefish, NOAA Fisheries has prioritized research projects and partnerships to address remaining challenges. One such challenge that impacts the production of this species is the disease furunculosis. This is currently the primary disease threat for farmed sablefish and is caused by atypical strains of *Aeromonas salmonicida*, a gram-negative bacterium. Although antibiotic treatments can be administered to reduce mortality following an outbreak, disease prevention through vaccination is desirable and has been identified as a high-priority need for further development of sablefish aquaculture. Recent vaccination projects have explored practical mass vaccination strategies along with more traditional approaches to prevent furunculosis. These include an oral vaccination study that assessed the efficacy of a killed whole-cell *A. salmonicida* vaccine administered orally via feeding alginate/gelatin micro-particles to juvenile sablefish. Another study that took a more traditional approach, assessed the efficacy of a primary immersion immunization and injection booster with and without adjuvant inclusion. Although more labor intensive, results indicated that this approach currently represents the most practical strategy to achieve long-term protection of sablefish against furunculosis. A final ongoing project that is discussed involves the development and testing of attenuated atypical *A. salmonicida* strains that could be administered via immersion during early juvenile stages. To produce such attenuated vaccine candidates, known virulent *A. salmonicida* strains were repeatedly passaged on tryptic soy agar (TSA) plates containing increasing concentrations of the antibiotics rifampicin and novobiocin. To achieve further attenuation, strains were acclimated to high incubation temperatures outside of their optimal growth range. Four isolates that grew under these conditions and showed resistance to these antibiotics have been confirmed as fully attenuated. These four strains now serve as potential vaccine candidates and are being assessed for their ability to protect sablefish from disease following immersion or injection immunization.

Key words: sablefish, furunculosis, vaccination, immunity

Accepted on Feb 26, 2026
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Introduction

Disease management in commercial aquaculture is critical to the success of most operations. Sablefish *Anoplopoma fimbria* represent an economically valuable marine species native to the Pacific Northwest of the United States and Canada. They are harvested commercially and have been farmed in net pens for nearly 20 years in Canada and more recently in the United States. Disease outbreaks do occur regularly in sablefish, resulting in direct mortality and reduced economic returns for farmed sablefish operations. The primary disease affecting this species is furunculosis, caused by an atypical strain of the bacterium *Aeromonas salmonicida* (Arkoosh *et al*. 2017; Goetz *et al*. 2021). Outbreaks of this disease can result in high mortality of fish during early juvenile stages and also reduce the marketability of later adult life stages due to clinical signs of the disease. Furunculosis is one of the oldest described bacterial diseases of fish and historically, *A. salmonicida* ssp. *salmonicida* was referred to as the 'typical' strain because of its early discovery and description in farmed salmonids (Wiklund and Dalsgaard 1998). However, 'atypical' strains and subspecies of *A. salmonicida* have since been isolated from non-salmonid hosts in freshwater and marine environments (Austin 2015). *Aeromonas salmonicida* can be identified phenotypically as a catalase and oxidase positive gram-negative, non-motile, non-encapsulated coccobacilli, that grows best between 22 to 25ºC (Austin *et al*. 1998; Gulla *et al*. 2016; Lian *et al*. 2020). Typical strains are generally diagnosed by the presence of brown pigmentation when cultured in the presence of tyrosine, and atypical strains are non-pigmented or exhibit reduced pigmentation (Donlon *et al*. 1983). Further genetic differences that separate typical and atypical strains based on gene expression and host affinity have been described (Vasquez *et al*. 2022).

In the 1980s, furunculosis vaccines were developed and implemented heavily in the Atlantic salmon industry. Midtlyng (2014) noted that in Scotland, salmon smolt survival to harvest increased from 65% to over 90% following the incorporation of injectable adjuvant-based bacterins. This shows that vaccination can be effective and protect against typical *A. salmonicida* strains in salmonids, but vaccines against atypical strains in non-salmonid fish species are less common. Early work to assess the potential of vaccinating sablefish has shown that certain injectable furunculosis vaccines can be effective against atypical *A. salmonicida* (Arkoosh *et al*. 2017). This is promising, but injection vaccination is not ideal due, in part, to the minimum size required for vaccination, labor costs, stress on the animal, and the inability to protect fish during susceptible early life stages. Therefore, alternative strategies that allow for mass vaccination of fish are desirable and include oral, immersion, or a combination of oral and immersion vaccinations with injection booster vaccinations when fish reach a larger size. This minipaper provides an overview of three studies aimed at developing new vaccines and practical delivery strategies to protect sablefish from disease caused by atypical strains of *A. salmonicida*.

In the first study, an oral vaccine platform that incorporated a formalin-killed *A. salmonicida* vaccine into liposome-containing complex alginate particles was developed and tested for its ability to elicit a protective immune response in sablefish. The primary objectives were to 1) encapsulate killed whole-cell *A. salmonicida* within alginate particles, and 2) evaluate experimental vaccines for protective immunity following pathogen challenge of sablefish.

The next study was aimed at the need to implement a practical vaccination strategy into production trials that are part of ongoing collaborations between NOAA Fisheries and the company Jamestown Seafood (operated by the Jamestown S'Klallam Tribe, Sequim, Washington, USA). To address this, a two-part study was conducted at NOAA's Manchester Research Station in Port Orchard, Washington (USA). This involved the semi-commercial scale grow-out of sablefish to market size in net pens or land-based tanks [flow through seawater or recirculated seawater (RAS)]. Previous production studies with sablefish at this site have resulted in furunculosis outbreaks and the need to treat fish periodically with antibiotics during the grow-out cycle. Vaccination of fish to prevent this disease has previously utilized commercial vaccines designed for salmonids and has met with limited success. Therefore, we evaluated a killed atypical *A. salmonicida* vaccine (utilizing strain KJ-1 – recently isolated from clinically diseased sablefish) by administering it to fish with or without adjuvant via an initial bath vaccination followed by an injection booster immunization. We hypothesized that bath immunization followed by an injection booster would provide long-term protection against furunculosis, and that vaccine efficacy would be enhanced by incorporating adjuvants. The primary objectives were to (1) vaccinate juvenile sablefish via immersion followed by a booster immunization at six weeks (approximately 50 g/fish), and (2) evaluate whether the inclusion of oil-based adjuvants during both immersion and booster injection administration enhanced the level of protection conferred.

The last project is ongoing and focuses on the development

of a live attenuated vaccine for sablefish with the goal of producing an efficacious immersion vaccine against atypical furunculosis. It was hypothesized that a live attenuated (non-virulent) atypical *A. salmonicida* vaccine could be developed and provide enhanced protection as a vaccine when compared to traditional killed whole-cell vaccine formulations. The primary objectives were therefore to 1) produce attenuated strains of atypical *A. salmonicida* using an antibiotic selection strategy, and 2) test live attenuated strains for their efficacy as vaccine candidates when delivered to sablefish by immersion or injection.

Overview of studies (objectives and methods)

1. Oral vaccination study

There were two trials conducted in this study to assess the potential of orally vaccinating sablefish using complex alginate particles (Fig.1). Alginate particles were produced using medium viscosity alginate and fish gelatin (Hawkyard *et al*. 2019). Liposomes containing compounds known to elicit feeding by marine finfish (betaine, alanine, and glycine) were included with these large alginate-gelatin particles resulting in 'complex particles'. In addition, the atypical *A. salmonicida* strain (T30), originally isolated from sablefish showing clinical signs of furunculosis (Arkoosh *et al*. 2017) was incorporated into alginate particles to achieve a high antigen dose. Several treatments were tested and included: 1) fish fed a sham control treatment consisting of alginate particles without killed-*A. salmonicida* cells (Sham_1); 2) fish fed a commercial diet (no particles) control (CC); 3) fish treated by injection vaccination (IV); 4) fish fed an oral vaccine particle (Oral_1) containing killed *A. salmonicida* and produced with the basic recipe described above; and 5) fish fed an oral vaccine particle (Oral_2) that integrated milled fish feed along with killed-*A. salmonicida* cells. All orally vaccinated treatments, including the sham, received the vaccine or sham particles as both primary and booster vaccinations. At 11 weeks post - primary vaccination, sablefish were challenged with atypical *A. salmonicida* strain T30, and mortality was monitored for 25 days to determine if vaccine treatments conferred protection. Relative percent survival (RPS) for each treatment was calculated and compared to the appropriate control/unvaccinated group using the following formula (Amend 1981):

RPS= 1 - [(% mortality in vaccinated fish) / (% mortality in unvaccinated reference fish)]*100

Following the initial trial, a second challenge trial was conducted using the atypical *A. salmonicida* strain with juvenile sablefish. This trial compared the protective efficacy of the oral vaccination methods described above with bath vaccination and combined oral–bath strategies.

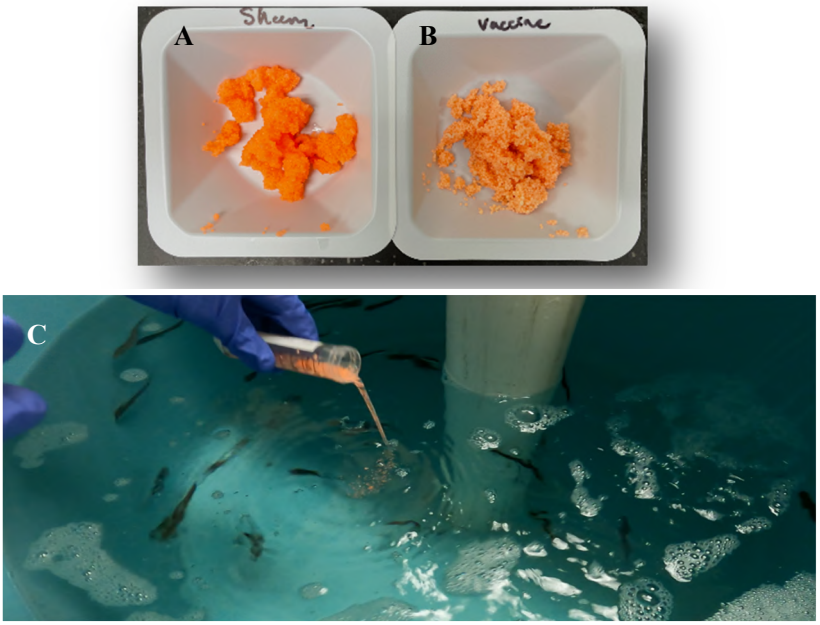

Fig.1 Complex alginate particles with or without killed whole-cell *Aeromonas salmonicida* incorporated as an oral vaccine

A, Sham particle (no vaccine); B, Alginate particle (with vaccine); C, Particles suspended in water and administered via feeding to fish (Photo Credit: Evan Jones).

2. Production scale fish vaccination study (adjuvant and booster assessment)

An initial small-scale experiment was designed that consisted of 1,500 fish (average 2.3 g/fish) split into three groups of 500 fish. **Group one** was immersion immunized for 30 minutes with a formalin-killed vaccine containing the KJ-1 *A. salmonicida* isolate diluted in seawater; **group two** was immersion immunized under identical conditions but with half the dose as the vaccine was mixed (50:50) with an adjuvant supplied by the company Seppic, Inc. (Fairfield, CT, USA); and **group three** was immersed in an equal volume of PBS diluted in seawater for 30 minutes and served as the sham control. At approximately six weeks post-primary immersion vaccination, 200 fish per group were injection-vaccinated with 100 µl of the killed KJ-1 isolate, with or without adjuvant. The negative control group received an injection of 100 µl of PBS. Groups were held in tanks receiving filtered seawater, and at 335 days post-vaccination, triplicate groups of fish from each treatment were challenged with the virulent KJ-1 strain of atypical *A. salmonicida*. Blood was collected from fish in each treatment throughout the study to determine specific serum anti-*A. salmonicida* antibody titers using an enzyme-linked immunosorbent assay (ELISA) recently developed by Jones *et al*. (2022).

In addition to the experiment described above, a production-level vaccination was implemented and 12,000 fish were immunized using the same vaccine treatments as described above, but an unvaccinated group was not included. Briefly, 6,000 fish received the primary bath and injection booster vaccinations with adjuvant and 6,000 fish received the vaccine treatments without adjuvant. These treatment groups were stocked (replicate pens or tanks/treatment) and grown to market size in either marine net pens (four pens with 2,000 fish/pen) or land-based tanks [four tanks (two flow through and two RAS) with 1,000 fish/tank] and monitored for mortality throughout the production cycle.

3. Development of a live attenuated atypical *A. salmonicida* vaccine

The methods for creating attenuated bacteria for use as vaccines are diverse (Ma *et al*. 2019). This study utilized an antibiotic-thermal selection strategy to induce random mutations, but did not involve genetic engineering. Briefly, four virulent parent strains of atypical *A. salmonicida* (T30, KJ-1, Spen-3, and K2-W), originally isolated from diseased sablefish, were selected and cultured in the presence of increasing concentrations of the antibiotics (rifampicin and novobiocin) as described by Pridgeon and Klesius (2011) with modifications. To achieve further attenuation, the same antibiotic-resistant strains were acclimated to incubation temperatures up to 30°C, which has been associated with the loss of virulence factors (Ishiguro *et al*. 1981). These thermal tolerant "resistant" strains were then tested to determine which were non-pathogenic (i.e. attenuated). Briefly, this was accomplished through *in vivo* challenge studies whereby, groups of juvenile sablefish were challenged by injection with either high doses of the mutant *A. salmonicida* strains or their corresponding virulent parent strain. Mortality was monitored for 14 days and mutant strains were considered completely attenuated if no infection-related mortality or clinical signs of disease occurred. Strains showing complete attenuation represent potential vaccine candidates and will be incorporated into future vaccination trials.

Results and Discussion

1. Oral vaccination in sablefish

To address the potential of an oral vaccine to be administered to sablefish and protect against atypical furunculosis, treatments were prepared and consisted of killed whole-cell *A. salmonicida* (T30 strain) incorporated into complex alginate particles. These particles were administered orally to fish as primary and booster immunizations in an initial trial. A second trial also combined alternating booster or primary bath immunization with the oral vaccine. Oral vaccines are challenging to develop and commercialize but are preferred because they are easy to apply and reduce handling-related stress to the fish. A key consideration for these vaccines is that for an effective immune response to be elicited in fish following oral vaccination, antigen degradation in the stomach must be minimized. Therefore, encapsulation methods using many materials have been described that can protect antigens until they are delivered to gut-associated lymphoid tissues (GALT) where antigen uptake and processing can occur (Ahmadivand *et al*. 2017; Jia *et al*. 2020). Results from the current study showed that fish fed a complex alginate particle vaccine had significant protection compared to negative control (unvaccinated) groups (Fig.2). However, the level of protection was significantly lower than that conferred by injection vaccination. The hybrid administration of the oral vaccine with a bath vaccine, either as a primary or booster vaccination, in a second trial, did not enhance protection compared to the oral primary and booster (data not shown). As expected, injection vaccination was highly protective

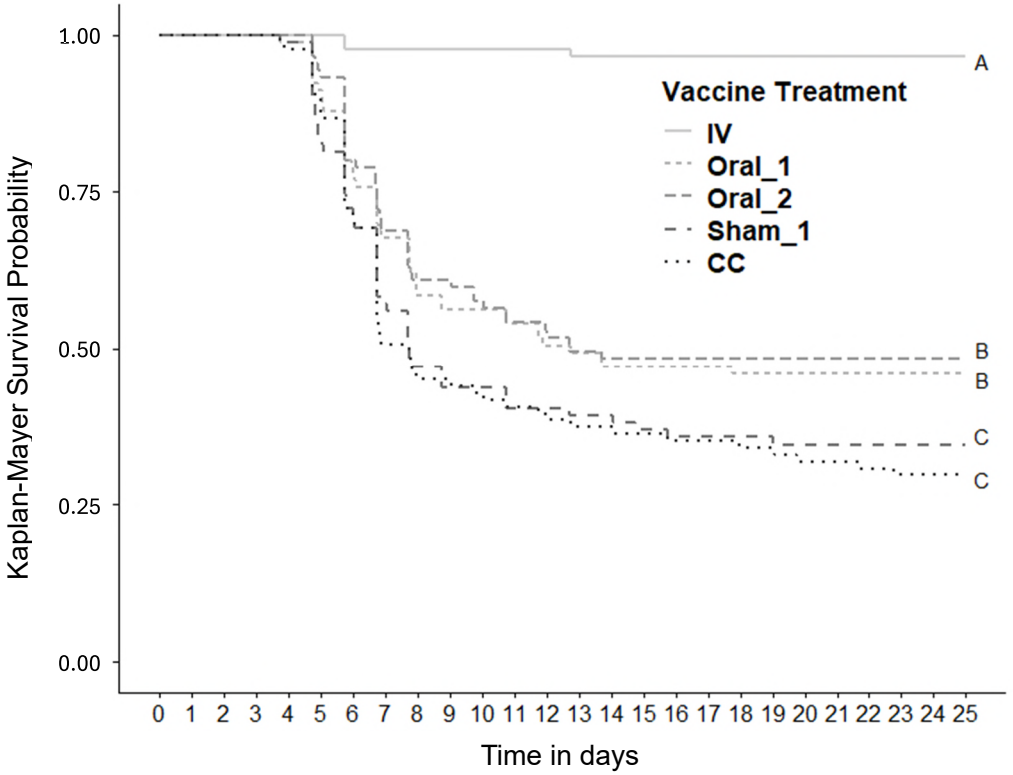

Fig. 2. Kaplan-Mayer survival curves following pathogen challenge for juvenile sablefish vaccinated by intraperitoneal injection (IV), orally using alginate particles (Oral_1, Oral_2), or unvaccinated (CC, Sham_1)

Different letters (A, B, or C) indicate significant differences between treatments.

with an RPS of 95%, whereas RPS values for oral vaccine treatments in these trials ranged from 17-21 % and were comparable to a bath vaccination with no booster, RPS 17% (data not shown). The study shows that oral vaccines can offer some protection against atypical furunculosis. The protection observed was similar to a bath-administered killed vaccine but was low compared to injection-based methods. Alternating the type of primary and booster administration between oral or bath vaccines did not enhance protection. The low RPS values in comparison to the injection vaccine indicate that additional research is required before such an oral vaccine would be commercially viable.

2. Production scale vaccination strategy

This study was designed to incorporate a bath vaccination when fish were small followed by an injection booster vaccination prior to stocking out into net pens or land-based grow-out tanks. Experiments were set up to test this approach in combination with or without the incorporation of newer adjuvants (Seppic, Inc) into the vaccine to determine if long-term disease protection could be conferred. Results from the experimental trials showed that sablefish vaccinated with or without adjuvant developed significant antibody responses by day 45 post-immersion vaccination. By day 90 (45 days post-booster) fish vaccinated with adjuvant showed significantly higher titers compared to fish not receiving adjuvant or fish in the control groups. In general, this trend continued out until day 335 (pre-challenge) and also held true for survivors of the pathogen challenge (Fig.3). Cumulative percent mortality following pathogen challenge showed that fish in groups vaccinated with or without adjuvant were highly protected (Fig.4) with RPS values of 77% and 85%, respectively. Interestingly, antibody titers were shown to be significantly enhanced in fish receiving the vaccine containing adjuvants; however, this was not reflected in higher protection for fish in these groups, at least when tested at nearly one year post-primary vaccination (Figs.3, 4). Furthermore, in the production-scale experiment, fish in the vaccine treatment groups never exhibited clinical signs of atypical furunculosis, and no *A. salmonicida* related mortality occurred in fish produced and grown to market size in marine net pens or land-based tanks. Overall, this strategy of vaccination proved highly effective at the both the experimental lab and field/production scale.

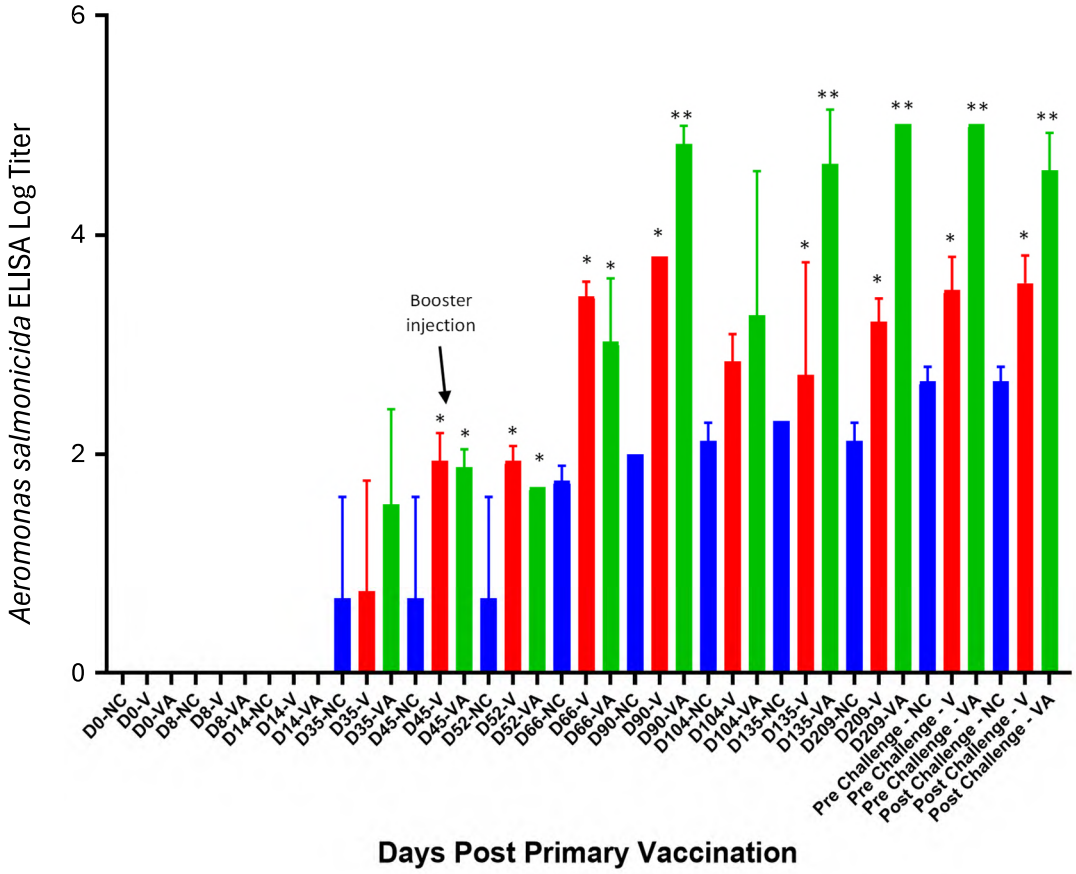

Fig.3 Specific serum anti-*Aeromonas salmonicida* antibody titers in sablefish following immersion vaccination and injection booster (day 45)

Antibody titers (as determined by ELISA) were determined from day 0 to day 335 (pre-challenge) and in surviving fish following pathogen challenge (post-challenge). Treatment groups included NC (unvaccinated groups receiving only PBS), V (vaccine only), VA (vaccine plus adjuvant). Different asterisks indicate significant differences from the control NC groups (*) or both the V and NC groups (**) at specific time points.

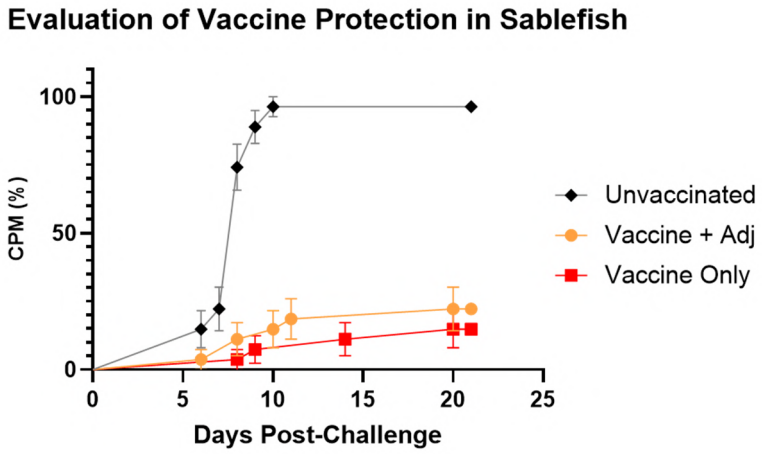

Fig.4 Cumulative percent mortality (CPM) of unvaccinated fish or fish vaccinated with a formalin killed-*Aeromonas salmonicida* vaccine with or without adjuvant

Fish were challenged at 335 days post primary vaccination and CPM calculated (triplicate groups) 21 days post-challenge with a virulent *A. salmonicida* strain.

3. Live attenuated *A. salmonicida* vaccine candidates

The final study described in this minipaper addresses the continued need for an effective vaccination strategy that does not require injecting large numbers of fish. The development of a live attenuated vaccine for atypical furunculosis in sablefish is being explored given the minimal protection previously observed with immersion vaccines comprised of killed whole-cell *A. salmonicida*. The use of live attenuated vaccines in the aquaculture industry can be effective and is not a new concept (Shoemaker *et al*. 2009). Since a live (non-pathogenic) pathogen is administered to fish, attenuated vaccines tend to stimulate a stronger immune response than killed vaccines, resulting in greater efficacy when delivered by immersion (LaFrentz *et al*. 2008; Ma *et al*. 2019). Here, an antibiotic-thermal selection method was applied to four virulent parent strains of atypical *A. salmonicida*. After multiple passages in the presence of rifampicin, novobiocin, and increased temperatures, 12 resistant strains were produced. These 12 strains were then used in a challenge study to determine if any had been partially or completely attenuated. Results from this challenge trial demonstrated that four of these resistant strains were completely attenuated; two stains produced from the T30 parent strain, one from the KJ-1 strain, and one from a Spen3 strain (Fig.5). These four strains now serve as potential vaccine candidates and will be further tested in upcoming vaccine trials to determine if long-term protection can be elicited in sablefish following immersion and injection vaccination. Preliminary trials with the KJ-1 resistant strain showed high protection following injection delivery and moderate protection via immersion vaccination (data not shown). Ongoing and future work will focus on evaluating all strains, quantifying dose requirements, duration of immunity, and developing alternative culture conditions to enhance vaccine efficacy.

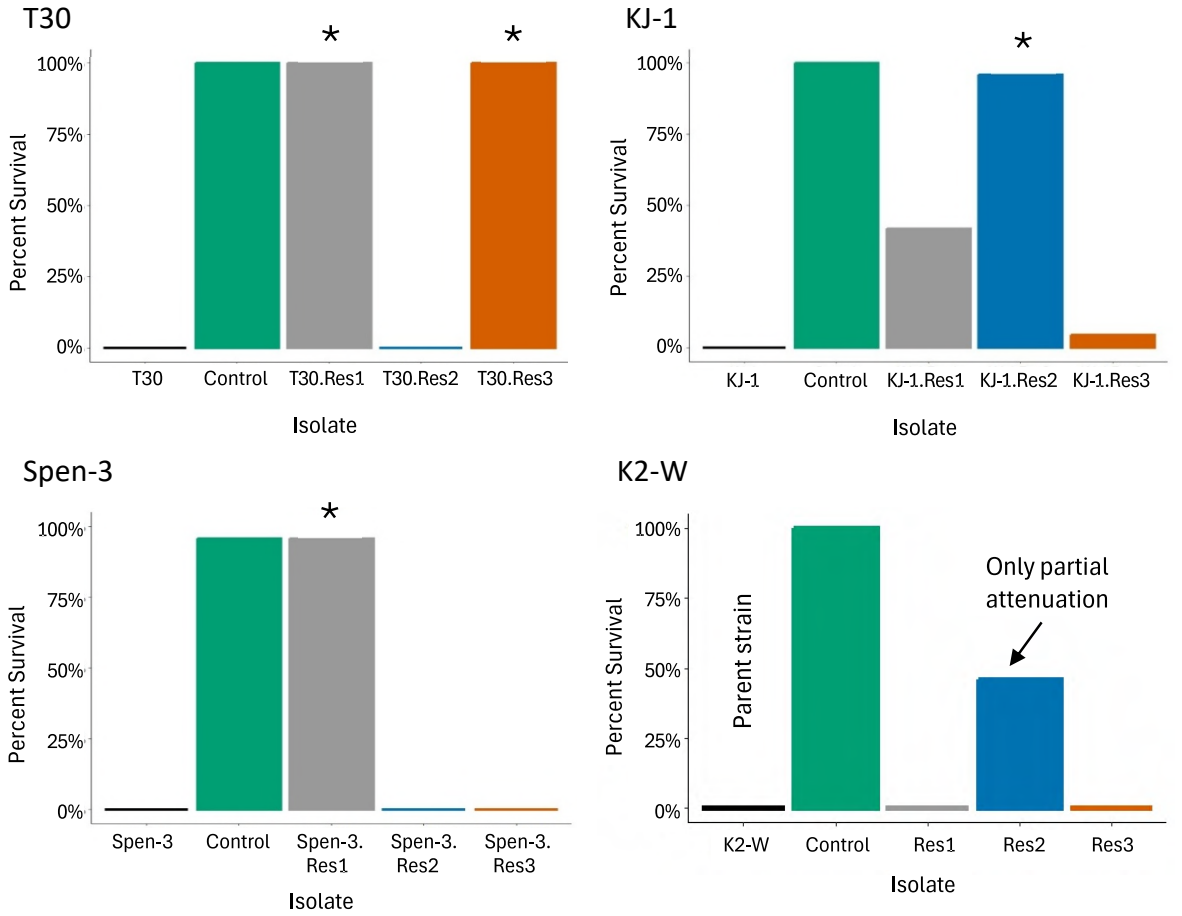

Fig.5 Challenge results showing percent survival of fish following challenge with four selected virulent atypical *Aeromonas salmonicida* parent strains (T30, KJ-1, Spen3, and K2-W) and "resistant" strains derived from each parent strain

Those strains that exhibited 100% survival are considered completely attenuated (*) and represent potential vaccine candidates for future evaluation.

Conclusion

The three studies described in this minipaper represent ongoing work that is aimed at developing novel approaches that can minimize the incidence of disease in sablefish caused by atypical strains of *A. salmonicida*. The goal is to provide a cost-effective and low-stress vaccine that can be easily administered to fish at both early and later life stages. The work here lays a foundation for this but also demonstrates that long-term immunity can be achieved in the interim with more traditional approaches if relevant atypical *A. salmonicida* strains are incorporated into killed immersion and injection vaccines. Newly developed attenuated *A. salmonicida* strains may enhance the effectiveness of immersion vaccines against atypical furunculosis in sablefish; however, further studies are needed to confirm their potential as vaccine candidates.

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Annotated Bibliography of Key Works

(1) Arkoosh MR, Dietrich JP, Rew MB, Olson W, Young G, Goetz FW (2017) Exploring the efficacy of vaccine techniques in juvenile sablefish, *Anoplopoma fimbria*. *Aquac Res*., **49**, 205-216. https://doi.org/10.1111/are.13449

This paper evaluates delivery strategies (immersion and injection) and tests multivalent vaccines for their ability to protect against atypical furunculosis for sablefish. Sablefish vaccinated by immersion at ~1.5 or ~4.5 g with a whole-cell multivalent vaccine were not protected against either typical or atypical *Aeromonas salmonicida*. However, the relative percent survival (RPS) or potency of the whole-cell multivalent vaccine injected i.p. in juvenile sablefish at ~50 g against typical and atypical *A. salmonicida* was 94.3% and 81.7%, respectively. The high RPS values indicated that the vaccine successfully initiated an immune response in sablefish.

(2) Goetz FW, Anulacion BF, Arkoosh MR, Cook MA, Dickhoff WW, and 10 other authors (2021) Status of sablefish, *Anoplopoma fimbria*, aquaculture. *J World Aquac Soc*., **52**, 607-646. https://doi.org/10.1111/jwas.12769

This is a key review article on sablefish, *Anoplopoma fimbria* (also called black cod), which is a long-lived marine species that is found in the Pacific from Baja California to Alaska, the Bering Sea, and through to the eastern coast of Japan. The value and feasibility of commercial aquaculture development along with important research needs are discussed. Advances in many research areas have been significant over the last 20 years and there are a few companies producing sablefish. Research advances include early life stage rearing along with production of all-female monosex offspring that grow faster than male or mixed sex populations.

Econometric models suggest that internal rates of return are 11-15% higher for monosex relative to mix-sex stocks over a 10-year period under typical cage culture conditions. Work showing that sablefish are susceptible to diseases (furunculosis and vibriosis) brought on by atypical *Aeromonas salmonicida* and *Vibrio anguillarum* is highlighted, but commercial vaccines (developed for salmonids) are only protective when given by injection. Long-term protection offered by vaccination has not been defined. Key takeaways from this paper are that more research is needed in relation to effective vaccine development and that improvements in methods for vaccine delivery would be beneficial.

(3) Jones EM, Oliver LP, Ma J, Leeuwis RHJ, Myrsell V, Arkoosh MR, Dietrick JP, Schuster CM, Hawkyard M, Gamperl KA, Cain KD (2022) Production of a monoclonal antibody specific to sablefish (*Anoplopoma fimbria*) IgM and its application in ELISA, western blotting, and immunofluorescent staining. *Fish Shellfish Immunol.*, **130**, 479–489. https://doi.org/10.1016/j.fsi.2022.09.038

This work addresses the issues with polyclonal antibodies and their limitations for important assays designed to monitor specific antibody kinetics or identify important immune cells in tissues. Sablefish (*Anoplopoma fimbria*) are an emerging aquaculture species and such new tools are needed to determine antibody response following vaccination or disease outbreaks. In this paper, a monoclonal antibody, UI-25A, specific to sablefish IgM was produced in mice. Western blotting confirmed that UI-25A recognizes the heavy chain of IgM and does not cross-react to proteins or carbohydrates in serum of four other teleost species. An ELISA was developed to measure *Aeromonas salmonicida* specific IgM in the plasma of sablefish, and UI-25A was used in Western blot analyses to identify immunogenic regions of *A. salmonicida* recognized by IgM from vaccinated sablefish. Immunofluorescent staining also demonstrated the ability of UI-25A to recognize membrane-bound IgM and identify IgM + cells (presumably B cells) in sablefish head kidney. Results demonstrate the usefulness of UI-25A as a tool to improve the understanding of antibody-mediated immunity in sablefish. This product will be valuable for vaccine development and the expansion of sablefish aquaculture efforts.

(4) Vasquez I, Cao T, Hossain A, Valderrama K, Gnanagobal H, Dang M, Leeuwis RMJ, Ness M, Campbell B, Gendron R, Kao K, Westcott J, Gamperl AK, Santander, J (2020) *Aeromonas salmonicida* infection kinetics and protective immune response to vaccination in sablefish (*Anoplopoma fimbria*). *Fish Shellfish Immunol*., **104**, 557-566. https://doi.org/10.1016/j.fsi.2020.06.005

This study addresses the need for effective vaccine programs against Aeromonas salmonicida, which have been identified as a high priority area for sablefish (*Anoplopoma fimbria*) aquaculture. This study established an *A. salmonicida* infection model to evaluate commercial vaccines and an autogenous vaccine preparation.

Using a clinical isolate of *A. salmonicida* (J410) they estimated a median lethal dose (LD50) of ~3 × 105 CFU/dose, and determined that the relative percent survival (RPS) for the autogenous bacterin mix was 65.22%, for commercial Forte Micro 4® vaccine it was 56.52%, and for Alpha Ject Micro 4® it was 30.43%. The RPS trends agreed with *A. salmonicida* tissue colonization levels at 10 days post-challenge. They measured total IgM titers, which peaked at 6-8 weeks post-immunization, but determined that the *A. salmonicida* A-layer binds to immunoglobulins F(ab)' in a non-specific fashion and affects immune assays and potentially vaccine efficacy. These results show that vaccine design influences sablefish immunity and provides a guide for sablefish vaccine programs.

水研機構研報, 第 53 号, 99 – 101, 令和 8 年
Bull. Jap. Fish. Edu. Agen. No. 53, 99 - 101, 2026

Hygiene management is important to prevent red sea bream iridovirus transmission between net pens: Insights from a case study assessing cross-contamination in a fish farm

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Extended Abstract: Red sea bream iridovirus (RSIV) infection, which is currently listed as a notifiable disease by the World Organization for Animal Health (WOAH), has caused significant economic damage in Japanese mariculture since the 1990s. Although formalin-inactivated vaccines are commercially available to control RSIV outbreaks, fish farmers do not use the vaccine when the vaccination cost is not acceptable compared to the market value of a given cultured fish. Therefore, basic biosecurity management such as hygiene procedures could be important to control RSIV outbreak. Nevertheless, in the case of mariculture using net pens or cages which is defined as semi-open system aquaculture by WOAH, the hygiene procedures have been considered less effective than those applied to land-based aquaculture because there is no physical barrier to prevent pathogens from moving via environmental water between aquaculture units. Our latest study suggested that RSIV transmission via seawater is highly associated with the distance between net pens and that the environmental water could function as a potential barrier to prevent viral transmission. Hence, we hypothesized that the biosecurity management could effectively reduce the risk of RSIV transmission even in semi-open system aquaculture where environmental water can move freely.

For implementing aquaculture biosecurity, the significance of fomite transmission in fish farms has been described, especially in salmonid aquaculture. However, the studies for aquaculture biosecurity are based on epidemiological data and questionnaire results for fish farms. To the best of our knowledge, there is no previous study that directly assessed the intensity of contamination in each aquaculture equipment associated with the fomite transmission. In the present study, cross-contamination of RSIV in aquaculture equipment and facilities in a fish farm where RSIV outbreak occurred was investigated by surface swabbing tests and an environmental DNA technique. Based on the results in this case study, we assessed the risk factors for transmission of RSIV between net pens to identify an effective hygiene procedure in the semi-open system aquaculture.

The investigation was performed in a fish farm where the RSIV outbreak occurred between September and October in 2022. The outbreak was initiated from juvenile red sea bream *Pagrus major* and transmitted to juvenile Pacific bluefin tuna *Thunnus orientalis*. Our investigation during the RSIV outbreak in the fish farm demonstrated that landing nets and gloves associated with collecting dead fish (carcasses) were highly contaminated with RSIV. The viral load of the contaminated equipment was > 10^8 copies of the RSIV genome. Because the equipment for collecting fish carcasses were used at all net pens without disinfection, the risk of fomite transmission was

Accepted on Feb 26, 2026
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considered to be higher than the transmission via environmental seawater if the distances among net pens are appropriately secured. On the other hand, we could not request fish farmers to undertake a strict hygiene procedure that is often implemented in land-based aquaculture because disinfection on boats is generally difficult owing to limited space of boats and the direct influences of weather. Therefore, we proposed mitigation measures against RSIV transmission between net pens and the following actions were initiated in the fish farm. A daily operation for collecting dead fish started from the net pen where the disease had not occurred and moved to the net pen where RSIV outbreak occurred to ensure that RSIV was not transmitted to the clean net pens by cross-contamination. In addition, the landing nets used for collecting fish carcasses were disinfected at the end of each day to avoid carryover of the virus to the following day. As a result, RSIV was not transmitted to the clean net pens in the fish farm for more than 30 days. However, once the RSIV outbreak occurred in the net pen upstream in the operation for collecting dead fish, RSIV was transmitted to all net pens in one week, suggesting that the transmission was caused by cross-contamination.

This study indicated that appropriate hygiene management is important to reduce the risk of RSIV transmission between net pens, even in semi-open system aquaculture. However, careful attention to the sequence of operation could not be a sufficient strategy when the RSIVD outbreak suddenly occurred in the upstream of operation as shown in the present study. For the future study, we need to seek disinfection procedures targeting the high-risk equipment such as landing nets and gloves for collecting dead fish, which can be undertaken on the boat considering cost-effective and labor-efficient method. We believe that the present study represents the first step for implementing appropriate biosecurity management in semi-open system aquaculture, which could be expanded for other viral or bacterial infections in aquaculture.

This study was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan (grant number JPJ007159).

Key words: red sea bream iridovirus, RSIV, cross-contamination, aquaculture biosecurity, hygiene management

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(1) Kawato Y, Ito T, Kamaishi T, Fujiwara A, Ototake M, Nakai T, Nakajima K (2016) Development of red sea bream iridovirus concentration method in seawater by iron flocculation. *Aquaculture*, **450**, 308–312.

It is the first report that the iron flocculation technique was applied to concentrate a virus causing fish disease. Since environmental DNA (eDNA) was directly extracted from the iron flocculation-trapped filter without an elution step, the procedure until real-time PCR assay became simpler and time effective.

(2) Kawato Y, Mekata T, Inada M, Ito T (2021) Application of environmental DNA for monitoring red sea bream iridovirus at a fish farm. *Microbiol. Spectr.*, **9**, e0079621.

Environmental DNA (eDNA) could be applied in monitoring waterborne viruses of aquatic animals. However, there are few data for practical application of eDNA in fish farms to control disease outbreaks. The results of their field research over three years targeting eDNA in a red sea bream (*Pagrus major*) fish farm implied that red sea bream iridovirus (RSIV) outbreaks in juveniles originated from the virus shedding from asymptomatically virus-infected broodstocks. Their work identified the infection source of RSIV in a fish farm by eDNA monitoring, and it could be applied as a tool in aquaculture to control fish diseases.

(3) Kawato Y, Takada Y, Mizuno K, Harakawa S, Yoshihara Y, Nakagawa Y, Kurobe T, Kawakami H, Ito T (2023) Assessing the transmission risk of red sea bream iridovirus (RSIV) in environmental water: insights from fish farms and experimental settings. *Microbiol. Spectr.*, **11**, e0156723.

This study aimed to understand the actual transmission risk of RSIV through environmental water among fish farms. The results indicated that the viral loads in the seawater were low, except for the net pens where RSIV outbreaks occurred. Furthermore, their experimental infection model indicated that the infection risk of RSIV-contained seawater with less than 10^3 copies/L was extremely low. These results suggest that the transmission of RSIV among fish farms via seawater is highly associated with the distance between the net pens, and the

environmental water is not always an infection source for the transmission of RSIV between fish farms.

(4) Kawato Y, Mizuno K, Harakawa S, Takada Y, Yoshihara Y, Kawakami H, Ito T (2024) Risk assessment of wild fish as environmental sources of red sea bream iridovirus (RSIV) outbreaks in aquaculture. *Dis. Aquat. Organ*., **158**, 65–74.

RSIV in wild fish near aquaculture installations was surveyed to evaluate the risk of wild fish being an infection source for RSIV outbreaks in cultured fish. In total, 1102 wild fish, consisting of 44 species, were captured from 2 aquaculture areas in western Japan between 2019 and 2022. Eleven fish from 7 species were confirmed to harbor the RSIV genome using a real-time PCR assay. Based on the diagnostic records of RSIV in the sampled area of wild fish, the RSIV-infected wild fish appeared during or after the RSIV outbreak in cultured fish, suggesting that RSIV detected in the wild fish was derived from the RSIV outbreak in cultured fish. Therefore, wild fish populations near aquaculture installations may not be a significant risk factor for RSIV outbreaks in cultured fish.

水研機構研報, 第 53 号, 103 – 105, 令和 8 年
Bull. Jap. Fish. Edu. Agen. No. 53, 103 - 105, 2026

Disease control measures in hirame hatchery: A case of hirame aquareovirus infection

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Extended Abstract: Japanese flounder *Paralichthys olivaceus*, commonly known as "hirame" in Japan, is a widely produced fish species mainly for stock enhancement in Japan. Approximately 20 million juveniles are produced annually for stock enhancement and aquaculture. Outbreaks of various diseases, such as viral nervous necrosis, birnaviral infection, and bacterial enteritis, have been hampering the production of hirame in hatcheries. In addition, mass mortality events associated with reovirus-like pathogens have been reported in some hirame hatcheries since the 2000s, which drastically impacts the production. Gross signs of the diseased fish are dark body color, abdomen swelling, and/or opaque viscera (clouding of the intestinal tract). The disease occurs in 20 to 50-day-old juveniles with cumulative mortality rates exceeding 80%. In contrast, mortality in adult fish has not been reported. The water temperature at which the outbreak has been reported is broad, ranging between 14.5 and 21°C. Hatchery staff members attempted to develop measures to mitigate mortalities associated with the disease by rearing fish in semi-saline water, lowering fish density, or treating them with sodium nifurstyrenate, however, none of them was effective.

Our research team has identified the causative agent as hirame aquareovirus (HAqRV) and developed several diagnostic tools. HAqRV is classified into the genus *Aquareovirus* of the family *Reoviridae*. This virus has double-stranded RNA as the viral genome in a non-enveloped virion. The virus actively replicates in the intestinal tract and liver, forming syncytia in which multiple cells are fused. Furthermore, we obtained findings suggesting that the vertical transmission from broodstock to juveniles is a primary transmission route, resulting in mass mortality events in juveniles. So, this study aimed to develop measures to prevent the vertical transmission of HAqRV.

To estimate the risk of vertical transmission, we checked the production process in hirame hatcheries. In hatcheries, wild fish are used as broodstock to maintain genetic diversity. Our previous study revealed that HAqRV was detected in approximately 30% of the wild fish caught for the broodstock. In general, hirame broodstock in a hatchery are maintained in a tank for years, so that we speculated that most of the broodstock are horizontally infected with HAqRV and become carriers of the viral agent. In addition, fertilized eggs, naturally spawned in a broodstock tank, are collected from the rearing water, and hence, the fertilized eggs could be contaminated with HAqRV released from the carrier broodstock. Based on these presumptive risks, we developed a series of control measures against HAqRV infection.

Our first approach was to develop a sublethal testing method for broodstock selection to detect and eliminate the virus-infected broodstock because our previous testing methods needed to kill broodstock. Our previous findings indicated that the intestinal tract was a major target organ of the virus. So, a medical swab was inserted

Accepted on Feb 26, 2026
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from the anus for about 5 cm into the intestine of anesthetized broodstock and rubbed the intestinal wall several times to scrape the epithelium and syncytia of the intestinal tract. In order not to collect feces, fish were fasted prior to sampling. RNA was extracted from the swab specimens and a viral gene was detected by reverse transcription real-time PCR method. The intestinal swab test successfully detected the virus-infected fish, but we realized that the diagnostic sensitivity of this method was lower than that of the lethal sampling method which sacrifices broodstock and uses a portion of the intestine. Thus, the effectiveness of the broodstock selection using the intestinal swab test was assessed in an experimental setting by detecting and quantifying the viral loads in raring water and fertilized eggs from broodstock that had received the test. Wild hirame (approximately 1.0-4.0 kg), which were candidates for the broodstock, were separated into HAqRV-negative (n = 33) and HAqRV-positive (n = 43) groups by the intestinal swab test. These fish were housed in a 50-t tank, respectively. Then, spontaneous spawnings of both groups were induced by elevating water temperature. The HAqRV was detected in the raring water and fertilized eggs from the HAqRV-positive group, but not from the HAqRV-negative group, suggesting that broodstock selection using the intestinal swab test was effective in reducing viral loads in fertilized eggs and rearing water in broodstock tank.

The second approach was to disinfect fertilized eggs using electrolyzed seawater. Although the intestinal swab test was able to eliminate virus-infected broodstock shedding a large number of viral particles, the disinfection procedure for fertilized eggs was still required to minimize the risk of viral transmission from virus-infected broodstock that cannot have been detected by the broodstock selection. Electrolyzed seawater mainly contains oxidants of hypochlorous acid produced by electrolysis equipment and has strong bactericidal and virucidal effects. Electrolyzed seawater has been used in some hirame hatcheries in Japan as it has been reported to be effective in inactivating some fish pathogens. Hence, we decided to focus on optimizing a condition of electrolyzed seawater for treating fertilized eggs, which inactivates HAqRV but does not compromise the hatching of the fertilized eggs. To determine the concentration for inactivating the virus, the infectivity of HAqRV was confirmed using a cell line after treatment with electrolyzed seawater at a range of oxidant concentrations. HAqRV was inactivated by the electrolyzed seawater at an oxidant concentration of 0.25 mg/L for 1 minute. Next, we determined an appropriate oxidant concentration and treatment time of the electrolyzed seawater that does not affect the hatching of the fertilized eggs. We set initial oxidant concentration supplied by the electrolysis equipment at 0.75 mg/L based on previous reports. The oxidant concentration drastically decreased to approximately 0.25 mg/mL immediately when fertilized eggs were immersed in the electrolyzed seawater due to organic substances derived from the eggs and the seawater. The oxidant concentration was not recovered to the initial concentration although the electrolyzed seawater was continuously supplied for at least 5 min at 1.0 L/sec which was the maximum condition of the water supply in this study. In any case, up to 5 min of treatment time did not affect the hatching of the fertilized eggs. Given the significant decrease of oxidant concentration, the fertilized eggs should be treated with electrolyzed seawater for maximum treatment time at optimum oxidant concentration to reduce the risk of vertical transmission of HAqRV. Thus, the optimum condition for disinfection of hirame fertilized eggs with electrolyzed seawater was determined to be as 5 min immersion in electrolyzed seawater at the nominal oxidant concentration of 0.75 mg/L with continuous supply at 1.0 L/min.

Finaly, we applied a comprehensive biosecurity plan against HAqRV in hirame hatcheries. In the plan, our newly developed measures were incorporated into conventional disease control protocols, which includes the use of UV-treated seawater and appropriate zoning in hatcheries. To confirm the effectiveness of the plan, we chose a hirame hatchery where an outbreak of HAqRV infection had occurred in the spring of 2021. We provided technical advice to the staff members in the hirame hatchery regarding the preventive measures against HAqRV, and the tools and equipment for the intestinal swab testing and disinfection were prepared. In November of the same year, all incoming and resident broodstock were tested by the intestinal swab test, and positive individuals were eliminated. Broodstock started to spawn in the winter, and the fertilized eggs were disinfected by the method developed in this study. In this batch, hirame were successfully raised to juvenile stages and released into the ocean for stock enhancement without an outbreak of HAqRV infection. The measures were further disseminated to seven additional

fish hatcheries, and we successfully prevented the outbreak of the virus. These results indicate that our disease control measures are highly effective in preventing outbreaks of HAqRV infection in hirame hatcheries.

Key words: hirame, aquareovirus, broodstock selection, electrolyzed seawater

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This valuable paper summarized fish and shellfish diseases that had occurred in hatcheries in Japan. The highest number of cases were reported in hirame, and the types of diseases that occurred in hirame hatchery were diverse.

(2) Nishioka T, Fujimoto H, Oka M, Arimoto M (2009) Diseases of marine fish and shellfish in hatcheries in Japan. *J. Fish. Technol.*, **2(1)**, 57-65 (in Japanese with English abstract).

As the above article, this paper also summarized fish and shellfish diseases that had occurred more recently (FY, 2000-2006) in hatcheries in Japan. The highest number of reports were recorded again in hirame, indicating that the diseases in hirame continued to be serious.

(3) Kawato Y, Mekata T, Nishioka T, Kiryu I, Sakai T, Maeda T, Miwa S, Koike K, Sadakane M, Mori K (2021) Isolation and characterization of hirame aquareovirus (HAqRV): A new Aquareovirus isolated from diseased hirame *Paralichthys olivaceus*. *Virology*, **559**, 120-130.

They isolated a novel *Aquareovirus* (hirame aquareovirus: HAqRV) from hirame and determined the complete genome of this virus. A comparison of the entire genome between the new virus and other aquareoviruses suggested that HAqRV is likely a new aquareovirus species. Virulence of HAqRV was demonstrated by experimental infection using hirame juveniles and HAqRV was reisolated from diseased fish. In immunohistochemistry, syncytial cells in the intestinal tract and liver of the experimentally infected fish were stained with the antiserum against HAqRV.

(4) Kawato Y, Maeda T, Nishioka T, Kiryu I, Mekata T, Matsuyama T, Tensha K, Yamashita I, Kawamura Y, Raku A, Senbokuya K, Yanagi S, Hayashi K, Kumagai A, Mori K (2022) Asymptomatically infected broodstock are a potential infection source for aquareovirus outbreaks in hatchery-reared Japanese flounder *Paralichthys olivaceus*. *Fish Pathol.*, **57(1)**, 11-19.

This paper estimated the route of transmission in hirame hatcheries where outbreaks occurred due to HAqRV. Quantitative PCR analysis of organs from broodstock revealed that more viral genes were detected in the intestinal tract than in the gonads. In addition, the viral sequences from broodstock and juveniles died by HAqRV were consistent among facilities. These results suggest that vertical transmission is the primary route of transmission, and the virus released from the intestinal tract of the broodstock might be the source of infection in the juveniles.

水研機構研報, 第 53 号, 107 – 114, 令和 8 年
Bull. Jap. Fish. Edu. Agen. No. 53, 107 - 114, 2026

Opportunities and challenges for Alaska kelp aquaculture

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Abstract: Kelp aquaculture is a nascent and growing industry in Alaska where cold, nutrient-rich, and clean waters result in high-quality biomass. There is considerable support from regional and federal initiatives to expand the blue economy sector, and in Alaska much of that support is focused on kelp and shellfish aquaculture. This paper reviews the opportunities, challenges, and ongoing research in three select topics that are key to sustainable industry expansion: (1) aquaculture site suitability, (2) ecosystem interactions with aquaculture, and (3) kelp stabilizing and processing. Regarding site suitability, there are ample opportunities for aquaculture expansion in the extensive coastal zone of Alaska, however low population densities and vast distances between inhabited communities result in infrastructure and logistical challenges for cultivating kelp and getting it to target markets. Investigations are underway that will inform optimized farm placement from both marine spatial planning and organismal physiology perspectives. Ecosystem interactions can constitute a service or a detriment depending on the nature of the interaction, which may also vary spatially and temporally. We are conducting research to understand how species of interest are interacting with kelp aquaculture and are developing *in situ* strategies to maximize benefits and regulatory efficiencies while minimizing interactions seen as harmful. Finally, stabilizing cultivated kelp in a cost-effective manner has proved challenging in Alaska's cold and wet climate. Research is underway to develop processing methods suitable to the climate and economies of Alaska, and local products are being developed to incorporate kelp into local manufacturing that may require less transport and stabilization. The expansion of the kelp aquaculture industry is full of exciting possibilities and formidable challenges. Knowledge exchange with regions that have mature kelp aquaculture industries, such as Japan, will likely aid in sector growth in Alaska.

Key words: Site suitability, ecosystem interactions, stabilization, processing

Introduction

Kelp aquaculture is an extremely new industry in the United States, especially when compared to countries such as Japan that have been consuming kelp for millennia and farming it for over half a century (Tanaka *et al*. 2020). Kelp are large, brown macroalgae in the Order Laminariales with a global temperate distribution. Millions of metric tons of two genera of kelp, *Saccharina* and *Undaria*, are commercially cultivated for food and other uses in China, Korea, and Japan (FAO 2024). Cultivation efforts in the West are at a much smaller scale and farms can be found in the United States, Canada, Ireland, Faroe Islands, and Norway, among others (Canvin *et al*. 2025). The initial motivation for kelp aquaculture development in the United States was for energy production in the 1970s, with a focus on biomass cultivation for methane production (Kim *et al*. 2019). Attempts at cultivation for food production in the country are even more recent, with the first commercial farms becoming operational in the 2010s in the State of Maine (Kim *et al*. 2019). Attempts to commercially cultivate kelp in Alaska

Accepted on Feb 26, 2026
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began with the first experimental operation in 2016. In the past decade, the industry in Alaska, Washington, and the U.S. Northeast, including Maine, has grown rapidly, in part due to federal and state investment in research and development.

The Alaska kelp industry has grown substantially in recent years, and it has benefitted from investments in research and development from state and federal sources. In 2014, the Alaska Fisheries Development Foundation received a Saltonstall-Kennedy Grant of $217 thousand from NOAA Fisheries for the Alaska Mariculture Initiative, kick-starting the Alaska Mariculture Task Force through Governor Walker. In 2016, the U.S. Department of Energy Advanced Research Projects Agency awarded over $3 million to the University of Alaska Fairbanks as part of the Macroalgae Research Inspiring Novel Energy Resources program to develop methods to scale up kelp cultivation for biofuel production (ARPAE 2025). This resulted in important innovations and jump-started the industry in Kodiak, Alaska. In 2022, the U.S. Economic Development Administration awarded a $49 million grant to Southeast Conference, a regional economic development organization, to catalyze development of the kelp and shellfish aquaculture industry as part of the Build Back Better Regional Challenge grant competition (Alaska Mariculture Cluster 2025a). Concurrently, the Exxon Valdez Oil Spill Trustee Council awarded over $32 million of grant funds over ten years to kelp and shellfish aquaculture research (EVOSTC 2022) and the State of Alaska developed a revolving loan fund for kelp and shellfish aquaculture operations to lower economic barriers to entry (AS 16.10.900). These investments, among other workforce development efforts, have resulted in substantial industry growth since the first kelp farm in 2015. In 2023, there were 30 kelp-only farms (out of 87 total aquatic farm operation permits) that were permitted and active during the year. Of that total, 15 kelp-only along with other multi-species farms, harvested a total of approximately 384,000 pounds, which was a decrease from 2022 when almost 872,000 pounds were harvested (ADFG 2025). Current market targets are human food, animal food, fertilizer and biostimulants, and commodity markets (biofuel, industrial additives).

Investment in and expansion of the kelp aquaculture industry has revealed important considerations in recent years, including where new farms should be sited and how to ensure environmental and economic sustainability. Kelp aquaculture operations must obtain leases and permits from local, state, and federal organizations and agencies with the goal of limiting negative or incompatible interactions with other industries and navigation, and ensure compliance with laws such as the Endangered Species Act (16 U.S.C. 1531-1544) and the Magnuson-Stevens Fishery Conservation and Management Act (16 U.S.C. ch. 38 § 1801 et seq.). However, little research exists on kelp aquaculture, generally, and kelp aquaculture in Alaska, specifically, to aid in regulatory decision-making. Likewise, little guidance exists for farmers to aid them in selecting optimal sites to maximize both likelihood of permitting success and biomass quality for the desired markets. Access to non-local markets is likewise necessary and a challenge for industry growth given the remoteness and rainy climate of coastal Alaska. Despite these challenges, there remains immense opportunity for growth of this sector.

This paper examines the opportunities, challenges, and ongoing research of three of the major topics arising as the industry expands. The first is the question of site suitability, or where to put new farms to maximize success for the farmer. The second is how to ensure environmental sustainability by improving our understanding of ecosystem interactions, and therefore compliance with aforementioned federal laws. The third is how to most efficiently stabilize and process cultivated kelp to best access non-local or year-round markets. This topic list is non-exhaustive and the three were selected based on extensive conversations with industry and regulators, as well as the authors' personal observations.

Site suitability

Kelp aquaculture is limited to the coastal Gulf of Alaska where sea ice does not form. This vast area includes over 50,000 km of coastline with limited coastal development and low human population densities. Coastal Gulf of Alaska is a geologically diverse and highly biologically productive region. Areas with aquaculture operations vary from exposed, marine habitats, most commonly found around the Kodiak Archipelago or outer coast of Southeast Alaska, to sheltered coves or inlets that can vary from marine to brackish. Farms in Kachemak Bay, Prince William Sound, and parts of Southeast Alaska can also be influenced by glacial runoff. This vast area and variability creates opportunities and challenges for siting kelp farms.

1. Opportunities

Low population densities and limited industrial and agricultural activities limit sources of potential anthropogenic terrestrial pollutants that could enter the nearshore realm

and influence farming activities. Surveys by the Alaska Department of Environmental Conservation (ADEC) consistently find bacterial and heavy metal levels to be well below regulatory thresholds in sites where monitoring occurs (ADEC 2024). Nearshore nutrient concentrations, necessary for kelp growth, are consistently high during winter and spring before massive phytoplankton blooms reduce summer nutrient concentrations in most nearshore areas (Cates *et al*. 2025; Weingartner *et al*. 2009). While lower-latitude states on the U.S. West Coast have observed significant declines in wild kelp biomass and canopy cover in the past few decades, kelp populations in Alaska remain robust (Berry *et al*. 2021; Hollarsmith *et al*. 2024; Nicholson *et al*. 2024). Stable wild kelp populations are both an indication of suitable environmental conditions for kelp growth, and they are necessary for the industry as kelp spores must be collected annually from within 50 km of the farm site to seed it out (Gruenthal and Habicht 2022).

2. Challenges

While the remote nature of coastal Alaska can mean limited input of anthropogenic nutrients and contaminants, it also presents logistical and workforce challenges. Many kelp farms are in sites or communities that are not connected to the broader road system so materials and harvested biomass must be shipped in or out via boat or plane, which is often a costly endeavor (State of Alaska Geoportal 2025). Likewise, small and isolated communities can result in workforce challenges, especially with the seasonal nature of kelp aquaculture (Alaska Sea Grant 2023). Despite low population densities and vast distances, there are many uses of the nearshore zone which permitted farms must be compatible with. These uses include commercial fisheries, tourism, subsistence harvests, military operations, navigational channels, among other potential uses that permitting agencies must consider. Ecological interactions are also considered, including marine mammal migration corridors and feeding grounds, fish spawning areas, Essential Fish Habitat, and other interactions with endangered or protected species (Schillaci *et al*. 2025). The oceanographic diversity of the nearshore zone of the Gulf of Alaska, including variations in exposure, salinity, temperature, and nutrient concentrations, means different sites are likely more suitable to some kelp species over others.

3. Research

Research to aid in site suitability falls broadly into two categories: determining where it makes logistical sense to site farms and investigating how potential farm production may vary across different oceanographic contexts. Regarding logistical considerations, the National Oceanic and Atmospheric Administration (NOAA) is identifying Aquaculture Opportunity Areas (AOAs) in Alaska in partnership with the State of Alaska (Schillaci *et al*. 2025). AOAs are defined geographical areas that may be suitable for commercial aquaculture development based on economic, environmental, and social considerations. To identify these areas, NOAA solicits extensive public input, conducts spatial suitability modeling that includes multiple data layers of other uses of the nearshore zone that may be fully or partially incompatible with aquaculture, and conducts an environmental review through the National Environmental Policy Act (NEPA). Suitability modeling in Alaska also includes logistical considerations such as proximity to population centers, ports, and processing infrastructure. One of the resulting products will be a peer reviewed atlas with geospatial planning information and maps that identify areas of higher and lower suitability for aquaculture. This will be one source of information to assist NOAA and the State of Alaska in developing alternatives for consideration in the NEPA process.

There are also multiple research efforts completed or underway to better understand where to site farms from a species perspective. The Mariculture Research and Restoration Consortium (Mar ReCon) is a multi-partner research group funded by the Exxon Valdez Oil Spill Trustee Council. Researchers within the consortium include state and federal agencies, a non-governmental and non-profit regional science center, academic institutions, and an Alaska Native tribe. The aim of the consortium is to better understand how aquatic farms interact with the surrounding environment and biological communities across the diverse oceanographic and ecological conditions found in the areas impacted by the 1989 Exxon Valdez oil spill, namely Prince William Sound, Kachemak Bay, and the Kodiak Archipelago (PWSSC 2025). Where relevant, results will be incorporated into the permitting process to minimize negative ecological interactions and maximize ecosystem services. Studies within this consortium also investigate regional variation in farm production to improve understanding of how the productivity and tissue chemistry of cultivated kelp and oysters are influenced by oceanography. These results will aid current and prospective farmers in selecting sites to maximize the traits and growth rates they desire in their products. Research has also revealed how differences in seawater nitrogen can impact kelp farm productivity. The authors concluded that sites should be

selected where the total seawater nitrogen does not dip below 0.6-1.0 μM during the farming season and if a site does experience seasonally reduced nitrogen levels, the farmer should increase the spacing between the grow lines to maximize the flow and nutrient transfer (Stephens *et al*. 2024).

Additional research is underway to optimize farm design for a given site, species, and desired morphology. Bull kelp (*Nereocystis luetkeana*) is of particular interest given the diverse morphological forms it can take, and it is being coordinated by the Bull Kelp Research Squad (BKRS 2025), a group of growers and researchers with the shared goal of improving methods for growing this locally valuable species that is endemic to the northeast Pacific. Other projects focusing on growing methods tailored to specific site conditions include focusing on optimizing line spacing, depth, and orientation, exploring the down-stream effects of hatchery conditions, and the effects of combining species of different morphologies (e.g. floating and non-floating) within the same farm site (Alaska Mariculture Cluster 2025a).

Ecosystem interactions

Alaska's aquatic farms are indelibly connected to the nearshore environment where they have the potential to interact with and be influenced by the surrounding flora and fauna. The nearshore zone provides nursery habitat for juvenile phases of economically important fish and invertebrate species, including the Pacific halibut (*Hippoglossus stenolepis*), walleye pollock (*Theragra chalcogrammus*), Pacific cod (*Gadus macrocephalus*), Pacific herring (*Clupea pallasii*), Pacific salmon (*Oncorhynchus* spp.), Dungeness crab (*Metacarcinus magister*), and king crab (*Paralithodes camtschaticus*), among many other species (Johnson *et al*. 2003). It also serves as a migration and feeding ground for multiple species of whales and pinnipeds, all protected under the Marine Mammal Protection Act (16 U.S.C. 1361-1407). Regulators recommend siting farms away from known Essential Fish Habitat (16 U.S.C. ch. 38 § 1801 et seq.), biologically important areas, and Pacific herring spawning locations, among other biological and physical siting considerations. However, much remains unknown about how these species may interact with aquaculture infrastructure and where these species are distributed in Alaska's vast coastline. Likewise, evidence suggests that kelp farms may provide valuable habitat for some organisms, therefore aquaculture siting could be done in a way that maximizes these ecosystem services (Theuerkauf *et al*. 2022).

1. Opportunities

Kelp aquaculture introduces complex midwater structures that may serve as a source of food and shelter for benthic and midwater fish and invertebrate species, especially during vulnerable early life stages. A meta-analysis of available literature found that seaweed farming was associated with a small increase in wild fish and mobile invertebrate abundance and a large though variable increase in species richness (Theuerkauf *et al*. 2022). Notably, the meta-analysis included mainly tropical seaweed farms, which highlights the dearth of ecological studies on temperate kelp aquaculture. In Alaska, kelp is usually harvested in the late spring which can coincide with the timing of salmon outmigration into the nearshore, the presence of schooling fish species such as Pacific herring, and the post-settlement juvenile phases of gadids and crabs (Laurel *et al*. 2016; Park and Shirley 2008; Vulstek *et al*. 2024). More information is needed to understand the true spatial and temporal overlap of kelp aquaculture and these organisms and the impact of harvest on the survival and distribution of these species. At the time of writing, the authors could find no evidence from published literature or other data sources of instances of negative interactions between kelp aquaculture and protected species, such as marine mammals, though reporting requirements differ widely between countries and regions. A risk assessment conducted by the Australian Seaweed Institute and based on best available data determined the risk of negative impacts from the kelp farm to habitat or protected species to be low to negligible (Kelly 2023).

Aside from potential ecosystem services of habitat provisioning and food, there is interest in the potential of kelp farms to capture and store carbon, thereby creating local refugia from ocean acidification and potentially reducing atmospheric CO_2 levels. A recent field study of multiple seaweed farm sites around the world found that organic carbon stocks beneath the farms was positively correlated with farm age, suggesting that burial and storage of carbon was occurring. The authors concluded that these farm sites act similarly to Blue Carbon habitats (Duarte *et al*. 2025). Experimental studies have also found that the commonly cultivated sugar kelp (*Saccharina latissima*) removed measurable levels of dissolved inorganic carbon and altered pH and saturation state, and this effect increased as CO_2 levels increased, suggesting this species has a high potential for creating local refuges from low-pH conditions (Ricart *et al*. 2023).

2. Challenges

While hypotheses on ecosystem interactions can be formed based on existing researchers, the fact remains that the available data are obtained from studies conducted in environments and ecosystems outside of Alaska and often based on growing methods that differ from the preferred catenary array design often used on Alaska farms, a design that maintains all grow lines at high tension. Alaska is also home to a particularly high concentration of protected species, including whales, seabirds, and pinnipeds (Young *et al*. 2024). This may increase opportunities for negative interactions, such as gear entanglement, though the authors could find no reports to date of any instances of entanglement in the region or outside. There is one record of a potentially negative ecological interaction with a fish species when a Southeast Alaska farm experienced a mass spawning event of Pacific herring. Alaska regulations prohibit the harvest of kelp if herring have spawned on the farm. The result was an almost complete loss of crop for the farmer, and further monitoring of the site by farm staff suggested low hatch rates of the herring eggs (Hollarsmith *et al*. 2025). Pacific herring numbers remain high in coastal Alaska and herring can shift their spawning grounds unpredictably, introducing the chance for additional interactions between farms and spawning fish.

3. Research

Research is underway to address known challenges, such as methods to deter Pacific herring from spawning on kelp farms, and to improve understanding of general interactions with surrounding flora and fauna. Using captive herring, researchers tested the effects of various deterrent strategies focused on the fish's behavior, including bubble curtains, active acoustics, strobing lights, and moving and suspended mid-water objects. Of all methods tested, only the bubble curtain had a deterring effect on the fish. This method mimics the predation strategy of humpback whales, a major predator of herring in Alaska (Hollarsmith *et al*. 2025). The logistics of installing this type of technology onto remote farms is considerable, so research efforts are ongoing to optimize these methods. Various projects are also underway to record species use of kelp farm infrastructure and how that use differs seasonally and across regions. The Mar ReCon project includes various monitoring efforts to track species presence or absence around farm sites, including marine birds, pinnipeds, mid-water and benthic fish, and benthic invertebrates, focusing on Southcentral Alaska and using primarily visual boat-based or SCUBA techniques, with some acoustic monitoring in select sites (PWSSC 2025). Researchers in this consortium are also investigating the carbon dynamics inside and outside of farm sites to assess whether the carbon uptake rates and ocean acidification amelioration reported from other areas is present in the highly tidal and dynamic oceanographic context of Southcentral Alaska. In Southeast Alaska and the Kodiak Archipelago, NOAA's Alaska Fisheries Science Center researchers are using visual surveys and environmental DNA to assess species use of kelp farms in the inner waterways. Results from all efforts will aid in permitting and siting to enhance positive ecosystem services and minimize negative ecosystem impacts.

Kelp stabilizing and processing

Cultivated kelp degrades rapidly after harvest, requiring harvesters and processors to stabilize the kelp in order to bring it to market in sufficient quantity and quality. Freezing and drying are currently the two primary stabilization methods for processing kelp in Alaska (Good *et al*. 2021; Serin 2024). Stabilizing cultivated kelp in a cost-effective manner has proved challenging in Alaska's cold and wet climate, and it is a major industry concern (Carovano *et al*. 2025). Cold-chain export of frozen kelp is an expensive shipping option, and drying kelp is extremely energy-intensive in a region with high energy and operational costs (McDowell Group 2017). Without preparation methods suitable for Alaska's often remote coastline and high energy costs, Alaska's kelp industry is at an economic disadvantage to other kelp growing regions. Providing kelp farms in Alaska with affordable and innovative stabilization technology is key to extending the shelf life of their product and increasing access to markets.

1. Opportunities

Alaska offers numerous processing opportunities for cultivated kelp including developing new products, leveraging existing seafood processing infrastructure and workforces, and exploring specialized markets. Currently, most cultured kelp in Alaska is sold into minimally processed whole food markets but there is potential to capitalize on more specialized markets such as food additives, cosmetics, dietary supplements, animal feeds, and biostimulants (Serin 2024). Potential synergies with existing seafood processing infrastructure may prove important to the industry's development. Repurposed fish-processing facilities and other uses of pre-existing infrastructure during the kelp harvest season align with the off-season of other fishing industries and

can provide freezer space and resources, including labor, to process cultivated kelp (Good *et al*. 2021). Cost structures and distance from markets limit current opportunities, but it may be offset by technological innovation, coordination among growers and markets, and other opportunities to share costs and pool resources (Carovano *et al*. 2025; Serin 2024). Local Alaskan start-ups prioritizing direct sourcing of kelp from farmers to produce kelp-based products have successfully tapped into local, online, and wholesale markets (i.e. Barnacle Foods and Kachemak Kelp Hub). These businesses are orientated around small volumes of seaweed processed into an evolving line of specialty food products.

2. Challenges

There are significant challenges that farmers face to access markets including high perishability of kelp, high energy and operational costs, and distance to market (Barnacle Foods and Spruce Root 2025). Kelp stabilizing in many regions, including Japan, relies on manually laying kelp on shorelines and drying from the sun (N. Yotsukura, Hokkaido Univ., Japan, personal communication). The large biomass, cost of labor and wet climate of coastal Alaska make this an unfeasible option and energy costs for non-solar drying are too high in many areas to be cost-effective without further innovation. Each coastal community in Alaska often operates on its own electrical grid, with varying energy sources including diesel which are expensive to operate (U.S. Energy Information Administration 2024). The cost of energy can thus impact the types of processing techniques that are feasible for individual farms. Cold chain export in order to access out-of-state or otherwise non-local markets can be cost-prohibitive. Alaska has few dedicated kelp processing facilities, making it difficult and expensive to handle the large volume of kelp produced, and the lack of local processing facilities can lead to increased transportation costs and time delay. As the kelp aquaculture industry in Alaska continues to grow, developing efficient, cost-effective processing systems will be key to increasing kelp's marketability and reducing operational costs. However, several key factors, including high energy costs, remote geographic locations, and labor constraints must be taken into account when selecting equipment.

3. Research

Research is underway to develop processing and stabilizing methods suitable to the climate and economies of Alaska. Investments into processing technologies have increased greatly and include an on-water mobile seaweed processor, an open-source primary processing line, and a kelp processing facility housed in a freight container. New processing technologies highlight innovative mobile designs that can be deployed in rural Alaskan communities and/or affordable stabilization technology. Recent testing of kelp stabilization methods include fermentation, bioactive compound extractions, high pressure processing, freeze drying, high-tunnel drying, and creating intermediate stabilized “slurries” (Alaska Mariculture Cluster 2025a). Additionally, new species are being grown and products are being developed to incorporate kelp into local manufacturing chains that may require less transport and stabilization, and expand upon existing markets (i.e. Kachemak Kelp Hub).

Additional research is being conducted to improve understanding of kelp nutritional value and bioactive compounds as these can vary depending on kelp species, life history, tissue type, season, and environmental conditions (Jardell 2024). Improved understanding of seaweed tissue has many benefits. It can support product innovation, diversify income streams, enable processors to refine extraction techniques for high-value compounds, inform future seaweed farming and harvesting strategies, establish industry standards for seaweed quality and composition, and attract investment and market interest (Alaska Mariculture Cluster 2025b).

Conclusion

Despite considerable challenges, the opportunities and potential remain high for growing a successful kelp aquaculture industry in Alaska. Research is underway that will help to determine where future farms should go from both a logistical and organismal perspective. Ecosystem interactions are a major regulatory concern and research efforts are beginning to fill knowledge gaps in how kelp aquaculture interacts with nearshore flora and fauna. Finally, cost-effective methods to stabilize kelp and improve access to markets are a high priority for the industry. Continued collaborations with other kelp-growing regions in the U.S. and the world, such as Japan, will aid Alaska in overcoming challenges and seizing opportunities for industry growth.

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水研機構研報, 第 53 号, 115 – 119, 令和 8 年
Bull. Jap. Fish. Edu. Agen. No. 53, 115 - 119, 2026

Present status and future scope of seaweed aquaculture in Japan

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Abstract: Around 1,500 seaweed species are distributed in the area near Japan, providing one of the most biodiverse marine environments globally. Seaweeds have played crucial roles in Japanese culture and food for centuries. Seaweed beds, known as "MOBA," provide ecological benefits such as nutrient cycling, water purification, and coastal protection while also support fisheries and cultural traditions. However, natural seaweed beds in Japan have declined by 30–40% since the 1990s due to overgrazing by sea urchins and herbivorous fish, rising water temperatures, and increased water turbidity. This decline threatens marine ecosystems and traditional practices like AMA dive fishing and Shinto rituals. Seaweed aquaculture, particularly of nori, kombu, and wakame, forms the foundation of Japanese seaweed industry. However, recent warming waters have shortened the cultivation periods and increased fish grazing, which has reduced the seaweed yields. Alternative strategies to maintain stable growing conditions of seaweeds include cultivation of them in land-based tanks and use of deep ocean water. While small seaweed species could be cultivated in tanks, cultivation of large species like kombu under artificial conditions is still in the process of development. Restoration of natural seaweed beds involves the removal of grazing animals and employing novel cultivation techniques that establish the seaweed clusters. Artificially restored seaweed beds, termed "SHIN-MOBA," could help sustain the biodiversity and conditions of fishery resources. Our expertise in seaweed cultivation and seaweed bed restoration will offer hope for adapting to climate change and taking over rich marine ecosystems worldwide.

Key words: aquaculture, climate change, MOBA, seaweed bed, SHIN-MOBA

Introduction

Seaweed and seagrass grow in various marine environments worldwide. Seaweed types vary depending on the environmental conditions of the area where they grow. Although seagrasses are distributed globally, large cold-water kelp species are found in localized cold marine areas. Temperate kelp species are distributed in the mid-latitudes, and brown algae of the genus *Sargassum* are primarily distributed in low-latitude tropical zones. Japan, located in the mid-latitudes, has an exceptionally diverse range of seaweed species, and thus is one of the world's richest regions for marine seaweed biodiversity (Fig.1).

Approximately 15,000 species of seaweed exist worldwide, approximately 1,500 species of which are distributed in Japan. Surrounded by the sea where seaweeds are abundant, Japanese people have been utilizing seaweeds into their daily life since ancient times. Seaweeds and seagrasses are also used as fertilizers for agriculture. Seaweeds have long been used as food ingredients in various regions in Japan (Fig.2). However, seaweed beds in Japan are changing due to the effects of climate change (Terada *et al*. 2021).

Current State of Seaweed Beds in Japan

The current state of seaweed beds, which support a wide variety of seaweed species, is critical in Japan. Seaweed beds play various ecological roles in marine ecosystems. The first is their supportive role in photosynthesis and nutrient cycling. The second is their regulatory role, which includes water

Accepted on Feb 26, 2026
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Fig.1 Differences in seaweed and seagrass distribution by latitude

Fig.2 Seaweed has been used in Japan for a long time

purification and coastal protection. The third is their spatial role as nursery grounds where fish and benthos gather and grow, and the seaweeds themselves serve as food for them, some of which are harvested by commercial fishery. Finally, seaweed beds play a cultural role by providing spaces for recreation and environmental education.

Seaweed beds therefore have a wide range of ecological functions. In Japan, seaweed beds are referred to as “MOBA” in Japanese. The term "MOBA" in Japan does not simply refer to a cluster of seaweeds; it encompasses various roles since seaweed beds support marine ecosystems and human livelihoods. As certain Japanese words such as "sushi," "tempura," and "samurai" have become globally recognized, we hope that the term "MOBA" will also become popular worldwide.

Fig.3 Japan's seaweed beds are in decline

Natural Seaweed Beds in Japan

Natural MOBA, which is vital to marine ecosystems and human life, had covered approximately 2,000 km^2 of the Japanese coastal waters until the 1990s. However, about 30-40% of these algal beds are thought to have disappeared so far (Fig.3). In addition to the decline in natural seaweed beds, poor growth and reduced production have been observed in seaweed aquaculture. The decline in seaweed beds not only degrades marine ecological functions but also threatens Japanese traditional culture.

Traditional Japanese female divers, referred to as “AMA,” have been significantly affected by the decline in seaweed beds. As turban shell (*Sazae*), abalones (*Awabi*), and spiny lobster (*Iseebi*) have become more difficult to harvest due to the decline in seaweed beds, the number of AMA has halved over the past decade. Furthermore, seaweeds have long played crucial roles in Japanese religious and cultural traditions. Many Shinto shrines use seaweeds in their traditional religious rituals. However, the decline in seaweed beds has made it difficult to conduct the traditional ceremonies (Kimura and Kudo 2011). At the coastal areas of the Seto Inland Sea, there used to be approximately 400 rock cave saunas that used the steam generated by burning a seagrass (*Zostera marina*). However, as the seagrass population declined, these saunas have been closed. Thus, the resent decline in seaweed beds in Japan has been affecting not only the marine ecosystems but also our traditional culture.

Causes of the Decline in Seaweed Beds in Japan

The largest factor contributing to the decline in seaweed beds in Japan is the grazing by sea urchins, followed by the grazing by herbivorous fish. Grazing by the marine animal is estimated to account for approximately half of the decline. Other contributing factors are the rise in water surface temperature, increased turbidity, and sediment accumulation on the bottom (Fig.4). Warmer water temperatures have increased the activity of sea urchins and herbivorous fish, thereby accelerating the decline in the seaweed beds (Kumagai *et al*. 2018).

The decline in seaweed beds in Japan's temperate zones typically follows the pattern below:

1. Extremely high temperatures inhibit the seaweed growth.
2. Fish grazing also increases, causing a rapid decline in the seaweed populations.
3. As the seaweeds become shorter in length, sea urchins then begin to consume them.
4. After the seaweeds disappear, seaweed spores newly settled on the bare rocks begin to grow, but they are rapidly consumed by the sea urchins.

Once the seaweed bed disappears, restoration becomes extremely difficult. In addition to seaweeds, seagrasses are also grazed by marine animals. In subtropical areas such as Okinawa, seagrasses have declined because of the grazing by sea turtles.

As the grazing increases, seaweed biomass gradually decreases. However, seaweed beds tend to collapse suddenly once the impact of grazing reaches a certain point. If the grazing could be controlled (for example, by removing fish), the seaweed bed will be regenerated. However, due to the extreme difficulty in the restoration of a collapsed seaweed bed, it is crucial to monitor the situation of seaweed beds and take an immediate action for restoration of seaweed beds at the first sign of decline.

Seaweed Aquaculture in Japan

In addition to harvesting wild seaweeds from natural shores, various techniques have been developed for stable seaweed cultivation in Japan. The primary seaweeds cultivated in Japan are nori (laver), ma-kombu (kelp), wakame (undaria), sujiao-nori (green laver), hitoegusa (monostroma), and okinawa-mozuku (*Cladosiphon okamuranus*). Among the cultivated seaweeds, nori has the highest production volume, followed by kombu and wakame. Nori and wakame cultivation constitutes

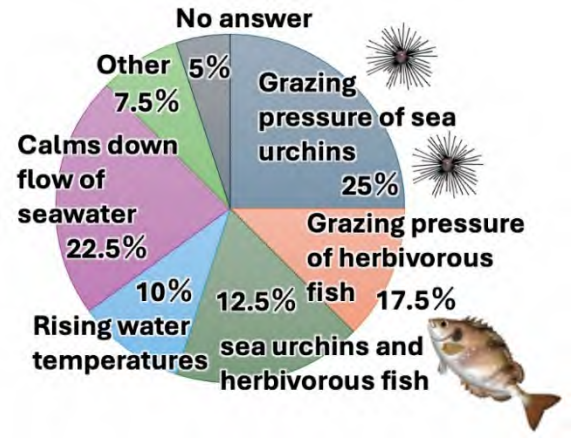

Fig.4 Factors contributing to the decline of seaweed beds in Japan

the foundation of seaweed aquaculture industry in Japan.

Every year, both of nori and wakame cultivation begin in autumn when the water temperature reaches 23°C, with a full-scale production commencing when the water temperature drops to 18°C. The seaweed aquaculture schedule is governed by water temperature; therefore, rising water temperatures by global warming not only affect the seaweed growth but also delay the start of cultivation and shorten the duration of the cultivation. These changes lead to the reduction in production yields.

In addition to the rising water temperatures**,** fish grazing has become a major issue in seaweed aquaculture, too. Most herbivorous fish prefer warmer water; therefore, when water temperatures rise, they continue to feed on cultivated seaweeds, even in autumn and winter, which also reduces the production yields.

Strategies for Adapting Seaweed Aquaculture to Climate Change

Both the area of wild seaweed beds and production in seaweed aquaculture in Japan have been declining due to climate change. Therefore, we must develop novel technologies of seaweed cultivation against these changes.

As for seaweed aquaculture, one adaptation strategy is to cultivate seaweeds in land-based tanks. Aonori (green laver) is the most widely cultivated seaweed in land-based tanks in Japan (Hiraoka and Oka 2008) (Fig.5). However, high water temperatures are inevitable problems even in the land-based tanks. Therefore, selective breeding of seaweed varieties that can withstand the high temperatures is a potential solution.

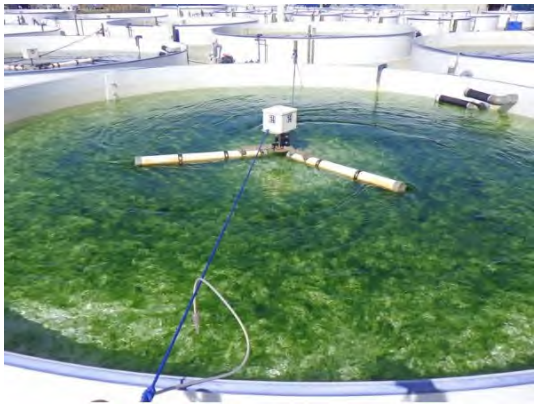

Fig.5 Seaweed cultivated in tanks on land

Fig.6 MOBA is a wild seaweed bed, and SHIN-MOBA is a new type of seaweed bed growing on artificial structures

Another strategy involves the use of deep ocean water, which is colder and rich in nutrients. Fourteen deep-sea water facilities are running in Japan, some of which are being used for seaweed cultivation. Recently, since cultivation techniques for several species of seaweeds have been established, seaweed production through the land-based cultivation has been increasing.

However, land-based tanks are primarily used to cultivate small seaweed species. Large species, such as kombu and wakame, have not yet been cultivated in the land-based tanks. This is due to technical problems, as well as the fact that large, beautiful kelp is popular in Japan. However, small kombu that could be cultivated in tanks will be consumed in salads. To develop Japanese seaweed culinary culture further, it is necessary to change the perception of the ideal appearance of seaweeds.

Restoring Natural Seaweed Beds to Adapt to Climate Change

Unlike land-based aquaculture, it is not meaningful to transplant natural seaweed beds onto land; seaweed beds must be established in the ocean. Since the primary cause of seaweed bed decline is the grazing by marine animals, eliminating these factors would theoretically allow seaweeds to grow healthily. In fact, there have been several cases where Japanese fishermen successfully restored seaweed beds by continuously removing the sea urchins.

If the removal of herbivorous animals alone is insufficient to restore the seaweed beds, advanced cultivation technology developed in Japan could be employed to establish seaweed clusters in the ocean. In the areas with heavy grazing pressure, seaweed beds could be protected by surrounding them with nets or hanging seaweed-filled baskets on them. Another approach is to cultivate seaweeds offshore or in deep waters, where herbivorous fish are less abundant. Even in such cases, it is necessary to cultivate sufficient amounts of seaweeds to exceed the grazing capacity of the fish.

Would the seaweeds growing on artificial structures, such as ropes or cages, be regarded as MOBA? Ideally, natural seaweed beds must grow on rocks; however, given the current challenges posed by climate change, it may be necessary to accept such artificially cultivated seaweed beds as a solution to recover the ecosystem.

Traditionally, “MOBA” refers to naturally growing seaweed beds. However, it is proposed that artificial seaweed beds be referred to as. “SHIN-MOBA”, with “SHIN” meaning "new" in Japanese. To conserve the biodiversity and ensure the sustainable use of marine resources, it is essential not only to protect existing seaweed beds but also to establish SHIN-MOBA in the barren areas (Fig.6). By applying the expertise in seaweed bed protection and seaweed cultivation in Japan, the goal is to create MOBA rich in seaweeds and marine life throughout the coastal areas in Japan.

Acknowledgements

I would like to greatly thank the research group to which I belong for their cooperation in my daily work.

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水研機構研報, 第 53 号, 121 – 125, 令和 8 年
Bull. Jap. Fish. Edu. Agen. No. 53, 121-125, 2026

Production improvement of nori aquaculture using biostimulants

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Abstract: Nori aquaculture is one of the most significant fisheries industries in Japan. However, the production of dried nori sheets has continuously been decreasing since 2000's due to high water temperature in an early aquaculture period (Oct. to Nov.), oligotrophication throughout the season and predation by herbivorous fish and birds. The genetic diversity of nori strains used for aquaculture is low, making it difficult to breed new beneficial strains against the climate change. So, we focused on "biostimulants," which have been known to give various plants some tolerance to abiotic stresses. In a series of our experiments, amino acids were evaluated as biostimulants for nori culture. We conducted three experiments based on the scheme of land plants using biostimulants: 1) screening of amino acids that are effective for the growth and survival of axenic nori protoplasts, 2) laboratory culture experiments in the presence of bacteria, and 3) field aquaculture trials. Moreover, prior to the field trials, we confirmed the effectiveness of a method of soaking the conchospores in tank seawater where each amino acid was dissolved to avoid using large amounts of amino acids when they are directly added into the area of aquaculture practice. As a result of the screening test, nori protoplasts treated with arginine or ornithine showed rapid growth. Under the condition of bacteria's existence, these two amino acids enhanced the adherence strength of nori thalli just by once soaking for 15 h. In the field trials, soaking conchospores in arginine or ornithine for 24 h enhanced not only the adherence strength but also the growth of nori blades, which increased the production of nori sheets by 1.1 to 1.3 times. We clarified that arginine and ornithine are the useful biostimulants in nori aquaculture, especially for enhancing the adherence strength and growth in an early aquaculture period and increasing the nori production.

Key words: adherence strength, amino acids, high water temperature, *Pyropia yezoensis*, soaking treatment

Introduction

High water temperature caused by the global climate change has influenced the nori aquaculture in an early period of cultivation in Japan (October to November), and oligotrophication occurred in a late aquaculture period (January to March) also affected the productibility. Recently, high water temperature and oligotrophication have become the most serious problems throughout the aquaculture period, while the predation by herbivorous fish and birds is becoming another great issue in nori aquaculture.

Breeding has been carrying out for the improvement of production and settlement of some environment-induced troubles occurred in nori aquaculture for a long time. However, the genetic diversity of nori strains used for aquaculture is low, making it difficult to breed new beneficial strains against the climate change. It is necessary to find or develop novel techniques for stable production of nori. So, we focused on "biostimulants" (BS), which have been known to give various plants some tolerance to abiotic stresses. European

Accepted on Feb 26, 2026
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Biostimulants Industry Council (EBIC) defined plant BS as "a product which stimulates plant nutrition processes independently of the product's nutrient content, with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: nutrient use efficiency, tolerance to abiotic stress, quality traits, or the availability of confined nutrients in soil rhizosphere." Moreover, EBIC also described as "biostimulant components include microorganisms, plant and algae extracts, amino acids, humic substances, mineral salts and some chemicals with biostimulant properties. Unlike fertilizers, which directly supply nutrients to plants, biostimulants stimulate the plant's own processes to better utilize nutrients and water" (https://biostimulants.eu/plant-biostimulants/).

Fig.1 shows a conceptual diagram of the harvest yield in land plants. The potential yield, which is 100%, could decrease to 20% due to various biotic and abiotic stresses in the process of growth from seedling to mature plant (Yamauchi and Kawai 2020). Application of pesticide and fertilizer, for example, can increase the yield to 40%, and further use of BS can increase the yield by 1.2 times. There are plenty of BS materials for land plants, e.g., seaweed extracts, polyphenols, humic substances, amino acids, bacteria and so on as above mentioned. For application of BS to macro algae, there are several studies reported recently (Abe *et al*. 2021a, 2021b, 2022; Han *et al*. 2022; Hurtado and Critchley 2018; Umanzor *et al*. 2019, 2020, 2022). Land plants could continuously be supplied with BS materials in an economical way at the right timing during the cultivation. On the other hand, for seaweed aquaculture, we have to consider how to use BS effectively due to disperse of BS when they are applied to the coastal area of nori farm. In this paper, we introduce a novel efficient method for using BS in nori aquaculture.

Short Materials and Methods

Experimental procedure to use BS for nori aquaculture

In the case of experiments on land plants, the effects of BS are evaluated by three steps. The first step is screening of candidate materials under an axenic culture condition. The second step is effect confirmation of BS selected in the first step under the presence of bacteria The third step is the final effect confirmation of BS in a field trial. We carried out our experiments following these procedures.

1. Screening of beneficial amino acids

We used 18 amino acids as candidate BS and carried out the screening of beneficial amino acids (1 mM) using axenic protoplasts of nori *Neopyropia yezoensis* (*Pyropia yezoensis*, Abe *et al*. 2021a, 2021b).

2. Effect of soaking conchospores in closed amino acid-dissolved seawater

To use BS efficiently, it was thought that the artificial BS treatment should be conducted in a closed system on land rather than their application in coastal nori farms. In the practical nori aquaculture, there is a process of soaking the net with nori seedlings being attached using land apparatus for 5 to 15 h in approximately 5 ton tank filled with clear seawater to enhance the adherence of conchospores. In this context, we tested the effect of soaking the net in tank seawater with amino acid being dissolved (Abe *et al*. 2022). Vinylon yarns with conchospores were soaked in seawater containing arginine (Arg) or ornithine (Orn) (1 mM) for 10 min, 1 h and 15 h. Then, we carried out an ordinal laboratory culture for about 2 months. We measured the adherence strength of young nori thalli to the nori nets in each treatment time.

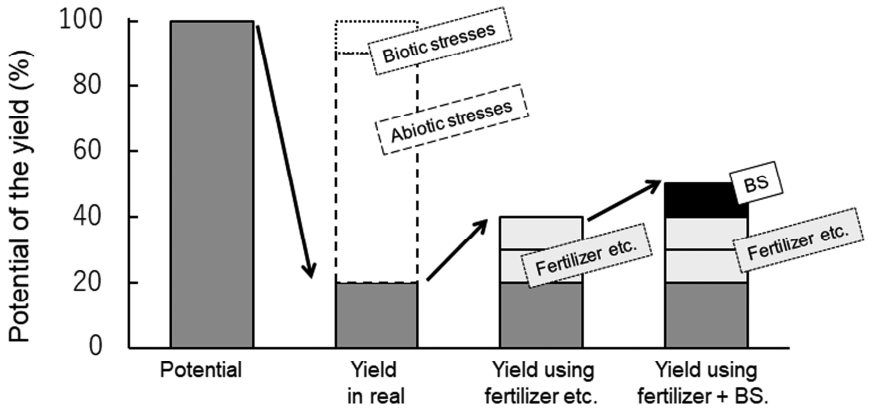

Fig.1 A conceptual diagram of the harvest yield in land plants. This figure was redrawn by the authors referencing that in Yamauchi and Kawai (2020)

3. Field culture trial

We conducted field culture trials over three years. After seedling by a common method on land, nori nets with conchospores being attached were soaked in seawater containing Arg or Orn (1mM) for 24 h. Then, nori farmers carried out an ordinal nori aquaculture in their own farms until harvest.

Results and Discussion

1. Amino acids beneficial to axenic nori protoplast

Fig.2 shows the results of survival rate (a) and thallus area (b) of axenic nori protoplasts cultured in a medium supplemented with each amino acid. Survival rate of nori protoplasts without amino acids (Control) was 57.1 ± 5.7%. The rates treated with each amino acid were at levels similar to or less than that of Control. On the other hand, thallus area of Control was 1237.5 ± 222.3 μm^2. The areas treated with Arg, glutamine (Gln), histidine (His), Orn and phenylalanine (Phe) were larger compared with that of Control. In terms of survival and growth, Arg and Orn were selected as effective BS for nori culture.

2. Effect of soaking conchospores in closed seawater containing Arg or Orn

Fig.3 shows the results of adherence strength measurement in each treatment time (a, 10 min; b, 1 h; c, 15 h). Soaking nori conchospores for 10 min did not change the adherence strength

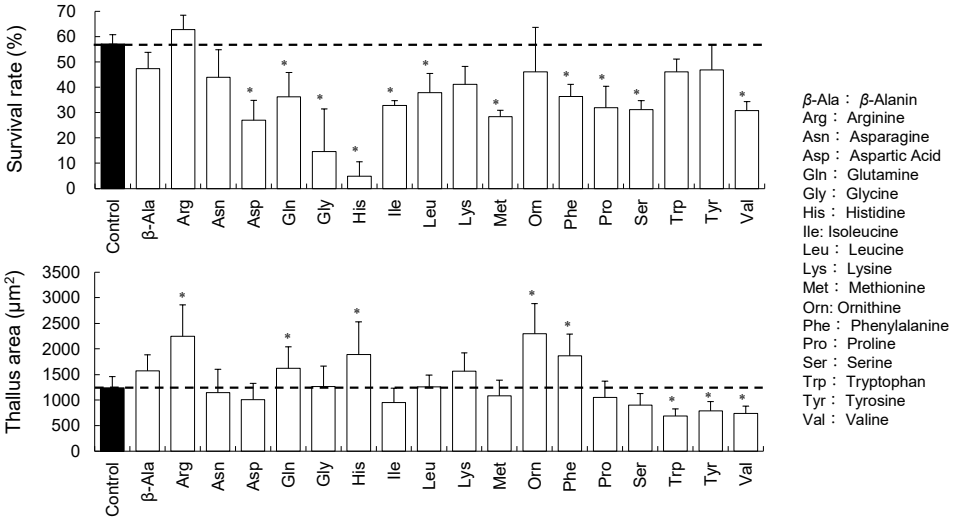

Fig.2 The results of survival rate (a) and thallus area (b) of axenic nori protoplasts previously treated with amino acids

Dotted lines indicate the average value of Control. An asterisk indicates a significant difference relative to the value of Control ($P < 0.05$).

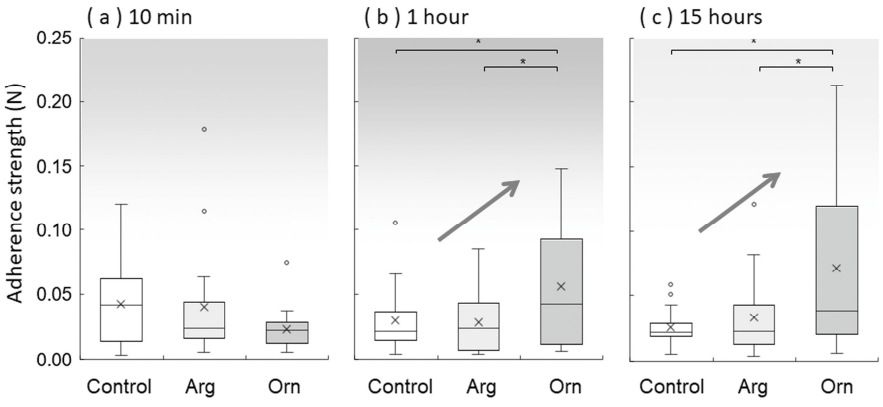

Fig.3 The results of adherence strength of nori thalli previously treated with arginine or ornithine in each treatment time (a, 10 min; b, 1 h; c, 15 h)

An asterisk indicates a significant difference between the groups ($P < 0.05$).

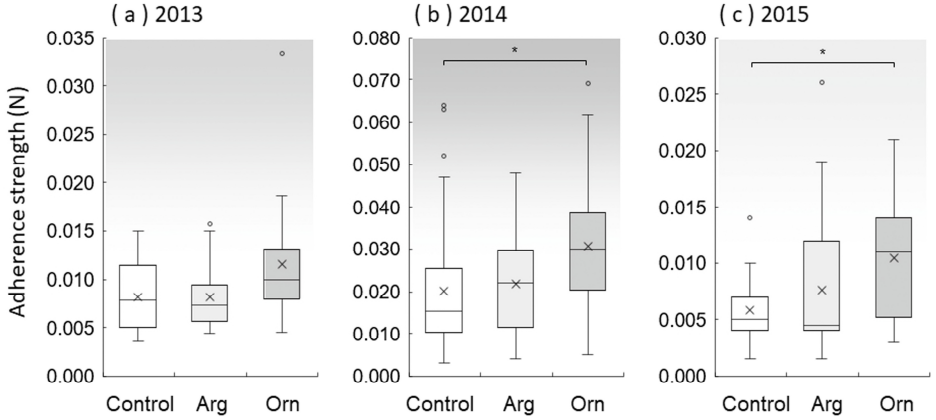

Fig.4 The results of adherence strength of nori blades previously treated with arginine or ornithine in each year of the field trials (a, 2013; b, 2014; c, 2015)

An asterisk indicates a significant difference between the groups by Tukey's multiple comparison test ($P < 0.05$).

Fig.5 The photographs of nori blades of each soaking treatment taken in the field trial (Nov. 2013)

of nori thalli among treatments. However, 1 h and 15 h of soaking, especially soaking in Orn, enhanced the adherence strength of thalli. These results suggest that soaking treatment with Arg or Orn dissolved in seawater after seedling has positive effects on nori culture.

3. Production improvement in field culture trials

Fig.4 shows the results of adherence strength measurement in each treatment in each year (a, 2013; b, 2014; c, 2015). In the field trials, soaking conchospores in Arg or Orn for 24 h enhanced the adherence strength of nori thalli in each year as found in the laboratory experiment. Fig.5 shows the photographs of nori blades in each soaking treatment group taken in Nov. 2013. In the Arg and Orn treatments, thalli grew better compared with that of Control. Additionally, Fig.6 shows representative daily yields of nori sheets in the 2015 season. The result shows that soaking nori conchospores in Arg or Orn for 24 h could increase the nori sheet production by 1.1 to 1.3 times.

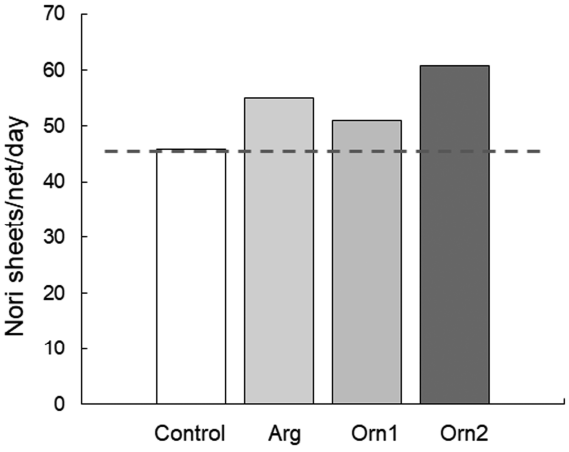

Fig.6 Representative daily yields of nori sheets in the 2015 season

Orn1 and Orn2 indicate the production in two different areas with the same ornithine soaking treatment. Dotted line indicates the average value of Control.

4. Potential of BS in seaweed aquaculture

It is important to use suitable materials specific to a given plant or seaweed as BS at the correct time. In the previous studies that examined the effects of BS for seaweeds, Han *et al*. (2022) and Umanzor *et al*. (2022) reported nori soaked in a medium containing brown alga extracts as BS for 10 days enhanced the tolerance for higher water temperature. Moreover, the brown alga extracts have another useful effects on the improvement of growth and vegetative propagation of nori. However, soaking or dipping of seaweeds as a temporal treatment of BS has only limited effects compared with a constant application of BS to land plants throughout the cultivation.

On the other hand, most of seaweed aquaculture are carried out in coastal areas, and it is practically difficult to soak or dip immature seedlings in seawater containing BS for a long time like 10 days. Moreover, we have to consider how to use BS effectively for seaweed aquaculture because BS materials are soon spread out by the water currents in the ocean. In this paper, we introduced a novel method of soaking conchospores in closed seawater containing Arg or Orn immediately after seedling of nori (Abe *et al.* 2022). This method can be carried out on land with a limited amount of BS (Arg or Orn) and to be incorporated in the ordinal process for seedling in Japanese nori aquaculture. It should be noted that repeated soaking was not necessary in this treatment, although it took 24 h. It is interesting to note that these two amino acids, Arg and Orn, were effective for conchospores, while application of Arg or Orn to adult nori thalli were ineffective (unpublished). This means that we have to use Arg or Orn at the correct time during nori cultivation.

As previously described, suitable materials should be used as BS at the correct time specific to a given plant or seaweed. The previous and present studies have shown that BS have a lot of potential for enhancement of environmental tolerance and production improvement in seaweed aquaculture. However, the number of BS materials, the effectiveness of which has been elucidated so far, are not enough for seaweeds. Thus, it is necessary to find another BS materials beneficial for seaweed culture.

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Bull. Jap. Fish. Edu. Agen. No. 53, 127 - 132, 2026

Seaweed seedling culture technique using LEDs and feeding behavior of herbivorous fish to suppress fouling by other seaweeds - in the case of "*hiziki*" *Sargassum fusiforme* culture -

Tsutomu NODA*[1, †], Tatsuru KADOTA*[1], Tomoyuki SHIKATA*[2], and Yuichiro FUJINAMI*[3]

Abstract: A brown seaweed *Sargassum fusiforme,* called "*hiziki*" in Japanese, is an important fishery resource in Japan. During the artificial seedling production of *hiziki*, fouling by other seaweeds (e.g. *Ulva* spp.) has become a great problem. In this study, we developed an efficient *hiziki* seedling culture technique using LEDs and the feeding behavior of herbivorous fishes: *Girella punctata* and *G. leonina*. *Hiziki* germlings (approximately 0.15 mm length) were cultured for 30 days under the LED light of four colors: green, blue, red, and white. At the end of the experiment, the respective mean length of the germlings in the green, blue, red, and white LEDs treatments were 1.95 mm, 1.44 mm, 0.25 mm, and 1.46 mm. The respective mean coverage of fouling by other seaweeds were 29.1%, 96.8%, 29.1%, and 87.2%. These findings indicate the green LED light is adequate for higher growth of *hiziki* and for growth suppression of fouling seaweeds. Furthermore, another experiment was conducted using herbivorous fish to remove other seaweeds. We placed *hiziki* germlings and other seaweeds propagating on a concrete block with 10 juveniles of *G. punctata* or *G. leonina* in a 100 L tank. After 4.5 h, the coverage of fouling by other seaweeds decreased from 23.5% to 0.5% in the *G. punctata* tank, and from 15.5% to 0.6% in the *G. leonina* tank. The lengths of *hiziki* on the block, those detached from the block, and those in the stomach contents of the fish were, respectively, 2.04 mm, 1.47 mm, and 0.86 mm in the *G. punctata* tank, and 2.37 mm, 1.54 mm and 1.25 mm in the *G. leonina* tank. These results demonstrate that larger *hiziki* seedlings are less susceptible to the browsing by *G. punctata* and *G. leonina*. Consequently, juvenile *G. punctata* and *G. leonina* were found to be effective for removing fouling seaweeds during the *hiziki* seedling production.

Key words: *Sargassum fusiforme*, LEDs, *Girella punctata*, *Girella leonina*, seaweed seedling production

Introduction

Seaweeds have lots of ecological functions such as absorption of dissolved nutrients and carbon, and some of them are fisheries-important species, too, such as human foods and industrial materials. Recently, their functions are attracting particular attention. A brown seaweed *Sargassum fusiforme* ("*hiziki*" in Japanese) is distributed on rocky shores in intertidal zones of Japan, Korea, and China (Yoshida 1998). In western Japan, *hiziki* grows up to 1-2 m in spring and early summer, and matures during May-June. *Hiziki* is regarded as resistant to high temperatures. Therefore, the *hiziki* habitat has extended from subarctic to subtropical areas. *Hiziki* is an important fishery resource in Japan, but wild *hiziki* production has been decreasing due to the changes in coastal environments and predation by herbivorous fish and benthos. In 1996, the

Accepted on Feb 26, 2026
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production was about 5,000 tons in Nagasaki Prefecture, Japan. In recent years, it has decreased to less than 500 tons (Fig.1, Nagasaki Prefecture, 1989, 2017). More than 90% of *hiziki* products recently distributed throughout Japan are imported from China and Korea. *Hiziki* has also been produced by aquaculture mainly using the seedlings collected from coastal areas, which has adversely affected the wild resources.

An artificial seedling production method of *hiziki* has been studied. Growth inhibition of *hiziki* occurs mainly due to green algae contamination during the seedling production. Noda *et al*. (2022) reported a method of removing fouling seaweeds using the feeding behaviors of juvenile herbivorous fish. Their study was performed with approximately 1.0 cm *hiziki* because smaller *hiziki* germlings might be consumed by the herbivorous fish. Thus, other methods are needed to remove the fouling seaweeds for smaller *hiziki* germlings. Brown algae (including *hiziki*) and green algae (including fouling seaweeds, e. g. *Ulva* spp.) have photosynthetic pigments of different types. The growth of both algae can be controlled by changing the light wavelength as demonstrated by Murase *et al*. (2018) and others. Both brown algae and green algae have chlorophyll a, which absorbs blue and red light. Green algae have chlorophyll b, and brown algae have chlorophyll c, both of which absorb blue light. By contrast, fucoxanthin in brown algae absorbs blue and green light, as described in Bricaud *et al*. (2004) and others. Thus, an experiment was planned to ascertain whether green light selectively promote the *hiziki* germling growth. As described in this report, we introduce an efficient *hiziki* seedling culture technique using LEDs. A suitable size of *hiziki* germlings that could be applied to the removal of fouling seaweeds by juvenile herbivorous fish *Girella punctata* (largescale blackfish) and *G. leonina* (smallscale blackfish) was also elucidated.

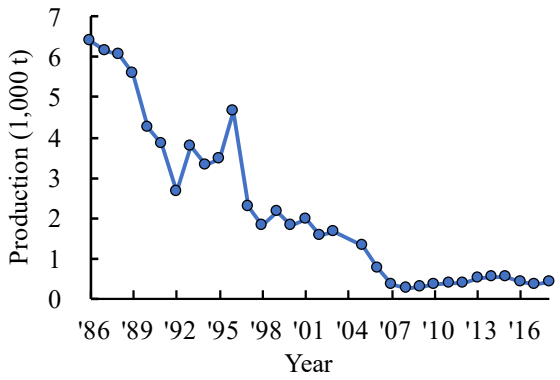

Fig.1 Change of *hiziki* production in Nagasaki Prefecture (Nagasaki Prefecture 1989, 2017)

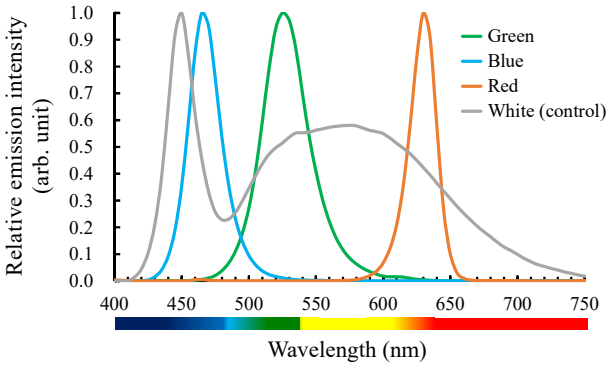

Fig.2 Relative emission intensity of LEDs used for the experimentation

Green, NCSG119 (Nichia); Blue, #182521 (Stanley Electric); Red, NCSR119 (Nichia); White, NVSW119F (Nichia). The relative emission intensity (arb. unit) was measured using a Spectromaster C-7000 (Sekonic).

Materials and Methods

Test I

Hiziki germling culture experimentation using LEDs was performed at Goto Field Station, Fisheries Technology Institute, Japan Fisheries Research and Education Agency (FRA), Nagasaki, Japan. After adult stage *hiziki* were collected from the coastal areas of Goto city during May and June in 2021, they were stocked in tanks filled with sand-filtrated seawater until maturation. On June 9, 2021, we collected the germlings (approximately 0.15 mm) from mature *hiziki* in the tanks and cultured them on eight concrete blocks (about 10,000 germlings per block, 39 × 19 × 10 cm). These germlings were cultured for 30 days, meanwhile they were exposed (100 μmol/m^2/s; 12L:12D light regime) to four colors of LED light: green, 526 nm central wavelength; blue, 466 nm; red, 631 nm; and white, complex light as a control (Fig.2). The product numbers of green, red, and white LEDs were NCSG119, NCSR119, and NVSW119F (Nichia, Tokushima, Japan), and that of blue LEDs was #182521 (Stanley Electric, Tokyo, Japan), respectively. The relative radiant intensity (arb. unit) was measured using a Spectromaster C-7000 (Sekonic, Tokyo, Japan). We used black 100 L polyethylene tanks (2 tanks for each color). The test tanks were covered with a lid to prevent outside light from entering, and sand-filtrated seawater was supplied (50 L/h). The water temperature was 20.6–25.2°C (no water temperature control). On June 16 (during cultivation) and July

Table 1 Concentrations of nutrients in the *hiziki* culturing seawater in Test I

Data (2021)	NO_3-N (μmol/L)	NO_2-N (μmol/L)	NH_4-N (μmol/L)	PO_4-P (μmol/L)	SiO_2-Si (μmol/L)	Dissolved Inorganic Nitrogen (μmol/L)
June 16	2.801	0.119	0.119	0.280	6.410	3.039
July 9	4.649	0.124	0.194	0.377	6.074	4.967

9 (at the end of cultivation) in 2021, nutrient concentrations in the culturing water in the tanks were analyzed using an autoanalyzer QuAAtro (BLTEC, Osaka, Japan) (Table 1). Since the seawater had a possibility of including other seaweeds embryos, the experiment was designed to mimic the conditions of mass seedling production in natural environment. After 30 days of culture, we measured the germling growth (from tip to root, over 30 individuals), the remaining rate of *hiziki*, and the coverage of fouling by other seaweeds. The remaining rates were calculated using the number of individuals at the start and that survived at the end of the examination by photographing the center of each block using a digital camera TG-5 (Olympus, Tokyo, Japan). The coverage of fouling by other seaweeds was analyzed for the photographs of concrete block surfaces using image analysis software ImageJ (US National Institutes of Health, Schneider *et al*. 2012).

Test II

The experiment of removing fouling seaweeds using juveniles of herbivorous *G. punctata* and *G. leonina* was performed at Goto Field Station, FRA. These fish were collected from the coastal area of Goto city during February and June in 2021 (Ito *et al*. 2018) and reared in sand-filtrated seawater until experimentation. Then, on July 19, 2021, a tank experiment was conducted. The water temperature was 25.0 °C. The *hiziki* germlings and other seaweeds propagated on two concrete blocks, which had been cultivated under the green LEDs, were used for the experiment. The coverage proportions of fouling by other seaweeds (*Ectocarpus* spp.) on the two blocks were estimated as 23.5% and 15.5% using ImageJ as described above. We placed each block in a black 100 L polyethylene tank with 10 juveniles of *G. punctata* (60.4 ± 9.0 mm TL) or *G. leonina* (62.1 ± 5.9 mm TL). After 4.5 h, the fish were euthanized in 100 ppm eugenol to examine the fish stomach contents. Then, the lengths of *hiziki* on the block, those detached from the block by browsing of the fish, and those found in the fish stomach contents were measured using a profile projector V-12 (Nikon, Tokyo, Japan).

Results and Discussion

Test I

After 30 days of cultivation under LEDs, the respective mean length of the germlings used in the green, blue, red, and white LED light treatments were 1.95 mm, 1.44 mm, 0.25 mm and 1.46 mm. The respective rates of remaining *hiziki* were 33.9%, 28.8%, 16.6%, and 15.7%. *Hiziki* cultivated under green LEDs grew largest with the highest rate of remaining *hiziki*. The respective coverages of fouling by other seaweeds in the green, blue, red, and white LED light treatments were 29.1%, 96.8%, 29.1%, and 87.2%. The appearances of the blocks showed no difference until 10 days. After 20 days, the fouling by other seaweeds had begun to propagate under blue and white LEDs. After 30 days, the blocks under blue and white LEDs were covered by other seaweeds. Green algae *Ulva* spp. increased mainly under the blue LED light, and brown algae *Ectocarpus* spp. increased mainly under the white LED light. By contrast, fouling by other seaweeds was slow on the blocks under green or red LEDs. These results demonstrate that the green LED light is adequate for higher growth of *hiziki* and for growth suppression of fouling by other seaweeds (Fig.3). In addition, the propagation of *Ectocarpus* spp. started to increase later during the 30 days of cultivation even under green LEDs. These results suggest that the green light cannot completely suppress the seaweed fouling.

Test II

In the fouling seaweeds removal experiment using juvenile herbivorous fish, the coverage of fouling by other seaweeds decreased from 23.5% to 0.5% in the *G. punctata* tank, and from 15.5% to 0.6% in the *G. leonina* tank. The mean length of *hiziki* on the block was longer than that detached from the block or that found in the fish stomach contents. These trends

were similar between both fish species: more than 80% of *hiziki* detached or eaten by fish were smaller than 2.0 mm (Fig.4). These results suggest that larger *hiziki* were less susceptible to the browsing by the fish. Therefore, *G. punctata* and *G. leonina* were found to be effective for removing the fouling seaweeds during the production of *hiziki* seedling that had grown larger than 2 mm. Noda *et al*. (2022) had previously reported that the use of *G. punctata* had a sufficient effect on the removal of other seaweeds without *hiziki* predation by the fish. The present results suggest that *G. leonina*, which is phylogenetically similar to *G. punctata*, can also be used similarly in *hiziki* seedling production.

Conclusion

Our study suggests that the use of LEDs and feeding behaviors of juvenile herbivorous fish is important for effective production of *hiziki* seedlings. During an early period of *hiziki* seedling production, green LEDs should be used for their growth promotion and the growth suppression of fouling

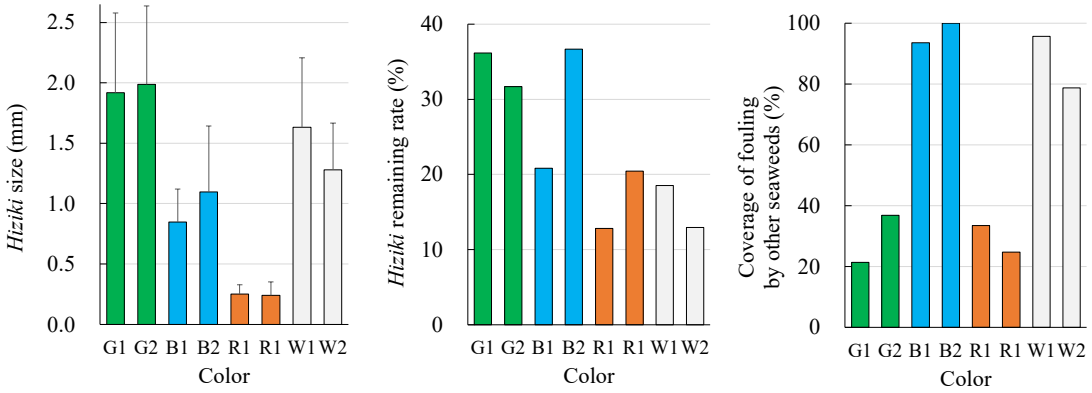

Fig.3 *Hiziki* size, remaining rate, and coverage of fouling by other seaweeds after the 30 days of culture

Vertical bars represent the standard deviations.

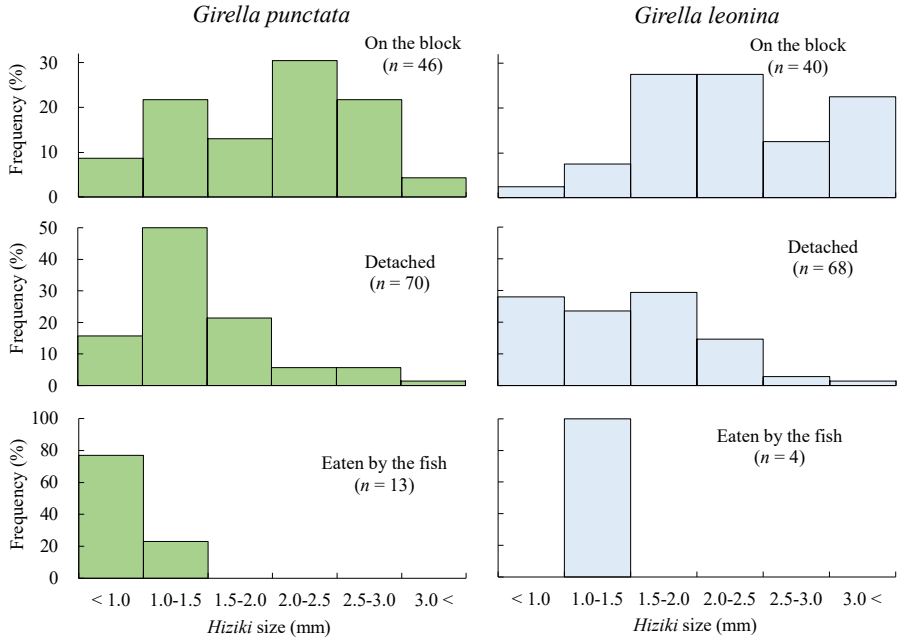

Fig.4 Histograms of the size of *hiziki* on the block, detached from the block, and eaten by *Girella punctata* and *G. leonina*

Fig.5 Appearances of concrete blocks before and after the introduction of juvenile *Girella punctata* and *G. leonina* during *hiziki* seedling production

by other seaweeds. After *hiziki* grew to 2 mm length, the feeding behavior of *G. punctata* and *G. leonina* can be applied to the removal of fouling seaweeds. Our method could be applied during practical *hiziki* seedling production (Fig.5). We are performing a test practice for artificial seedling production using this method. Further examinations must be conducted to assess the appropriate light intensity for *hiziki* growth, suitable sizes of *G. punctata* and *G. leonina* for fouling seaweeds removal, and potential use of other herbivorous fish species.

Acknowledgments

The authors deeply appreciate the assistance of members of Nagasaki Field Station and Goto Field Station, Fisheries Technology Institute, for cultivation of *hiziki* and rearing of *G. punctata* and *G. leonina*. We thank Dr. N. Murase, Dr. M. Abe, Dr. K. Abe, and Dr. S. Kitatsuji for their discussion and support during the experiments.

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Ito T, Iino Y, Nakai S, Itoi S, Sugita H, Takai N (2018) Distribution patterns of settlement-stage juveniles of *Girella punctata* and *Girella leonina* on the rocky coast of the Kanto–Izu region, Japan. *Fish. Sci*., **84**, 627-640.

Murase N, Abe M, Noda M (2018) Growth and maturation of gametophyte in *Undaria pinnatifida* under different light quality from light emitting diodes (LEDs). *J. Natl. Fish. Univ.*, **67 (2)**, 91-97 (in Japanese with English abstract).

Nagasaki Prefecture (1989) The results of Nagasaki fishery census in 1989. Nagasaki Prefecture, Nagasaki, 195 p (in Japanese).

Nagasaki Prefecture (2017) The results of Nagasaki fishery census in 2017. Nagasaki Prefecture, Nagasaki, 187 p (in Japanese).

Noda T, Kadota T, Shimaoka K, Fujinami Y (2022) Removal of fouling seaweed on artificial substrate for *Sargassum fusiforme* seedling culture by juvenile *Girella punctata* grazing. *Aquacult. Sci.*, **70 (1)**, 113-117 (in Japanese with English abstract).

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods*, **9**, 671-675.

Yoshida T (1998) Marine algae of Japan. Uchida Rokakuho Publishing, Tokyo, pp. 367-368 (in Japanese).

Annotated Bibliography of Key Works

(1) Noda T, Kadota T, Shimaoka K, Fujinami Y (2022) Removal of fouling seaweed on artificial substrate for *Sargassum fusiforme* seedling culture by juvenile *Girella punctata* grazing. *Aquacult. Sci.*, **70 (1)**, 113-117 (in Japanese with English abstract).

Tank experiments were conducted to develop a technique to remove fouling seaweeds from seedling production of *Sargassum fusiforme*. They set a substrate with *S. fusiforme* and fouling seaweeds (*Ulva australis* and *U. intestinalis*) and 12 juvenile *Girella punctata* in replicate tanks (n = 4). The coverage and length of the fouling seaweeds decreased as time proceeded, while those of *S. fusiforme* did not decrease. In addition, *S. fusiforme* were not observed in the stomach contents of *G. punctata*. These results suggest that *G. punctata* can remove fouling seaweeds without apparent damage to *S. fusiforme* in its seedling production.

(2) Murase N, Abe M, Noda M (2018) Growth and maturation of gametophyte in *Undaria pinnatifida* under different light

quality from light emitting diodes (LEDs). *J. Natl. Fish. Univ.* ,**67 (2)**, 91-97 (in Japanese with English abstract).

The effects of light quality on growth and maturation of gametophyte in *Undaria pinnatifida* were examined in an indoor culture at 20℃ under the lighting condition of 12h light-12h dark cycle and 50 μmol/m^2/s using four different light emitting diodes (LEDs) and a fluorescent light. The relative growth rates of the male and female gametophytes under green LEDs showed higher values, but those under red LEDs showed lower values. The female gametophyte matured quickly under blue LEDs and the fluorescent light. On the other hand, they matured more slowly under white and green LEDs. Under the red LEDs condition, the maturation of female gametophytes was not observed at all.

(3) Ito T, Iino Y, Nakai S, Itoi S, Sugita H, Takai N (2018) Distribution patterns of settlement-stage juveniles of *Girella punctata* and *Girella leonina* on the rocky coast of the Kanto–Izu region, Japan. *Fish. Sci.*, **84**, 627-640.

The early life history of girellid fishes in Japanese waters is unclear, and little is known about their species-specific reproductive strategies. They examined seasonal changes of the distribution patterns for settlement-stage juveniles of *Girella punctata* and *G. leonina* on the rocky shore in the regions of Kanto and Izu, Japan, to infer the influence of the Kuroshio Current on their reproduction. They collected 813 settlement-stage juveniles mainly in Sagami Bay and genetically identified the species.

The juveniles of *G. punctata* were collected on the rocky shore in Sagami Bay from April to August, with abundant catches in May and June. Thus, they inferred that juvenile *G. punctata* ubiquitously inhabited the rocky shore in the area in spring and summer. By contrast, juveniles of *G. leonina* were rarely collected in Sagami Bay, with a total catch of only 66. Notably, no juveniles were collected during the wintertime in Sagami Bay, although an abundant catch of *G. leonina* had been previously reported in Sagami Nada off Sagami Bay during January to March. These clear-cut differences between the habitats of two species likely reflect the differences in their proximity to the path of the Kuroshio Current. They expected that the Kuroshio Current strongly influenced the reproductive success of *G. leonina*.

水研機構研報, 第 53 号, 133 – 139, 令和 8 年
Bull. Jap. Fish. Edu. Agen. No. 53, 133 - 139, 2026

Advantages of small-sized macroalgae in seaweed bed restoration in Kyushu, western Japan

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Abstract: Seaweed beds composed of large-sized macroalgae (i.e., *Sargassum* spp. and *Ecklonia* spp.) have been declining along the coast of western Japan, especially in Kyushu. Excessive feeding by herbivorous fish is likely responsible for the loss of macroalgal beds, but we have not yet developed effective techniques to remove these fish. Recent research started in Kyushu have focused on the advantages and roles of small-sized macroalgae in the restoration of seaweed beds because small-sized macroalgae may be less vulnerable to herbivory than large-sized macroalgae and may have value as food and habitat for fishery benthos such as purple sea urchin (*Heliocidaris crassispina*). This study discusses the results of recent research on the benefits of small-sized macroalgae in seaweed bed restoration in Kyushu and highlights: (1) the low vulnerability of small-sized macroalgae to feeding by herbivorous fish, (2) the functionality of small-sized macroalgae as food for *H. crassispina*, and (3) the results of field restoration trials of small-sized macroalgae in sites with high feeding pressure from herbivorous fish. (1) The vulnerability of small-sized macroalgae to feeding by brown chub (*Kyphosus bigibbus*), Japanese parrotfish (*Calotomus japonicus*), and mottled spinefoot (*Siganus fuscescens*) were examined in tank experiments. Small-sized macroalgae were found to be less vulnerable than large-sized macroalgae to feeding by all fish species examined. Additionally, vulnerability to feeding was found to vary among species of small-sized macroalgae; red algae such as *Gelidium elegans* and *Dichotomaria falcata* tended to be the least susceptible. (2) Tank experiments also revealed that various small-sized macroalgae were useful as food for *H. crassispina*, although the gonad index values (GI) of the sea urchin fed any of the small-sized macroalgae were lower than that fed large-sized macroalgae. In addition, GI of the sea urchin fed red algae (*G. elegans* or *Palisada intermedia*) was higher than those fed the other species of small-sized macroalgae. (3) Attempts to restore small-sized macroalgae were conducted at two sites (Nagasaki and Kagoshima) in Kyushu and succeeded in increasing small-sized macroalgae such as Gelidiales and Rhodomelaceae by removing sea urchins. However, large-sized macroalgae such as *Sargassum* spp. did not increase at either site. Furthermore, GI of *H. crassispina* which left unremoved or moved to the removal area increased in the restored seaweed bed at Kagoshima. These studies suggest that various small-sized macroalgae have lower susceptibility to consumption by herbivorous fish and serve as food resources for *H. crassispina*. Therefore, the use of small-sized macroalgal species could be an effective means of seaweed bed restoration, thereby increasing fishery resources such as *H. crassispina* in waters with high feeding pressure from herbivorous fish.

Key words: herbivorous fish, *Heliocidaris crassispina*, isoyake, Kyushu

Accepted on Feb 26, 2026
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Introduction

Recently, seaweed beds composed of large-sized macroalgae such as *Ecklonia* spp. and *Sargassum* spp. have been rapidly declining along the coast of western Japan, especially in Kyushu (Kadota *et al*. 2023; Kiyomoto *et al*. 2021; Tanaka *et al*. 2012; Yoshimura *et al*. 2009). It has been suggested that environmental changes such as rise in water temperatures have disturbed the balance between animal herbivory and seaweed production, and are causing the decline in seaweed beds (Fisheries Agency 2021). Therefore, removal of herbivorous animals and/or transplantations of mature seaweed individuals that supply large amounts of diaspores such as zoospores, are adopted to restore seaweed beds in Japan. So far, restoration of large-sized macroalgae using these methods has been successful at several sites (Agatsuma *et al*. 1997; Ishikawa *et al*. 2017; Fisheries Agency 2021; Taino and Hosogi 2011; Yotsui and Maesako 1993). However, such attempts to restore seaweed beds have failed at some sites, and these failures are attributable to feeding by herbivorous fish, which are more vagile than sea urchins. On the other hand, efficient methods for removing herbivorous fish have not been established yet.

For the reasons described above, small-sized macroalgae have attracted more attention in seaweed bed restoration in Kyushu (Miyazaki Prefecture 2014; Yoshimura *et al*. 2015). Small-sized macroalgae refer to all seaweeds except for large-sized macroalgae such as *Ecklonia* spp. and *Sargassum* spp., and include various species of brown, green, and red algae (Fujita *et al*. 2010). Among these, small-sized upright macroalgae other than coralline algae, such as *Dictyota* spp. and *Gelidium* spp., are expected to be the target species for restoration. Two advantages of using small-sized macroalgae have been suggested (Miyazaki Prefecture 2014; Yoshimura *et al*. 2015). First, they may be less vulnerable to predation by herbivorous fish than large-sized macroalgae. Second, despite their size, small-sized macroalgae may offer provision of food and habitat for fishery organisms. Therefore, it is expected that small-sized macroalgae can be propagated by removing sea urchins without removing herbivorous fish, thereby improving fishery resource conditions. However, it is not known whether small-sized macroalgae have lower vulnerability to feeding by herbivorous fish than large-sized macroalgae, and the effects of small-sized macroalgae as food and habitat are known only for a limited number of fishery resources in Kyushu. Furthermore, field trials of seaweed bed restoration targeting small-sized macroalgae have rarely been conducted.

Recently, studies on small-sized macroalgae for seaweed bed restoration have been conducted in Kyushu, where the ecological impact of herbivorous fish is expected to be particularly strong. This paper discusses recent studies on the benefits of small-sized macroalgae for seaweed bed restoration and highlights the following three points: (1) the lower vulnerability of small-sized macroalgae to feeding by herbivorous fish, (2) the functionality of small-sized macroalgae as food for purple sea urchin (*Heliocidaris crassispina*), and (3) the results of field restoration trials of small-sized macroalgae in two sites with high feeding pressure from herbivorous fish. The functionality of small-sized macroalgae had been reported by Yoshimura *et al*. (2015), who suggested that small-sized macroalgae provided an important habitat for juvenile spiny lobster (*Panulirus japonicus*) and food for abalones (*Haliotis* spp.) and turban shell (*Turbo sazae*). In this paper, we describe the benefits of small-sized macroalgae for *H. crassispina*, which have been studied following the Yoshimura's (2015) study.

Lower vulnerability of small-sized macroalgae to feeding by herbivorous fish

Mottled spinefoot (*Siganus fuscescens*), Japanese parrotfish (*Calotomus japonicus*), and various kyphosids including brown chub (*Kyphosus bigibbus*) are known to be typical browsers of seaweeds in western Japan (Vergés *et al*. 2014). To evaluate the vulnerability of small- and large-sized macroalgae to feeding by these three fish, tank experiments were conducted (Table 1). In these experiments, large-sized macroalgae and small-sized macroalgae were simultaneously offered to the herbivorous fish and the reduction in each alga was monitored over time. In the experiment with *K. bigibbus* (mean total length, 43.0 cm), one large-sized (*Sargassum alternato-pinnatum*) and six small-sized macroalgae (brown algae: *Padina arborescens*, *Dictyopteris undulata*, and *Dictyopteris prolifera*; red algae: *Gelidium elegans* and *Dichotomaria falcata*; green algae: *Codium intricatum*) were evaluated (Kadota *et al*. 2022). The results showed that *S. alternato-pinnatum* was the most vulnerable to feeding by *K. bigibbus*, followed by *P. arborescens.* In contrast, the other seaweeds were not so vulnerable to feeding by *K. bigibbus*. Analysis of stomach contents of wild *K. bigibbus* also showed that large-sized macroalgae such as *Sargassum* spp. and *Undaria pinnatifida* made up a significant portion of the stomach contents from spring to early summer when both large-sized and small-sized macroalgae are abundant (Yatsuya *et al*. 2015).

In the experiment with *C. japonicus* (mean total length, 36.8 cm), one large-sized (*Sargassum fusiforme*) and five small-sized macroalgae (brown algae: *P. arborescens* and *D. undulata*; red algae: *Palisade papillosa* and *G. elegans*; green algae: *Codium fragile*) were evaluated (Noda and Kadota 2024). The results showed that *S. fusiforme* was the most vulnerable to feeding by *C. japonicus*, followed by *P. arborescens*, *D. undulata* and *P. papillosa* were slightly damaged, while *G. elegans* and *C. fragile* suffered little damage.

Two tank experiments were conducted to evaluate the vulnerability to feeding by *S. fuscescens.* In the first experiment, the feeding selectivity of three size classes of *S. fuscescens* (mean total length, 8.7, 20.6 and 30.0 cm) was evaluated by providing *S. fusiforme* as the large-sized macroalga and *P. arborescens* or *Ishige okamurae* (brown algae) as the small-sized macroalga (Kiriyama *et al*. 2005). The results showed that *S. fuscescens* tended to prefer *S. fusiforme* to either of the small-sized macroalgae in all size classes. The second experiment was conducted on *S. fuscescens* with an average total length of 28.1 cm, using the same method as for *K. bigibbus* (Kadota *et al*. 2022). The results showed that *S. alternato-pinnatum*, *P. arborescens* and *C. intricatum* were the most vulnerable to feeding by *S. fuscescens*. Unlike the results from the other herbivorous fish, small-sized macroalgae were eventually damaged after the large-sized macroalga was lost. However, *G. elegans* was less vulnerable to the herbivory and *D. falcata* suffered little damage.

The results of a series of vulnerability experiments suggest that large-sized macroalgae are more vulnerable to feeding by all herbivorous fish examined, and that various small-sized macroalgae are less susceptible to feeding by herbivorous fish, although these experiments tested a limited number of seaweed species. Among small-sized macroalgae, brown algae such as *P. arborescens* tended to be more vulnerable to feeding, while red algae such as *G. elegans* and *D. falcata* tended to be less vulnerable. This suggests that red algae such as *G. elegans* will be promising small-sized macroalgae for seaweed bed restoration in Kyushu. However, a previous tank experiment showed that *G. elegans* and *D. falcata* were more vulnerable than brown algae to feeding by scalpel sawtail (*Prionurus scalprum*; Kadota *et al*. 2022). Although *P. scalprum* is not considered to have as great an impact on seaweed as the three species mentioned above (Fisheries Agency 2021), these red algae should be carefully considered as candidates for restoration of seaweed beds in the area with a large *P. scalprum* population.

The functionality of small-sized macroalgae as food for *H. crassispina*

Heliocidaris crassispina is an important fishery resource in Kyushu. The individuals in seaweed beds with abundant food can be used as a fishery resource, while those in barren sites do not develop gonads and cannot be used for fishery resource. Two tank experiments were conducted to evaluate the food values of small-sized macroalgae for *H. crassispina* (Table 2). Shao *et al*. (unpublished data) offered *H. crassispina* caught in barren sites salted *U. pinnatifida* as a large-sized macroalga and *D. undulata* and *G. elegans* as small-sized macroalgae for 2 months. The results showed that the gonad index values (GI) of *H. crassispina* fed the small-sized macroalgae were higher than that of the unfed group. When comparing the two small-sized macroalgae, *G. elegans* had a better food value than *D. undulata*. However, GIs of *H. crassispina* fed the small-sized macroalgae did not increase as much as that fed the large-sized macroalga.

Another experiment was conducted by Takada (2016) to evaluate the food values of different small-sized macroalgae. In this experiment, eight small-sized macroalgae (brown algae: *Colpomenia sinuosa*, *D. undulata*, *D. prolifera*, *Dictyota spinulosa* and *P. arborescens*; red algae: *G. elegans* and *Palisada intermedia*; green algae: *C. fragile*) were offered to *H. crassispina* and GIs were compared with *H. crassispina* that were fed salted *Saccharina japonica*. The results showed that these small-sized macroalgae had some food values for *H. crassispina,* but not as much as the large-sized macroalga. Additionally, the red algae had the highest food values among the small-sized macroalgae examined and GIs of the urchins fed the red algae reached about 80% of that fed the large-sized macroalga.

Furthermore, GI of *H. crassispina* was investigated in seaweed beds composed of small-sized macroalgae and the outside of the beds (areas with relatively few small-sized macroalgae) at two depths (2 and 7 m) in Nagasaki (Kadota *et al.* 2022). GI in the seaweed beds was higher than that in the outside areas at both depths. As a result, the time period when GI became over 6% (the lower limit to have commercial value) was longer in the seaweed beds than in the outside areas. When comparing the shallow seaweed bed composed of mainly red algae with the deep seaweed bed mainly composed of brown algae, GI was higher in the shallow seaweed bed than in the deep seaweed bed. However, GI peaked at 8.9% in the shallow seaweed bed in Nagasaki (Kadota *et al.* 2022), while GI peaked at 11.8% in the seaweed bed composed of *Sargassum*

Table 1 Vulnerability of large-sized macroalgae and small-sized macroalgae to feeding by herbivorous fish

Species	Relative vulnerability					References
	1 (high)	2	3	4	5 (low)	
Kyphosus bigibbus	***Sargassum alternato-pinnatum*** (B)	*Padina arborescens* (B)	*Codium intricatum* (G)* *Dictyopteris undulata* (B)* *Dictyopteris prolifera* (B)* *Dichotomaria falcata* (R)* *Gelidium elegans* (R)*			Kadota *et al*. (2022)
Calotomus japonicus	***Sargassum fusiforme*** (B)	*Padina arborescens* (B)	*Dictyopteris undulata* (B)	*Palisada perforate* (R)	*Codium fragile* (G)* *Gelidium elegans* (R)*	Noda and Kadota (2024)
Siganus fuscescens	***Sargassum fusiforme*** (B)	*Padina arborescens* (B)				Kiriyama *et al*. (2005)
	Sargassum fusiforme (B)	*Ishige okamurae* (B)				Kiriyama *et al*. (2005)
	Codium intricatum (G) ***Sargassum alternato-pinnatum*** (B) *Padina arborescens* (B)	*Dictyopteris prolifera* (B)	*Dictyopteris undulata* (B)	*Gelidium elegans* (R)	*Dichotomaria falcata* (R)*	Kadota *et al*. (2022)

Bold text, large-sized macroalgae; regular text, small-sized macroalgae
G, green algae; B, brown algae; R, red algae
An asterisk indicates the seaweeds that were particularly resistant to feeding damage (decrease rate of 20% or less).

Table 2 The effect of large-sized macroalgae and small-sized macroalgae on gonad index of *Heliocidaris crassispina*

Gonad index				References
1(high)	2	3	4 (low)	
Salted *Undaria pinnatifida* (B)	*Gelidium elegans* (R)	*Dictyopteris undulata* (B)	Non-feeding	Shao *et al*. (unpublished)
Salted *Saccharina japonica* (B)	*Palisada intermedia* (R) *Gelidium elegans* (R)	*Codium fragile* (G) *Padina arborescens* (B) *Dictyota spinulosa* (B) *Dictyopteris undulata* (B) *Dictyopteris prolifera* (B) *Colpomenia sinuosa* (B)		Takada (2016)

Bold text, large-sized macroalgae; regular text, small-sized macroalgae
G, green algae; B, brown algae; R, red algae

spp. in Kyoto (Yatsuya and Nakahara 2004). Therefore, GI would be higher in the seaweed beds composed of small-sized macroalgae than that in the barren sites, but not as high as GI in the seaweed beds composed of large-sized macroalgae.

The results of tank experiments and field surveys suggest that small-sized macroalgae are food resources for *H. crassispina*, although their food values are lower than large-sized macroalgae. In particular, red algae such as *G. elegans* had the highest food value among the small-sized macroalgae examined. Therefore, red algae could be promising candidates among small-sized macroalgae for the restoration of seaweed beds as food resources for *H. crassispina* in Kyushu.

Field trials of restoration of small-sized macroalgae in sites with high feeding pressure from herbivorous fish

Field experiments to restore small-sized macroalgae were conducted on two reefs in Nagasaki Prefecture and Kagoshima Prefecture, Kyushu (Igari *et al*. 2022; Kadota *et al*. 2022). At both sites, sea urchins (mainly *H. crassispina* in Nagasaki and *Echinometra mathaei* and *Echinostrephus aciculatus* in Kagoshima) were initially removed, and seaweeds were subsequently monitored. The results showed that coverage of small-sized macroalgae increased in the experimental areas compared with the control areas where sea urchins were not removed by the following spring. The small-sized macroalgae that increased were mainly red algae such as Gelidiales and Rhodomelaceae. However, large-sized macroalgae such as *Sargassum* spp. hardly increased at both sites. Change in GI of *H. crassispina* left removed or moving to the experimental area was also monitored in the Kagoshima site after the removal (Igari *et al*. 2022). The GI in the experimental area was higher than that in the control area and the GI only in the experimental area exceeded 6% in the following spring.

Conclusions and future research directions

Barren sites have persisted where herbivory of sea urchins and herbivorous fish are high. In such reefs, sea urchins have small gonads and are therefore not suitable for commercial catch. However, studies conducted in Kyushu suggest that the removal of sea urchins once from the areas could change such a situation via the following process. First, because small-sized macroalgae are less vulnerable to predation by herbivorous fish, small-sized macroalgae increase. Then, the propagated small-sized macroalgae serve as valuable food resources for *H. crassispina* left unremoved or moving to the removal areas, and lead to the increase in the GI , which becomes a good fishery resource. Once sea urchins become fishery resources, periodic harvesting of them would help maintain low sea urchin densities. Previous studies have shown that small-sized macroalgae provide an important habitat for juvenile spiny lobster and food for abalones and turban shell (Yoshimura *et al*. 2015). Other fishery resources are also expected to increase along with *H. crassispina* in the favorable cycle described above. Thus, we consider the use of small-sized macroalgal species to be an effective means of restoration of seaweed beds that have experienced high feeding pressure from herbivorous fish, and to thereby increase fishery resources such as *H. crassispina* in Kyushu.

Red algae, especially *G. elegans*, are thought to be promising candidates of small-sized macroalgae for seaweed bed restoration in Kyushu. *Gelidium elegans* is less vulnerable to feeding by herbivorous fish and a good food resource for *H. crassispina* (Kadota *et al*. 2022; Noda and Kadota 2024; Takada 2016). In addition, *Gelidium* and species of related genera create a better habitat for juvenile spiny lobster (Yoshimura *et al*. 2015). They have also been utilized as the major sources for commercial extraction of agar, making them important fishery resources. The characteristics of *G. elegans* could efficiently increase the fisheries resources in the areas with high feeding pressure from herbivorous fish. Furthermore, the maximum critical temperature for the growth of *G. elegans* is reported to be higher than those for many large-sized macroalgae (Baba 2010; Baba 2021; Komazawa 2017; Murase 2022). This characteristic could be another advantage in seaweed bed restoration by allowing adaptation to climate change. However, crops of *Gelidium* (mainly *G. elegans*) have been declining on the coast of Hachijo-jima Island, off the southern coast of Kanto, central Japan (Komazawa 2017), possibly due to the decreased nutrient concentrations in the water. Small-sized macroalgae encompass a diverse range of species, but only a limited number of them have been evaluated for their advantages in the resistance to herbivorous fish, food value for fishery resources, and other biological characteristics. Further studies are needed to evaluate more diverse small-sized macroalgae from a broader perspective, including environmental factors such as water temperature and nutrients on their growth.

Acknowledgment

We thank Dr. G. Yoshida and Dr. T. Yamamoto for their valuable comments on the manuscript.

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水研機構研報, 第 53 号, 141 – 151, 令和 8 年
Bull. Jap. Fish. Edu. Agen. No. 53, 141 - 151, 2026

Pacific oyster condition and mortality in a U.S. Pacific coast estuary: Can relationships with environment and reproductive state be utilized to sustain future production ?

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Abstract: Pacific oysters (*Magallana* [*Crassostrea*] *gigas*) were introduced to the US Pacific coast beginning in the early 1900's and have become the predominant cultivated shellfish species contributing substantially to US domestic production. While they were widely transported amongst locations, they only regularly spawned and became "naturalized" in several discrete estuarine locations like Willapa Bay, WA. Though culture methods differ, the US industry, like that in Japan, relied on "natural" set at these locations until the large-scale adoption of shellfish hatcheries in the late 1970's. A monitoring program was therefore established to examine both larval set and the condition of adult oysters in Willapa Bay during the mid 1950's and has been maintained since that time. The gametogenic cycle for oysters is relatively well studied and has been previously correlated with environmental triggers like temperature, salinity, and food which influence oyster growth but can also act as stressors and are linked to gametogenesis and summer mortality events. We summarize the results of an investigation into broad decadal temporal scale and Pacific Ocean basin spatial scale environmental forcing factors that influence oyster condition in Willapa Bay. We then, link these to seasonal shifts in oyster condition and reported summer mortality events that have occurred on an annual temporal scale within this estuary, including one such event in 2019 when a joint US-Japan study was being conducted. A comparison with similar observations and environmental data from the Seto Inland Sea in Japan that year suggests that multiple stressors are likely involved and differ by location but the response of oysters to local growing conditions (seagrass presence, on-off bottom culture) was similar. We provide a brief proposal and framework to further examine metabolic or energetic characteristics (glycogen content or anaerobic capacity) that could be used to either breed oysters for resilience to such stressors and/or provide the shellfish industry the ability to adapt to and mitigate for the effects of an uncertain future climate.

Key words: Pacific oysters, condition, reproductive state, mortality

Introduction

Pacific oysters (*Magallana* [*Crassostrea*] *gigas*) were introduced to the US Pacific coast beginning in the early 1900's and have become the predominant cultivated shellfish species along this coast. Pacific oysters are currently produced by about 175 farms that employ about 2,500 people in often economically depressed coastal communities with harvest valued at more than $149 million annually (United States Department of Agriculture 2024). While these oysters have been widely transported amongst locations, until recently they only regularly spawned and therefore became "naturalized"

Accepted on Feb 26, 2026
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populations at several discrete estuarine locations like Willapa Bay, WA (Lindsay and Simons 1997; McAfee and Connell 2021; Quayle 1988; Ruesink *et al*. 2005; Steele 1964). Though culture methods differ, the US industry relied on imports of seed from Japan or "natural" set at these locations until the large-scale adoption of shellfish hatcheries in the late 1970's (Chapman and Esveldt 1943; Chew 1984; Lindsay *et al*. 1959). A monitoring program was established to examine both larval set and the condition of adult oysters in Willapa Bay beginning in the mid 1950's and has been maintained since that time (Schoener and Tufts 1987; Schumacker 1999; Westley 1961). The gametogenic cycle for oysters is relatively well studied and has been previously correlated with environmental triggers like temperature, salinity, and food which influence oyster growth but can also act as stressors and are linked to gametogenesis and summer mortality events (Cheney *et al*. 2000; Garcia *et al*. 2011; King *et al*. 2021; Perdue *et al*. 1981; Solomieu *et al*. 2015).

We briefly summarize the results of an investigation into broad decadal temporal scale and Pacific Ocean basin spatial scale environmental forcing factors that influence oyster condition in Willapa Bay (Dumbauld *et al*. 2023). We then link these to seasonal shifts in oyster condition and summer mortality events that have occurred on an annual temporal scale within this estuary. This includes a brief summary and some additional results from a study conducted for joint UJNR research in 2019 and comparison with similar observations and environmental data from the Seto Inland Sea in Japan (Hasegawa *et al*. 2021) which suggest that multiple stressors are likely involved and could differ by location. Finally, we provide a brief proposal and framework to further examine metabolic or energetic characteristics (glycogen content or anaerobic capacity) that could be used to either breed oysters for resilience to such stressors and/or provide the shellfish industry the ability to adapt to and mitigate for the effects of an uncertain future marine environment.

Methods

Several different methods have been used to calculate bivalve condition that relate a measure of meat weight (wt) to that of the shell or the volume of the shell cavity. The condition index (CI) of oysters in Willapa Bay was historically measured using the Westley method calculated as: CI = dry body wt x 100 / [(whole wt in air − whole wt in water) – (shell wt in air − shell wt in water)] (all wts recorded in g) from 1954-1998 (Westley 1961). Biologists collected 20 individual

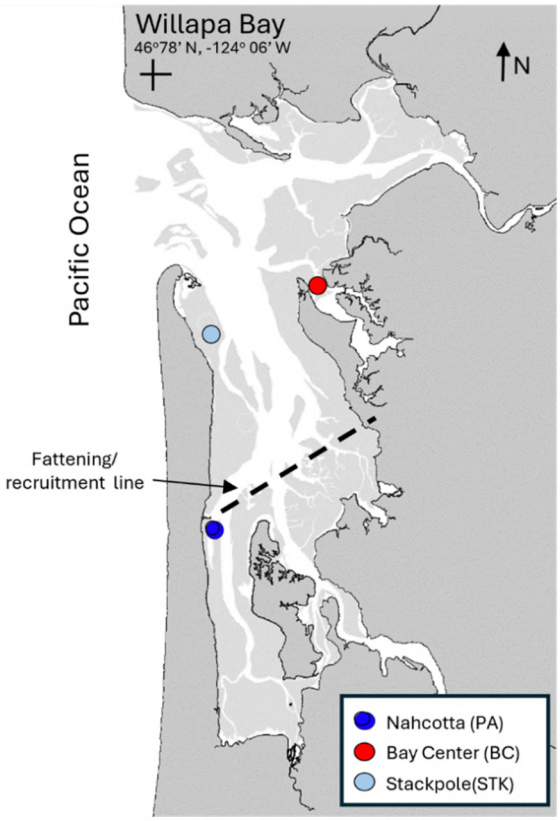

Fig.1 Map of Willapa Bay,Washington, USA showing 2019 transplant experiment sites (Nahcotta and Bay Center)

Nahcotta site (Parcel A, PA) is also a long-term oyster condition index monitoring location as is Stackpole (STK), a site closer to the estuary mouth. Also shown is a dashed line separating high quality oyster fattening beds in the northern portion of the estuary from southern portion of the estuary where growers usually plant oyster seed.

adult oysters of similar size (generally about 2 years old, volume = 125 cc.) at each of 4 locations in Willapa Bay on a monthly basis. After a detailed comparison with other methods, it was determined that a less error prone and more widely used gravimetric method where CI = dry meat wt *100 / (whole wet wt – dry shell wt), produced similar and directly related estimates (Lawrence and Scott 1982; Schumacker 1999). This standardized procedure that used individual instead of pooled oyster samples and thus allowed for error estimates about monthly means was adopted in 1998. We present a brief summary of a recent analysis of this long-term data record (1960-2021) which relates CI to basin wide ocean conditions (Dumbauld *et al*. 2023), and evaluate the seasonal pattern of CI comparing the average long-term record with that for years when mass mortality events were documented.

We analyzed experimental data collected as part of a UJNR study conducted in 2019 where adult oysters were collected

from one of these long term sampling locations in Willapa Bay (Nahcotta = Parcel A, PA) near the south end of the estuary and then transplanted back to this same location and to a second location (Bay Center, BC) closer to the estuary mouth (Fig.1) to evaluate the seasonal pattern of condition and gametogenesis (Hasegawa *et al*. 2021). Oysters were placed in bags on poles in two habitats (eelgrass, open mudflat), in two configurations (directly on sediment surface and 25cm above the surface) and a second set deployed from a nearby pier where they would be continuously submerged. This submerged treatment was designed to be similar to the typical method for hanging culture in Japan (Fujiya 1970; Hirata and Akashige 2004). Six sets of bags were deployed at each location in February and 3 sets replicate bags with individually labeled oysters retrieved in July. The other 3 sets of bags were retrieved and survival and condition index measured in May, June and August to capture a seasonal trend and compare condition with that measured at the Nahcotta and Stackpole (STK) long term monitoring sites in this estuary. Similar experimental data were collected at Hatsukaichi in Hiroshima Bay, a part of the Seto Inland Sea, Japan in 2019 (see Hasegawa *et al*. 2021 for description).

Results and Discussion

An analysis of the long-term data set for oyster condition index collected at four locations in Willapa Bay indicated consistent trends amongst locations. A single component of the variability in CI was most closely related to similar variability in the upwelling index for the nearshore coastal ocean which was positively correlated with condition index during the summer upwelling period (Dumbauld *et al*. 2023). Two large scale shifts in oyster condition occurred in 1977/1978 and 1999/2000 (Fig.2). The first shift corresponded with a previously well documented change from a cool to warm phase of the basin scale Pacific Decadal Oscillation (PDO), but the more recent shift instead correlated with local fluctuations in temperature and upwelling intensity. Differences in the average condition index measured at Nahcotta and Stackpole (closer to the estuary mouth, Fig.1) were evident and shifts in seasonal timing of peak condition also appeared to be linked to basin wide indices with higher condition and earlier peaks occurring after the most recent shift.

Oyster condition index followed similar seasonal trends at

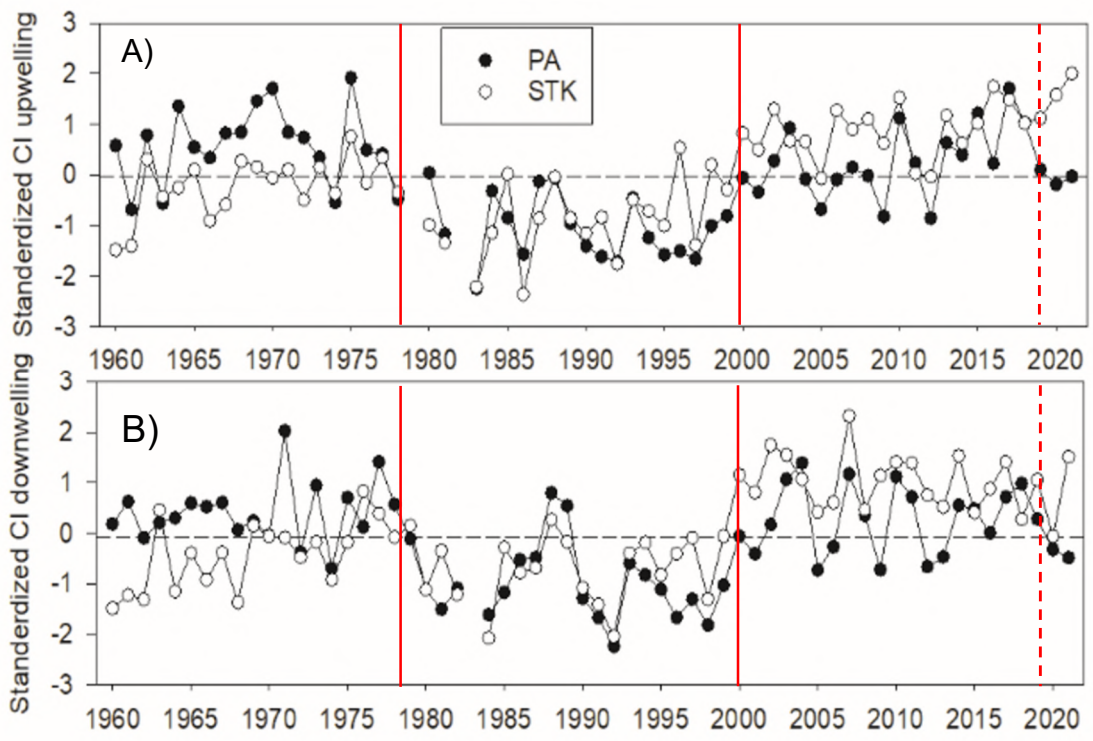

Fig.2 Long term record (1960-2022) of oyster condition index at Nahcotta (PA) and Stackpole (STK) locations averaged over A) the upwelling period (May-September) and B) the downwelling period (November-April) and standardized to the long term mean for those periods

Solid vertical lines represent years (1978/1979 and 1999/2000) when the index shifted and dashed line for 2019 when experimental transplant study was made (modified from Dumbauld *et al*. 2023).

both sites in Willapa Bay in the 2019 experiment, but condition was already higher at Bay Center than at Nahcotta when first measured in May and dropped significantly in August due to an apparent spawning event (Fig.3). By July condition was slightly higher for oysters suspended off bottom and trends differed for those continuously suspended at the pier with oysters in Bay Center having apparently spawned by July. Mortality was higher at Bay Center where it began in July but increased substantially in August with only 40% survival. Survival was higher at Nahcotta and generally higher for oysters deployed at the pier. Though slightly higher in eelgrass than open mud, neither oyster condition nor mortality were significantly influenced by habitat. (Fig.4).

The seasonal pattern of oyster condition index at the Nahcotta long-term monitoring site mirrored that recorded for the experimental oyster deployments and differed from the long-term average (1955-2019), with a large increase in condition occurring from April to June and then a sharp decline and apparent spawning event occurring by August followed by an extended period of recovery (Fig.5). Though condition was higher at the Stackpole long-term monitoring site closer to the estuary mouth, the seasonal pattern at that location was similar. Shellfish growers operating in Willapa Bay reported a significant summer mortality event in 2019, so we compared condition index with other years when mortality was similarly reported (Fig.5). Though not as dramatic, a similar pattern of higher-than-average condition in winter and a slightly earlier and larger increase in average condition with a significant drop (likely due to spawning events) occurred in these years as well.

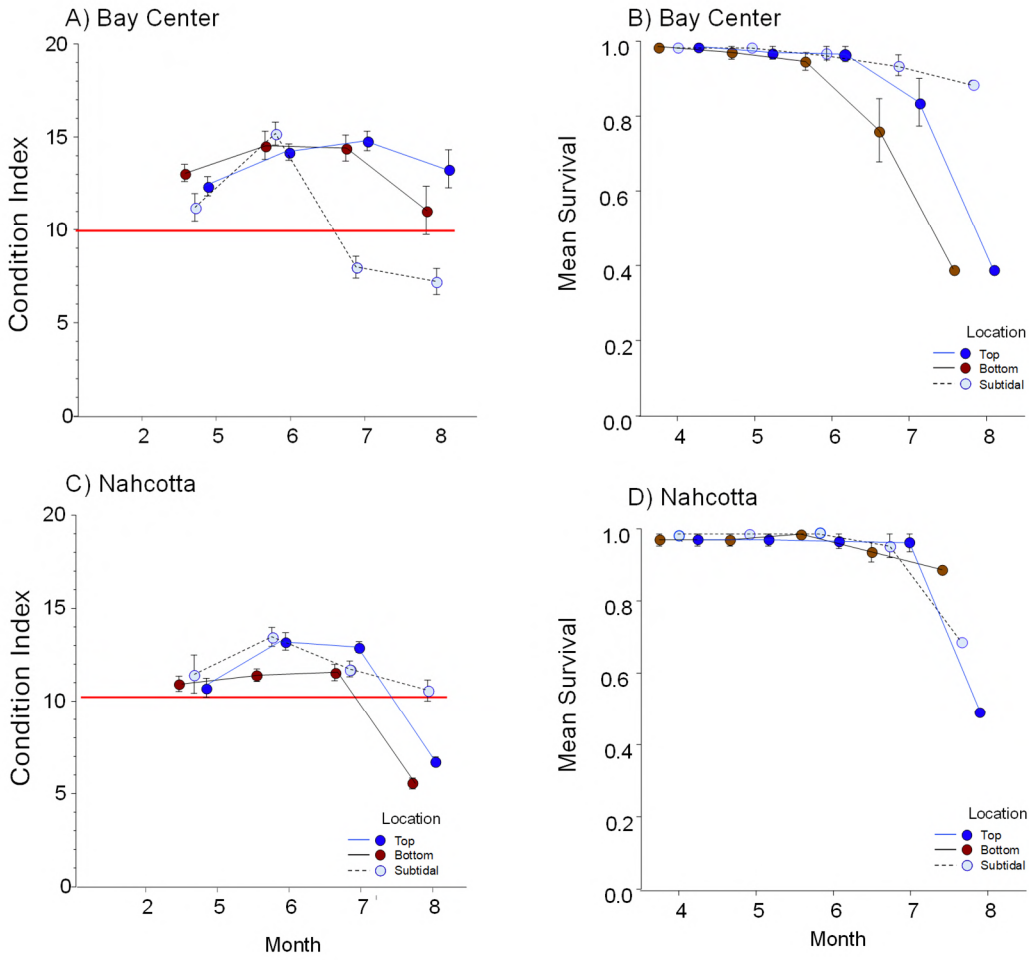

Fig.3 Results of transplant experiment conducted in Willapa Bay in 2019. Condition index and survival of oysters transplanted to Bay Center (A and B, respectively) and condition index and survival for those transplanted to Nahcotta (C and D, respectively)

Shown are oysters planted in bags deployed just above the sediment (Bottom), about 25 cm above the sediment (Top), and those deployed at a nearby pier (Subtidal). Red line at condition index = 10 added for visual reference.

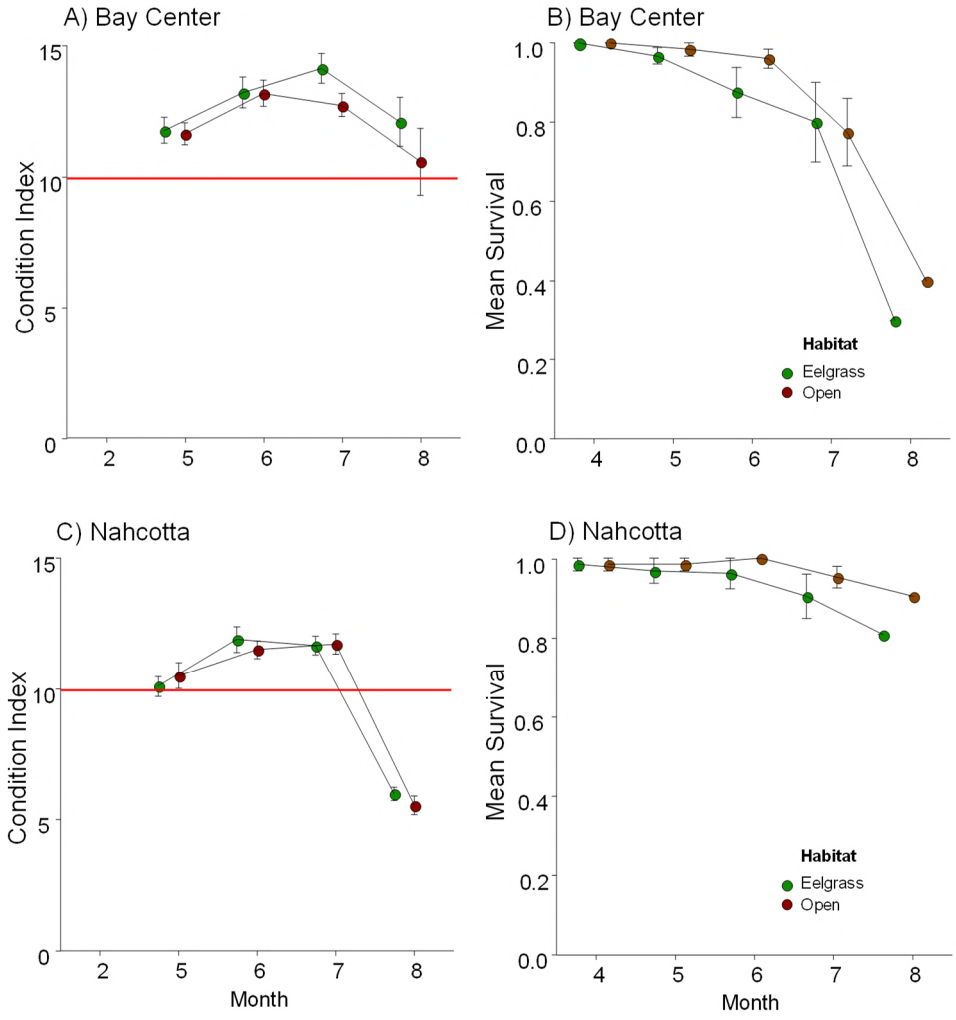

Fig.4 Results of transplant experiment conducted in Willapa Bay in 2019

Condition index and survival of oysters transplanted to Bay Center (A and B, respectively) and condition index and survival for those transplanted to Nahcotta (C and D, respectively). Shown are oysters planted in bags located within eelgrass and those planted in open habitat. Line at a condition index of 10 is added for visual reference.

We evaluated temperature and environmental records collected alongside the oysters in Willapa Bay during the 2019 experiment to compare with long-term records, but this year has since been shown to represent a significant warming event in the Northeast Pacific now recognized as marine heatwaves. These heatwaves are anomalously warm water events that extend over thousands of square kilometers in ocean basins and can be modulated by other climatic patterns like the PDO and El Niño-Southern Oscillation (ENSO) (Hobday *et al*. 2018; Ren *et al*. 2023). Though the scale of warming, distribution relative to the coast, and seasonal progression differed from a very large previous 2014-2016 event described as Blob 1.0, the 2019 heatwave became known as Blob 2.0 (Amaya *et al*. 2020; Chen *et al*. 2021). The effect of these events on nearshore coastal ocean conditions includes warmer seawater temperatures, potentially concentrated nearshore phytoplankton production and altered phytoplankton species composition that must also influence conditions for oysters in coastal estuaries (Stone *et al*. 2018, 2020).

Seasonal patterns of wind stress affect the transport of water and phytoplankton into coastal estuaries (Banas *et al*. 2007; Brasseale and Maccready 2025; Roegner *et al*. 2002). Phytoplankton distribution is subsequently influenced by water residence time both at the estuary scale, e.g. higher flushing and shorter residence = more frequent replenishing near the estuary mouth, but also across broad tide flats like those in Willapa Bay (Wheat *et al*. 2019). This estuary scale pattern is reflected in generally higher condition at the

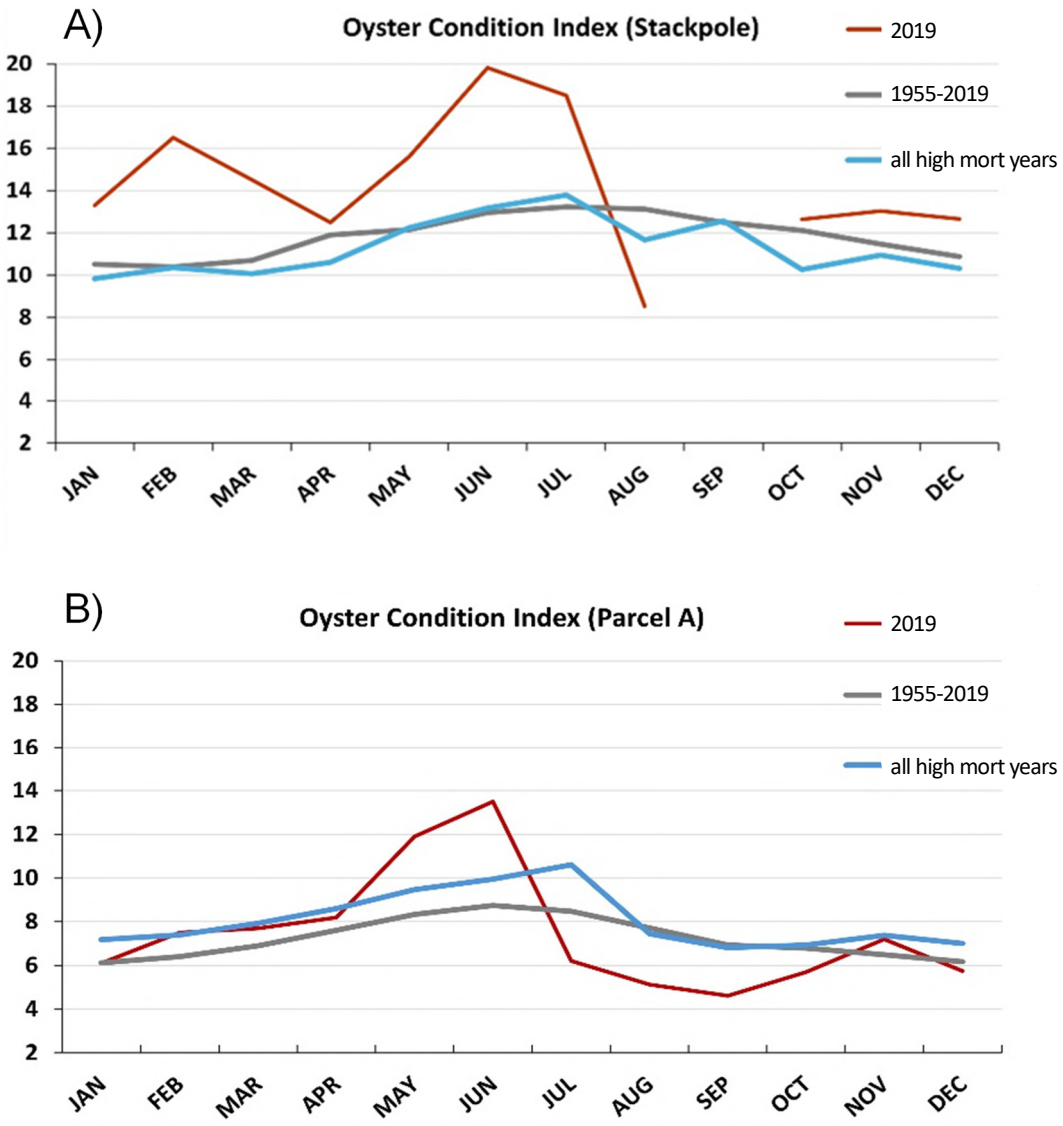

Fig.5 Seasonal trend of oyster condition index at A) Stackpole (STK) long term monitoring location and B) Nahcotta (PA) monitoring location in Willapa Bay

Shown are lines for 2019, the average condition index for 1955-2019, and a line representing the average condition index for years when shellfish growers reported significant mortality events in Willapa Bay.

Stackpole versus Nahcotta long-term monitoring sites across all years (Dumbauld *et al*. 2023) and at the Bay Center versus Nahcotta location in our 2019 experimental deployments. Shellfish growers have long taken advantage of this by planting and/or harvesting naturally caught seed near the south end of the estuary where larvae are retained and transplanting these oysters to fattening beds closer to the estuary mouth for harvest (fattening/recruitment line, Fig.1). Generally higher condition for oysters raised off the bottom observed at both locations in 2019 has also been previously documented, but most studies have been conducted on young oysters which devote less energy to reproduction. Results are site dependent and influenced by water flow, sediment conditions, distance to channel and even density of nearby oysters as competitors even when tidal elevation is held constant (Ruesink *et al*. 2003; Wheat and Ruesink 2013; Wheat *et al*. 2019). Nonetheless a recent study at numerous sites in Washington State, USA including several sites in Willapa Bay was consistent with our 2019 results with the condition of oysters positively influenced by raising them above the sediment surface but not influenced by the presence of eelgrass (Ruesink *et al*. 2023). Oyster survival was site dependent but also higher for oysters reared

off-bottom and only marginally negatively influenced by eelgrass.

Multiple investigations into factors leading to summer mortality events for Pacific oysters observed in the field have been conducted including initial work by researchers in Japan (Imai *et al*. 1968) and the US (Beattie *et al*. 1980; Cheney *et al*. 2000; Perdue *et al*. 1981), leading to perhaps the most substantial investigation by researchers in France (The MOREST project, Samain 2011). This and subsequent research suggest that when opportunistic pathogens (like the ostreid herpes virus OsHV-1 and several species of *Vibrio*) were present, they were important, but multiple environmental stressors otherwise contribute by influencing oyster metabolism, growth, and gametogenesis and thus ultimately resistance/ tolerance and gene response (de Lorgeril *et al*. 2020; Fleury *et al*. 2020; Pernet *et al*. 2019). Thus breeding programs have been developed and are advancing new genomic selection tools to identify traits and create disease resistant oyster broodstock that enable the shellfish aquaculture industry to avoid catastrophic losses due to OsHV-1 (Degremont *et al*. 2015; Delomas *et al*. 2023; Divilov *et al*. 2023; Gutierrez *et al*. 2020; Thompson *et al*. 2024), but pathogens are not the single cause of all mortality events. Recent evidence from laboratory stressor experiments suggests that food availability is an underlying and most significant factor affecting physiological response of juvenile seed oysters to disease, ocean warming, and acidity such that the lack of sufficient food limited growth, reproduction and energy reserves and thereby increased oxygen consumption and disease susceptibility (Caillon *et al*. 2023). Mortality of older oysters in the second summer has been previously linked to the gametogenic cycle and correlated with environmental triggers like temperature, salinity, and food which influence oyster metabolism and growth (Cheney *et al*. 2000; Garcia *et al*. 2011; King *et al*. 2021; Perdue *et al*. 1981; Solomieu *et al*. 2015). Long-term monitoring efforts of oyster condition and mortality in France point to the importance of feeding conditions during the winter/spring conditioning period where enhanced food and trophic conditions combined with warm temperatures lead to rapid gametogenesis and higher mortality risk in the presence of other metabolic stressors like low dissolved oxygen (Ernande *et al*. 2004; Gourault *et al*. 2019; Thomas *et al*. 2018).

Oysters were also investigated at Hatsukaichi in Hiroshima Bay in 2019 (Hasegawa *et al*. 2021), a part of the Seto Inland Sea in Japan, where oyster production has recently fluctuated markedly and mortality rates have increased (Hirata and Akashige 2004). Initial results indicated that the seasonal cycle of gametogenesis differed between the US and Japan. While the peak in sexual maturation occurred one month earlier in Japan (June versus July), oysters reached spawning stage over a similar period (600 cumulative temperature ^{o}C days) at locations in both countries. Spawning resulted in low condition and gonadosomatic indices and was more prevalent at Hatsukaichi when oysters were measured in June than in Willapa Bay in July, but mortality also increased in Willapa Bay in August. Relationships between environmental factors and growth in the Seto Inland Sea have also been recently investigated by Pang *et al*. (2024) who documented higher mortality in 2015-2021 than was observed in 1990. They found little difference in the temperature regime and attributed this to lower salinity and higher rainfall which has been previously shown to trigger mass spawning events in Matsushima Bay, Japan (Yokouchi *et al*. 2022). The Seto Inland Sea is much larger and less influenced by the nearshore coastal ocean than Willapa Bay with higher precipitation and lower salinities occurring during the summer months. Nonetheless, marine heatwaves of different origin are increasing in frequency (Sato *et al*. 2024), and what were once eutrophic conditions due to nutrient additions are now becoming more oligotrophic conditions with corresponding changes in the phytoplankton composition and abundance (Nishikawa *et al*. 2010). Despite these differences oysters displayed similar patterns with respect to deployment and habitat to those in Willapa Bay in 2019 with those grown off bottom having higher condition than those grown on bottom with a less obvious effect of seagrass presence.

Given significant increases in summer mortality events in both the US and Japan and recent advances in technology that might help resolve the mechanisms responsible and potentially allow shellfish industry participants to at least mitigate for losses, it seemed useful to briefly outline a new collaborative approach that we have proposed on the U.S. west coast. This proposed work involves addressing three research priorities outlined in a recent workshop on summer mortality (Virginia Institute of Marine Science 2024): 1) Develop a mechanistic understanding of mortality events, including energy budgets, pathobiology, etc., 2) Prepare oysters to withstand stress in all phases, for example through hardening or priming against future challenges, and 3) Breeding for increased, general resilience. The currently proposed work addresses priorities 1 and 3, but indirectly provides a foundation for addressing priority 2. The primary objective is to develop a generalized but quantitative resilience trait index that can be used by breeding programs to increase field survival and at the same

time enable participating farmers to assess the level of stress experienced by their oysters. The first step involves employing a suite of cost effective assays based on physiological and metabolic stress that can be determined in field reared oysters. An example is measuring NA^+/K^+ ATPase activity which evaluates cellular stress and has been shown to decrease in oysters that are susceptible to mortality (George *et al*. 2023), but separate evaluations will also be made for organismal metabolic rate, energy storage, and capacities for aerobic and anaerobic metabolism. Since it is difficult to attribute mortality to a single stressor measured in the field, the strength of this approach is then to also apply these tests to bi-parental families from an oyster breeding program as part of the Pacific Oyster Genomic Selection (POGS) project which is developing oysters that are resistant/tolerant to OsHV-1 and deploying these at multiple farmed sites in collaboration with growers.

Acknowledgements

The authors first wish to thank our colleagues from Japan with whom we are collaborating to complete and report the 2019 joint UJNR research project including N. Hasegawa, M. Hori, and S. Watanabe with support from the Japan Fisheries Research and Education Agency. The long-term study of oyster condition in Willapa Bay continues to be supported by the Washington Department of Fish and Wildlife with multiple contributors who collected data, analyzed, and discussed results over the years. The list of individuals is too long to cite here, but includes shellfish industry members and scientists at the University of Washington. Funding for some of the work was contributed by USDA-ARS (CRIS project # 2072-63000-005-00D) and WDFW. The authors also thank B. McIntyre, A. Bolm, A. J. Trimble, I. Durland, and N. Scott for assistance in the field and laboratory during the 2019 study.

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Annotated Bibliography of Key Works

(1) Cheney D, MacDonald BF, Elston RA (2000) Summer mortality of Pacific oysters, *Crassostrea gigas* (Thunberg): Initial findings on multiple environmental stressors in Puget Sound, Washington, 1998. *J. Shellfish Res.*, **18**, 456-473.

This manuscript presents the results of a study conducted to investigate Pacific oyster summer mortality events in Puget Sound, Washington, USA where no specific disease factors appeared responsible but instead mortality events linked to multiple environmental stressors including temperature extremes, low oxygen conditions and phytoplanton as food. An initial comparison of mortality amongst diploid and triploid oysters was also made.

(2) Dumbauld BR, Du XN, Hunsicker M, Forster Z (2023) Multi-decade changes in the condition index of adult Pacific oysters (*Crassostrea gigas*) in response to climate in a US west coast estuary. *J. Sea Res.*, **193**, 102383.

The authors present an analysis of almost seven decades of oyster condition data collected in Willapa Bay, Washington, USA. They identified two important and coherent shifts in oyster condition that can be associated with changes in ocean climate at basin wide and more local scales. They also charactrized patterns in oyster condition within Willapa Bay that had long been recognized by industry participants and associated with oyster gametogenesis and spawning.

(3) George MN, Cattau O, Middleton MA, Lawson D, Vadopalas B, Gavery M, Roberts SB (2023) Triploid Pacific oysters exhibit stress response dysregulation and elevated mortality following heatwaves. *Glob. Change Biol.*, **29**, 6969-6987.

These authors examined the physiological response of Pacific oysters to environmental conditions that are potentially responsible for summer mortality. Physiological assays included metabolic depression due to a reduction in sodium pump activity and dysregulated expression of genes associated with glucose metabolism and mitiochondrial function.

(4) Hasegawa N, Dumbauld B, Hori M, Watanabe S, Rust M, Forster Z (2021) Comparative study of the impact of environmental change on oyster culture between USA and Japan, as collaborative research under UJNR. *Bull. Jap. Fish. Res. Edu. Agen.*, **50**, 115-121.

This is an introduction to the collaborative study initiated between the USA and Japan. Data was collected, but some analyses partially reported on at this time were delayed primarily due to the global pandemic.

(5) Samain JF (2011) Review and perspectives of physiological mechanisms underlying genetically-based resistance of the Pacific oyster to summer mortality. *Aquat. Living Resour.*, **24**, 227-236.

This review of multiple years of data collected for the “MOREST” project in France remains one of the important references that suggests a link between summer mortality events, physiological mechanisms like reproductive effort and stressors like temperature. The authors also suggest avenues for investigating genetically-based resistance to stress.

水研機構研報, 第 53 号, 153 – 156, 令和 8 年
Bull. Jap. Fish. Edu. Agen. No. 53, 153 - 156, 2026

Image analysis for estimating soft body mass from shell morphology in *Crassostrea gigas*

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Abstract: Oyster farming is one of the oldest forms of aquaculture and one of the largest markets in the world. Oysters are sessile organisms whose shell morphologies are highly variable, and strongly adhere on host substrates. The relationship between shell morphology and soft body mass in oysters is poorly understood because of the difficulty in the morphological analysis of complex-shaped shells. The "ideal" shell shape for aquaculture production has not been determined for more than 2,000 years.

In this study, we tried to develop a new analytical approach to estimate the soft body mass from the shell morphology in Pacific oyster, *Crassostrea gigas*. We assumed that the whole shell morphology should be translated into numerical values that characterize the soft body mass, rather than traditional indices comprised of a limited number of linear distances such as shell length and width. We first collected the overhead photographic data from the shells. These data were transformed into numerical values that represent the morphological components using elliptic Fourier analysis. Then, we screened the morphological components that could affect the relative soft-body mass using a generalized linear model. Using these analytical results, we developed a numerical model suitable for evaluation of the oyster meat production by the shell morphology. Our results will help establish a new criterion for assessing the quality of oysters as seafood and promote oyster farming.

Key words: allometry, aquaculture, elliptic Fourier analysis, oyster farming

Introduction

Oyster farming is one of the oldest forms of aquaculture and believed that it had begun in ancient Rome (Laing and Bopp 2018). Global aquaculture production in oyster farming has continuously been increasing over the past 70 years, and the total production had increased 1.5-fold from 4 million tons in 2009 to 6 million tons in 2019 (FAO 2024). The major species used in oyster farming is Pacific oyster, *Crassostrea gigas*. With the increase in the market value of farmed oysters, the production value had doubled from 3 billion USD to 6 billion USD during those 10 years. In addition to the east Asian countries, culture of eating oysters, as represented by oyster bars in USA and Europe, has become popular. Oyster farming is now one of the central parts of the world's fisheries industry and has already been took root in our food culture.

Oysters are sessile organisms whose shell morphologies are highly variable, and strongly adhere on host substrates. These variations affect the size of the edible part of oysters; thus, shell morphology is an important factor in evaluating the oyster

Accepted on Feb 26, 2026
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quality (Brake *et al*. 2003; Mizuta and Wikfors 2019). Notably, the ratio of shell height/shell length (D/L) or shell width/shell length (W/L) could be useful indices for determining the oyster quality (Mizuta and Wikfors 2019). For example, desirable shell traits have been suggested to have D/L of 0.33 or D/L > 0.25 and W/L of 0.67 or W/L > 0.63 (Brake *et al*. 2003; Mizuta and Wikfors 2019). However, practical use of these proposed ratios for quality classification seems to be limited because the validity of them is still under discussion.

Based on the current situation, we expected that the difficulty in morphological analysis for estimating the oyster quality could be caused by the complex-shaped shell, which disturbs universal measurement of linear distances. We further considered that whole shell morphology should be translated into numerical values that characterize the soft body mass, rather than traditional simple linear indices. In this study, we developed a novel analytical approach to estimate the soft body mass from shell morphology in Pacific oyster.

Materials and Methods

Data collection

The experimental animals were collected from a local oyster farm in Uramura (34°26'N, 136°53'E; Toba, Mie, Japan). After measuring the total and soft body weights, the upper surface of the shell was photographed. To collect stable photographic data, the target oyster shell was placed in a transparent acrylic box (20 × 20 × 20 cm), and photographs were taken by fixing a digital camera above the upper side. Subsequently, the contour of the shell was manually traced using a tracing paper, and the scanned data were used for elliptic Fourier analysis. The contour at the upper side of the target was converted into a Fourier series using SHAPE ver. 1.3 (Iwata and Ukai 2002). The Fourier series was used for principal component analysis to calculate the variables that indicated independent morphological changes. The effective number of principal components (PCs) was selected in accordance with the Kaiser criterion (Jackson 1993).

Statistical analysis

The correlation between the soft body mass ratio (wet soft body weight/total body weight) and morphological indices was analyzed using a generalized linear model (GLM) with log-transformation and Gaussian distribution. Total body weight, PCs, and distance from the means of the PCs were used as explanatory variables (Table 1). To evaluate the validity of the conventional method and the method used in this study, D/L and W/L were also included as explanatory variables. The best model was selected using stepwise model selection in accordance with Akaike's information criterion (AIC). The significance of each parameter was tested using a likelihood ratio test. All statistical analyses were conducted using R software version 4.0.2 (R Core Team 2023).

Table 1 Variables used for the GLM analysis targeting the ratio of the soft body mass in the total body weight

Classification	Variable
Body size	Total body weight
Conventional indices for shell morphology	D/L (shell height / shell length)
	W/L (shell width / shell length)
Principal component scores by elliptic Fourier analysis targeting the top view of the shell	PC1
	PC2
	PC3
	PC4
	PC5
	PC6
	PC7
	PC8
	PC9
Distance from the means of the principal component scores above	dPC1
	dPC2
	dPC3
	dPC4
	dPC5
	dPC6
	dPC7
	dPC8
	dPC9
Cross terms of the body size and the indices characterizing the shell morphology	Total body weight × D/L
	Total body weight × W/L
	Total body weight × PC1
	Total body weight × PC2
	Total body weight × PC3
	Total body weight × PC4
	Total body weight × PC5
	Total body weight × PC6
	Total body weight × PC7
	Total body weight × PC8
	Total body weight × PC9
	Total body weight × dPC1
	Total body weight × dPC2
	Total body weight × dPC3
	Total body weight × dPC4
	Total body weight × dPC5
	Total body weight × dPC6
	Total body weight × dPC7
	Total body weight × dPC8
	Total body weight × dPC9

Results and Discussion

Elliptic Fourier analysis was used to convert the shell morphology data obtained from the overhead view into nine PCs (Fig.1). PC1, which represented the highest proportion, indicated the morphological changes represented by W/L. PC2 was considered to indicate the direction of asymmetry in shell morphology. The morphological changes that could not be quantified by the conventional distance measurements were detected, as in the other PCs. The correlation of the scores in these PCs and the relative soft body mass was examined by GLM analysis with stepwise model selection to determine which morphological variation changes the soft body size. The actual relative soft-body mass and that estimated by the best model obtained in that analysis showed a high correlation (Fig. 2; $R^2 = 0.4859$), indicating that the model obtained in this study could be useful for estimating the soft-body size in the target oyster population.

Simple indices, such as D/L or W/L, have often been used for oyster quality classification (Mizuta and Wikfors 2019), but the result of our GLM analysis implies that these conventional indices might not be useful for estimating the oyster quality. The three models used in our analysis did not include any conventional morphological indices. Instead, PC2 and PC9 were included in the model (Table 2). Only the coefficients in PC9 were significant in all the models (n = 33; likelihood ratio test; $P < 0.05$). This indicates the importance of PC9 in determining the soft-body mass estimated from the shell morphology, although PC9 represented the lowest proportion in explaining the variations in the shell morphology. Since the PCs that represented the major morphological variations were not significant or eliminated from the models, the use of major morphological variations could mislead us in estimating the oyster quality. The effect of PC9 on the soft-body size changes was classified into two categories. The relative soft-body size decreased as the distance between the PC9 score and the mean increased. This suggests that the mean shape is the best one representing the highest relative soft body mass. Secondly, the effect of PC9 described above decreased as the body size increased. In other words, body size is considered a negative factor for the effect of PC9.

Thus, this case study indicated the unreliability of conventional simple indices of shell morphology in determining the oyster quality and proposed a novel morphological factor to estimate the quality. The biological function that relates to the morphological changes determined by PC9 is not clear in this study; therefore, we are currently working on this issue. In the present study, it was suggested that the PCs that determine the relative soft body size seem to change depending on the environmental factors. Therefore, PC9 cannot always be used as a reliable morphological index to estimate the oyster quality. Reliable morphological indices that could practically be used for evaluation of the oyster quality have not been found for decades, since the morphology of oyster shells is highly variable. Further studies will help to establish a more efficient marketing strategy for oyster farming.

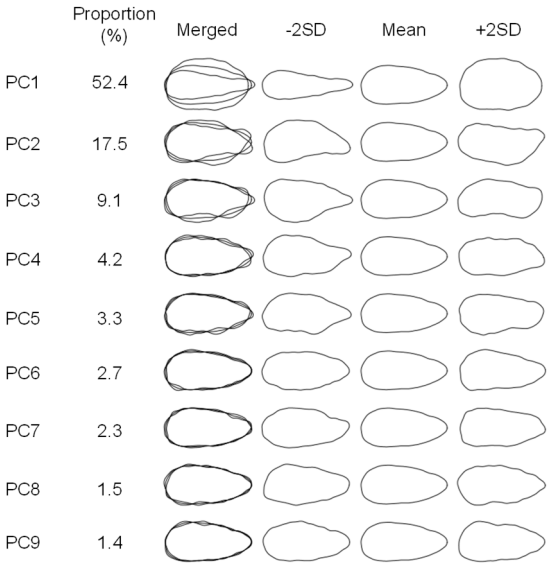

Fig.1 The results of the elliptic Fourier analysis and subsequent PC analysis

The respective figures at mean, −2SD and +2SD indicate the shape changes when each PC score took those mean, −2SD and +2SD values.

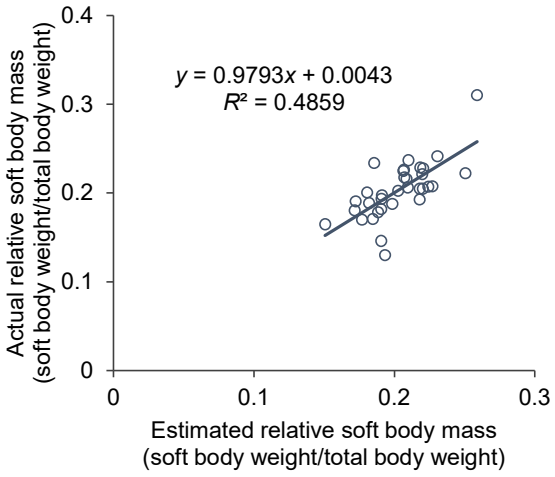

Fig.2 The correlation between the actual relative soft body mass and that estimated by the model obtained by the GLM analysis

Table 2 The results of the GLM analysis targeting the ratio of the soft body mass in the total body weight

		Model			
		1 (best)	2	3	Null
AIC		-142.22	-142.05	-142.04	-130.28
Estimated coefficient (SE)	Intercept	-1.067 (0.122)	-0.9737 (0.1395)	-1.074 (0.123)	-1.593 (0.027)
	Total body weight	-0.007567 (0.002022)**	-0.008506 (0.002082)**	-0.007145 (0.002020)**	
	PC2	-0.6472 (0.4406)	-0.8544 (0.4610)	-0.6165 (0.4505)	
	dPC5		-1.914 (1.569)		
	dPC9	-35.07 (11.23)***	-40.52 (11.86)***	-0.3078 (10.87)***	
	dPC7	2.561 (1.841)	2.810 (1.861)		
	Total body weight × dPC9	0.454 (0.1972)*	0.5307 (0.2034)*	0.4105 (0.1964)*	

The three models that represented lower AIC values and the null model were shown.
*, $P < 0.05$; **, $P < 0.01$; ***; $P < 0.001$ (n = 33; likelihood-ratio test)

Acknowledgments

We thank Mr. Y. Senda, Mr. D. Asao, Mr. I. Kimura, and Mr. T. Nakamura for their assistance during this study.

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Annotated Bibliography of Key Works

(1) Iwata H, Ukai Y (2002) SHAPE: A computer program package for quantitative evaluation of biological shapes based on elliptic Fourier descriptors. *J. Hered.*, **93**, 384-385.

In this study, the authors developed a software that transforms the morphologies of target objects from photographic data using elliptic Fourier analysis. This software enables an easy analysis using elliptical Fourier descriptors.

水産研究・教育機構研究報告

Bulletin of Japan Fisheries Research and Education Agency

令和 8 年 3 月 25 日　発行

発　行　所　国立研究開発法人　水産研究・教育機構
〒221-8529　神奈川県横浜市神奈川区新浦島町 1-1-25　GRC 横浜ベイリサーチパーク　6 階
National Research and Development Agency, Japan Fisheries Research and Education Agency
GRC Yokohama Bay Research Park 6F, 1-1-25 Shinurashima-cho, Kanagawa-ku, Yokohama-shi
Kanagawa 221-8529, Japan
http://www.fra.go.jp

発　行　者　中山一郎

ISSN 2758-3953

BULLETIN OF
JAPAN FISHERIES RESEARCH AND EDUCATION AGENCY

No. 53
March 2026

Bull.Jap.Fish.Res.Edu.Agen.

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