

The Opportunistic Pathogen *Chryseobacterium balustinum* WLT: Pathogenicity and Antibiotic Resistance

Won Joon Jung ^{1,†}, Sang Guen Kim ^{1,†}, Sib Sankar Giri ¹ , Sang Wha Kim ¹, Jeong Woo Kang ¹, Jun Kwon ¹, Woo Taek Oh ¹, Sung Bin Lee ¹, Young Min Lee ¹, Su Jin Jo ¹, Cheng Chi ², Jin Woo Jun ^{3,*} and Se Chang Park ^{1,*} 

- ¹ Laboratory of Aquatic Biomedicine, Research Institute for Veterinary Science, College of Veterinary Medicine, Seoul National University, Seoul 08826, Korea; cwj0125@snu.ac.kr (W.J.J.); imagine0518@snu.ac.kr (S.G.K.); ssgiri@snu.ac.kr (S.S.G.); blackcat9201@snu.ac.kr (S.W.K.); kck90@snu.ac.kr (J.W.K.); kjun1002@snu.ac.kr (J.K.); mike0202@snu.ac.kr (W.T.O.); lsbin1129@snu.ac.kr (S.B.L.); mushhama@snu.ac.kr (Y.M.L.); ssjjone@snu.ac.kr (S.J.J.)
- ² Laboratory of Aquatic Nutrition and Ecology, College of Animal Science and Technology, Nanjing Agricultural University, Nanjing 210095, China; chicheng0421@snu.ac.kr
- ³ Department of Aquaculture, Korea National College of Agriculture and Fisheries, Jeonju 54874, Korea
- * Correspondence: advancewoo@snu.ac.kr (J.W.J.); parksec@snu.ac.kr (S.C.P.)
- † These authors contributed equally to this work.

Abstract: This study aimed to re-evaluate the pathogenic characteristics of *Chryseobacterium balustinum*, generally known as a food spoilage bacterium. We observed mass mortality in a rainbow trout farm in Jeonbuk province, Republic of Korea. Fewer studies have reported on rainbow trout mortality or infections in Korea than in other countries; among these, few studies have reported cases of *Chryseobacterium* infections. Sequencing analysis revealed that this bacterium is closely related (99.24%) to *Chryseobacterium balustinum*. Bacterial identification assays, including the API test, sequencing, and phylogenetic analysis, found the 24 isolates to be *C. balustinum*. The strain showed multiple resistance to 18 of 25 antibiotics tested. Primary clinical symptoms of its infection are damage to the fins, necrosis, and cytoplasmic vacuolation in hepatocytes. The pathogenicity of the strain was determined following Koch's postulates and the challenge test. The present results suggest that *C. balustinum* WLT can be considered a multidrug-resistant zoonotic pathogen responsible for mortality and economic losses.

Keywords: *Chryseobacterium balustinum*; drug-resistant; rainbow trout; Korea; pathogenicity



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1. Introduction

Genus *Chryseobacterium* was first proposed by Vandamme et al. [1]. The genus was moved from the family *Flavobacteriaceae* to the family *Weeksellaceae* in 2019 [2]. *Chryseobacterium* spp. are generally present in the environment and are found in soil, water, and lake sediments, similar to their close relatives in *Flavobacteriaceae* [3,4]. *Chryseobacterium balustinum* was first isolated from halibut that was unfit for sale because of discoloration and the softening of skin and muscle tissue [5]. Until recently, the bacterium was considered as a dairy and seafood food spoilage organism [5,6]. Similar to *Flavobacterium* spp., some strains of *Chryseobacterium* spp. are pathogenic to plants and fungi, and they are present in food products such as cow milk and raw meat [7–9].

Recently, there have been emerging reports regarding clinical cases caused by *Chryseobacterium* spp. in different farmed animals, such as pond loach, yellow perch, and salmonid fish, and pets such as bullfrogs and ball pythons. *Chryseobacterium* spp. has not been considered as a common zoonotic pathogen; only a few studies have reported clinical infections in humans [10,11]. Despite the general belief that *Chryseobacterium* is an opportunistic pathogen that only causes secondary infection in immunocompromised patients, it has been reported to cause severe sepsis in an immunocompetent traveler [12].

Thus, the zoonotic possibilities of the *Chryseobacterium* should not be neglected as we may be in close contact with it in daily life.

Due to their relatively low pathogenicity, *Chryseobacterium* species have been kept out of the spotlight. However, the high antibiotic resistance profiles of *Chryseobacterium* have been sufficient to collect general interest in perspectives of clinical relevance. They have shown resistance to antibiotics approved for industrial purposes and last-resort antibiotics, and there have been increasing reports of infections in animals in South Korea. There are not many antibiotics to use among permitted drugs. Thus, it is of utmost importance to report every *Chryseobacterium* outbreak to trace the dissemination in aquaculture. The antimicrobial resistance and pathogenicity of the species must be studied to select suitable antimicrobial agents as a treatment. Many studies highlight the antimicrobial resistance of the *Chryseobacterium* species and warn of its zoonotic possibilities [13,14].

In this study, we investigated cases of infection with *Chryseobacterium* in rainbow trout (*Oncorhynchus mykiss*) from a farm in Jeonbuk province, Republic of Korea. Identification of the bacterial isolate was confirmed through sequencing and phylogenetic analysis of the 16S rRNA gene. The antibiotic susceptibility of the bacterial isolate and the pathogenicity were assessed. Moreover, histopathological changes in the liver of diseased fish were evaluated.

2. Materials and Methods

2.1. Fish Sampling

In March 2019, a rainbow trout farm in Jeonbuk province, Republic of Korea, requested a pathological diagnosis of trout based on common clinical symptoms displayed, which included anorexia and damaged dorsal and caudal fins (Figure 1). The farm has a closed circulating water system from groundwater and the water temperature was 15–16 °C. The farm reared 30,000 fish, distributed in six water tanks which contained 5000 L of water each. The estimated mortality rate was approximately 10%; however, continuous and visible clinical symptoms gradually intensified. The percentage of affected fish was >80% and the size of the fish was approximately 1 kg ± 100 g. Infected fish experienced common symptoms including loss of appetite and hemorrhage in the caudal fin. Ten fish with severe clinical symptoms were selected for the study. They were caught and transported to the Laboratory of Aquatic Biomedicine at Seoul National University, Seoul. These fish were inspected for any external lesions visually and microscopically for bacterial, viral, and parasitic diseases. The gills and fins of the fish were swabbed and smeared on glass slide for observation under a light microscope. Viral infection, infectious hematopoietic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV), and viral hemorrhagic septicemia virus (VHSV) were diagnosed using PCR and specific primers. Thereafter, the kidney, liver, and spleen were dissected for bacterial isolation.

2.2. Bacterial Isolation

The recovery of bacteria was performed from the liver, kidney, and spleen of rainbow trout. Each tissue sample was streaked directly on tryptic soy agar (BD Difco, Detroit, MI, USA) and cytophaga agar. Streaked plates were incubated at 20 °C and 25 °C for 72 h and bacterial growth was monitored. Colonies present in the largest proportion were sub-cultured. Recovered bacteria were stored at −80 °C in tryptic soy broth (BD Difco, Detroit, MI, USA) supplemented with 25% glycerol for further analysis.

2.3. Biochemical Characterization

The Gram reaction of bacterial isolates was determined using a Gram staining kit (BioMerieux, Seoul, Korea) following the manufacturer's protocols. The oxidase reaction and catalase activity were assayed using 1% tetramethyl-*p*-phenylenediamine (Merck, Madison, NJ, USA) and 3% (volume by volume) aqueous hydrogen peroxide solution, respectively. For bacterial identification, API 20NE and API ZYM kits (BioMerieux, Seoul, Korea) were used following the manufacturer's protocols.



Figure 1. Clinical symptoms of *Chryseobacterium balustinum* infection indicate severe damage on the dorsal and caudal fin.

2.4. Bacterial Identification

Sub-cultured bacteria were grown on TSA at 25 °C for 24 h and the bacterial DNA was extracted using a Qiagen DNeasy Tissue Kit (Qiagen Sciences, Valencia, CA, USA) for 16S rRNA gene analysis using PCR. For PCR amplification, universal primers (27F, 1492R) targeting 16S rRNA gene were used, as previously reported [15]. PCR products were sent to the genomics division of Macrogen (Seoul, Korea) for gene sequence analysis, where nucleotide sequencing was performed using ABI PRISM 3730XL Analyzer with BigDye[®] Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, Waltham, MA, USA).

2.5. Phylogenetic Analysis

As all the 16S rRNA genes of the isolates were 100% identical, the sequence of strain WLT was included in the phylogenetic analysis as a representative. The 16S rRNA gene sequence was used to assess the similarity with other *Chryseobacterium* spp., using BLASTn (NCBI, Bethesda, MD, USA) and EzBioCloud server (<https://www.ezbiocloud.net/>) to identify similar bacterial strains. Twenty closely related bacteria of the genus *Chryseobacterium* were selected for phylogenetic analysis and sequences were aligned using MEGA X software [16]. The 16S rRNA sequence of *Janthinobacterium tractae* SNU WT3 was included as an out-group [17]. A phylogenetic tree was constructed using the MEGA X software using the neighbor-joining method, and evolutionary distances were calculated using Kimura's 2-parameter model [18,19]. Tree topology was assessed using 1000 bootstrap replicates [20]. The 16S rRNA gene sequence of the *Chryseobacterium* WLT was deposited within GenBank (Accession number: MN317337).

2.6. Histopathological Analysis

Fish displaying severe clinical signs, including damaged fins and lethargic behavior, were selected for histopathological examination. The spleen, kidney, and liver were dissected and preserved in 10% neutral-buffered formalin to determine the pathological and degenerative changes resulting from the infection. Fixed tissues were trimmed, dehydrated using ethanol, embedded in paraffin blocks, and sectioned and stained with hematoxylin

and eosin. The specimens were examined using a light microscope and digitally scanned by Xenos Inc. (Seoul, Korea).

2.7. Antibiotic Susceptibility Test

The bacterial strain isolated from diseased rainbow trout was subjected to antibiotic susceptibility tests against 24 antibiotics using the disk diffusion method on Mueller–Hinton agar (BD Difco, Detroit, MI, USA). *Chryseobacterium* spp. is inherently resistant to various antibiotics including tetracycline, erythromycin, linezolid, aminoglycosides, chloramphenicol, and *beta*-lactams. The following antibiotics were assayed: amikacin, ampicillin, piperacillin, cefazolin, cefepime, cefotaxime, ceftazidime, ceftizoxime, aztreonam, imipenem, meropenem, gentamicin, kanamycin, streptomycin, tetracycline, doxycycline, ciprofloxacin, nalidixic acid, norfloxacin, ofloxacin, trimethoprim-sulfamethoxazole, chloramphenicol, and erythromycin. The results were determined as resistant, intermediate, and susceptible, and were assessed based on the diameter of the zone of inhibition (mm) [21].

2.8. Pathogenicity Challenge Trials

To estimate the pathogenicity of bacterial isolates, rainbow trout weighing 20 g on average were purchased from another farm in Chungbuk province, Republic of Korea. Fish were transported and stabilized for two weeks before the trial under the same conditions as the challenge trial. Thirty fish were intraperitoneally injected with 3×10^7 , 10^6 , 10^5 , and 10^4 CFU of bacteria per fish in each group. The control groups were injected with the same volume of PBS. The experiments were performed with 30 fish per group, and the average mortality rates were determined as days post infection. Each group of artificially infected fish was kept in 120 L water tanks, and the water temperature was regulated at 16 °C. The trials were conducted for 15 d after all fish groups were stabilized.

3. Results

3.1. Clinical Symptoms and Bacterial Isolation

The clinical symptoms observed herein, such as severe damage to the caudal fin in diseased fish, were similar to that of cases of *Flavobacterium psychrophilum* infection [22]. However, PCR-based bacterial detection yielded negative results [23]. Furthermore, no virus or parasitic infections were observed upon microscopic and macroscopic examination. Virus detection using PCR with specific primers found all samples were negative for IHNV, IPNV, and VHSV [24,25]. Moreover, since *Pseudomonas fluorescens* and *Aeromonas salmonicida* are considered major pathogens in salmonids, their detection was carried out and the results were negative [23,26–28]. After excluding some major rainbow trout pathogens, bacterial isolation was performed to identify the disease etiology. After incubation at 25 °C for 24 h, circular, yellow colonies of 34 mm diameter appeared on both TSA and cytophaga. For the isolation of pure colonies, colonies were individually sub-cultured. Notable differences between these bacteria and other *Flavobacterium* spp. were that culture duration was relatively short and bacterial alternation was not observed during sub-culturing [29]. Finally, a total of 24 isolates were recovered.

3.2. Biochemical Characterization

The strain was Gram-negative and oxidase- and catalase-positive. It did not produce indole nor reduce nitrate. With the API ZYM strip, the strain was negative for C4 esterase and β -glucosidase, but positive for C8 esterase lipid, valine arylamidase, trypsin, α -glucosidase, and N-acetyl- β -glucosaminidase. On the API 20NE strip, the strain was negative for arabinose, mannitol, and citrate but positive for maltose assimilation.

3.3. Phylogenetic Analysis

16S rRNA gene sequencing analysis revealed 98–99% similarity to other *Chryseobacterium* related species: 99.24% (*C. balustinum* DSM 16775T), 98.96% (*C. piscium* LMG23089T), and 98.75% (*C. scophthalmum* DSM 16779T). The phylogenetic tree (Figure 2) was con-

structed with 20 *Chryseobacterium* spp., indicating that the *Chryseobacterium* spp. isolated from the rainbow trout part of this study are the causative agents of mortality and can be considered *C. balustinum* bacteria.

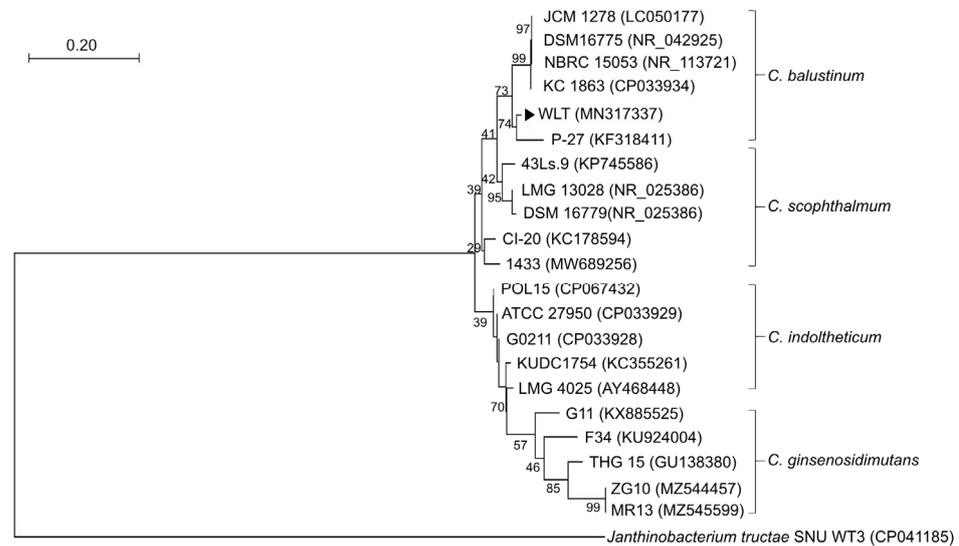


Figure 2. Phylogenetic tree constructed using 16S rRNA gene sequences of the 20 *Chryseobacterium* species and strain isolated in this study. The tree was constructed by MEGA X using the neighbor-joining method with a bootstrap analysis of 1000 replicates. (Genbank accession number: MN317337).

3.4. Pathogenicity Challenge Trials

The virulence of the strain WLT was determined by the challenge trial. The first mortalities occurred within 4 days of injection, and the 3×10^7 group showed 60% mortality (Figure 3). The pathogenicity trial revealed that the LD50 was estimated to be lower than that of other *Chryseobacterium* spp. One of the *Chryseobacterium* spp. isolated from multiple Great Lake salmonids (T28) had an LD50 of $>10^8$ CFU per fish; however, the estimated LD50 value of the present isolate was between 10^7 and 10^6 CFU per fish [30].

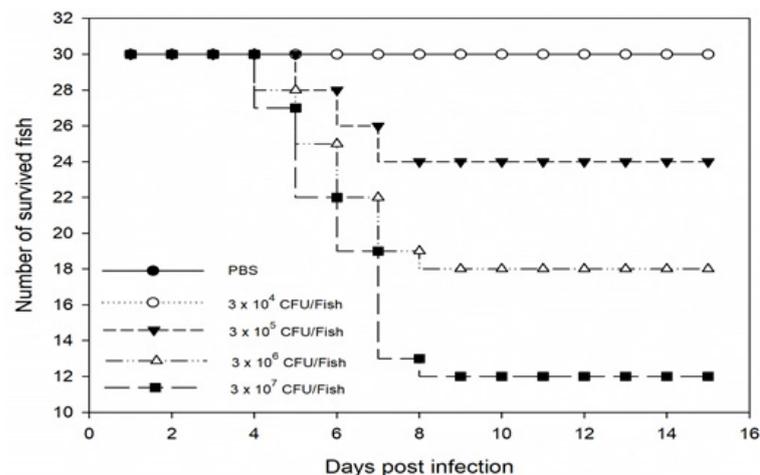


Figure 3. Challenge trial result showing the survival rate of the infected fish upon day post-infection.

3.5. Histopathological Analysis

Histopathological analysis revealed that the pathological lesions were more concentrated on the liver than on the kidney and spleen. Moderate hepatocyte degeneration and cytoplasmic vacuolation were observed throughout the liver (Figure 4). However, the degree of the damage was not proportional to the mortality rate of rainbow trout. In the present case, the kidney and the spleen were relatively normal.

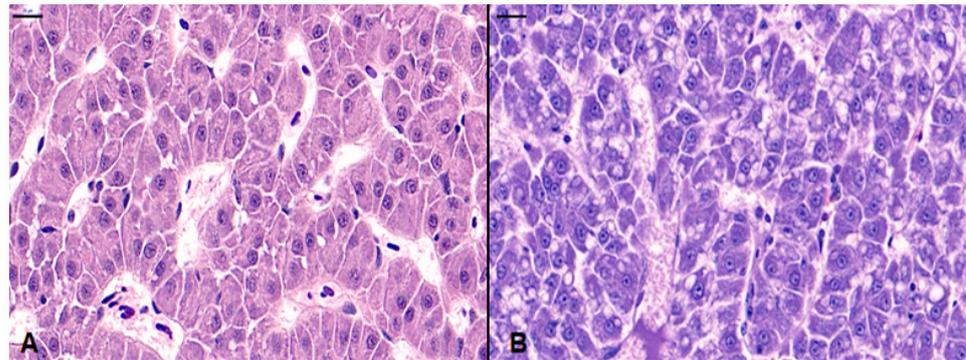


Figure 4. Histopathology of liver of healthy rainbow trout (A) and a moribund rainbow trout with *Chryseobacterium balustinum* WLT infection (B). Necrosis and cytoplasmic vacuolation can be observed in hepatic cells on liver of diseased fish. Slides were stained with hematoxylin and eosin. Scale bars = 20 μ m.

3.6. Antibiotic Susceptibility Test

The antibiotic susceptibility test indicated that, similar to other *Chryseobacterium* spp., the isolate WLT was resistant to different antibiotics (Table 1). *Chryseobacterium* sp. is inherently resistant to numerous antibiotics including tetracyclines, erythromycin, linezolid, polymyxins, and chloramphenicol [31]. The antibiotic susceptibility test revealed that the strain was resistant to ampicillin, piperacillin, cefazolin, cefepime, cefotaxime, ceftazidime, ceftizoxime, aztreonam, gentamicin, amikacin, kanamycin, streptomycin, tetracycline, doxycycline, nalidixic acid, trimethoprim-sulfamethoxazole, chloramphenicol, and erythromycin. However, it was susceptible to cefoxitin, imipenem, meropenem, ciprofloxacin, norfloxacin, and ofloxacin.

Table 1. Antibiotic susceptibility test performed on *C. balustinum* WLT by disk diffusion method (R: resistant, S: susceptible, I: intermediate).

Antibiotics	Susceptibility	Inhibition Zone Diameter (mm)	Antibiotics	Susceptibility	Inhibition Zone Diameter (mm)
Ampicillin	R	7	Piperacillin	R	9
Cefazolin	R	10	Cefepime	R	9
Cefotaxime	R	10	Cefoxitin	S	22
Ceftazidime	R	12	Ceftizoxime	R	3
Aztreonam	R	2	Imipenem	S	26
Meropenem	S	28	Gentamicin	R	11
Amikacin	R	4	Kanamycin	R	5
Streptomycin	R	2	Tetracycline	R	8
Doxycycline	R	11	Ciprofloxacin	S	24
Nalidixic acid	R	7	Norfloxacin	S	27
Trimethoprim-sulfamethoxazole	R	9	Ofloxacin	S	25
Chloramphenicol	R	13	Erythromycin	R	4

4. Discussion

There is a tendency that *Chryseobacterium* spp. is often neglected in clinical settings because of the low number of cases and decreased pathogenicity. However, severe antibiotic resistance makes them hard to control, especially in immunocompromised patients or stressed animals, and this has been the case for *C. balustinum*. The first isolation was reported in halibut and was recognized as a food spoilage bacterium rather than a pathogenic bacterium since it was isolated from the fin of the fish [5,6,30]. In this study, we observed the mortality of farmed rainbow trout and verified that it originated from the infection by *C. balustinum* designated as WLT.

As *Chryseobacterium* is prevalent in the natural environment, which includes river water and soil, there is a high possibility for individuals to be exposed to it. Indeed, a severe sepsis case was reported from those who had previous exposure to soil and water [12]. The causative agent was presumed to have infected the individuals through the skin. In the case of the present study, the pathogenesis of *Chryseobacterium* was presumed to proceed from a localized to a systemic infection. It was also observed in our previous report with *C. cucumeris* [32]. Furthermore, considering the production of proteolytic enzymes, including esterase, the zoonotic potential of *C. balustinum* through infection through the skin should not be excluded.

Generally, the pathogenicity of *Chryseobacterium* in farmed fish is comparably lower than the major bacterial pathogens (e.g., *Flavobacterium* and *Lactococcus*) and earlier studies reported 5 LogCFU/fish as the median lethal dose of *Chryseobacterium* [33,34]. The LD50 of *C. cucumeris* in pond loach was 7.8 LogCFU/fish [32], whereas the LD50 of *C. indologenes* in yellow perch was 8.1 LogCFU/fish [35]. The *C. scophthalmum* TPBLGL 18 infection in golden mahseer resulted in median lethal dose of 6.2 LogCFU/fish [36]. Although the pathogenicity of the *Chryseobacterium* was not much greater than the common pathogens, multidrug-resistant features of the strain WLT may pose a therapeutic challenge [33,34]. Therefore, *Chryseobacterium* could be considered as a fish pathogenic microorganism in addition to its food spoilage activity.

Since, *Chryseobacterium* sp. has been recognized as an emerging fish pathogen in South Korea in recent times, studies regarding antibiotic susceptibility-based treatment methods should precede [32,37]. In a previous *Chryseobacterium* infection case study, we failed to cure the diseased fish and we speculated that high prevalence of antibiotic resistance by *Chryseobacterium* was a reason. The *C. balustinum* WLT also revealed resistance to almost all the tested antibiotics (e.g., ampicillin, doxycycline, and nalidixic acid against Gram-negative bacteria) that are permitted by the National Institute of Fisheries Science, South Korea for aquaculture uses. Accordingly, an effective treatment protocol should be adopted to address the emerging *Chryseobacterium* outbreaks.

5. Conclusions

In conclusion, one of the isolates of *Chryseobacterium* spp. pathogenic to rainbow trout closely resembled *C. balustinum* on 16S rRNA gene sequencing analysis. Following Koch's postulates, the bacteria were confirmed to cause clinical symptoms such as damaged fins and mortality. Further studies on the pathogenicity of the bacteria are required since these bacteria can induce economic loss in other rainbow trout fisheries in Korea or other countries. Moreover, follow-up evaluation is required for this outbreak to control and prevent this disease.

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Institutional Review Board Statement: Ethical review and approval were waived for this study as the Institutional Animal Care and Use Committee of Seoul National University does not provide an ethical number for research using fish except laboratory fish (e.g., zebrafish) which are grown under specific purpose for research in laboratory animal facilities. The fish that were used in this experiment were not an object under the committee guideline and explanation. Therefore, our research was performed in the Department of Aquaculture, Korea National College of Agriculture and Fisheries, where they have authorization from the government regarding fish cultivation and experiment. So, we followed strict ethical guidelines in experiment procedure and handling fish using the facility.

Conflicts of Interest: The authors declare no conflict of interest.

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