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Efficacy of oral praziquantel treatment against the skin fluke infection of cultured chub mackerel, *Scomber japonicus*

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ABSTRACT

Outbreaks of skin flukes and associated mortalities have been observed in cultured chub mackerel, *Scomber japonicus*. Although freshwater bathing effectively removes the parasites, large post-treatment mortalities are occasionally observed especially under high water temperature conditions due to the stress from handling and confinement. Since outbreaks of the skin fluke infections often occur in high temperature seasons, development of an effective and safe control method is essential for the advancement of mackerel aquaculture. The present study aims to: 1) identify the skin fluke of cultured chub mackerel and 2) develop an alternative control measure using oral drug administration. The skin fluke was identified as *Neobenedenia girellae* by morphology and molecular data targeting ITS region of rDNA. Two trials of oral administration of praziquantel (PZQ), a common anthelmintic, were conducted and parasite intensity was compared before and after the treatment and between treated and untreated control fish. Fish rejected PZQ-coated commercial pellets, but oral administration was successfully achieved using frozen krill as a basal diet. The three-day administration with a dose of 150 mg·kg^{-1} BW·day^{-1} resulted in over 80% reduction in worm intensity. However, some proportion of the skin flukes survived the drug treatment. The resistance to PZQ does not relate to worm's developmental stage. Freshwater bathing was more effective and eradicated the parasite, but some post-treatment mortality of host fish was observed. Moreover, the parasite intensity drastically increased after the freshwater bathing, possibly due to stress and loss of mucus during the bathing. The study indicates the PZQ oral treatment is effective to control *N. girellae* in chub mackerel aquaculture when the drug is properly administrated with an appropriate feeding technique.

1. Introduction

Mackerel is one of the most important fishery resources around the world. Chub mackerel, *Scomber japonicus* have been highly popular and common seafood in Japan due to its low price and high yield. However, Japanese mackerel stock has significantly declined over the last few decades, from over 1.6 million tons annual catch to less than 500 thousand tons between 1977 and 2009 (data from The Ministry of Agriculture, Forestry and Fisheries of Japan, http://www.jfa.maff.go.jp). To meet the demands and to protect wild stocks, aquaculture of mackerel has recently been started in Japan. Since the establishment of the complete culture cycle of *S. japonicus* in 2002 (Murata et al., 2005), the mackerel culture receives growing attention and further development of the industry is expected. As in any other aquaculture, infectious diseases are one of the potential problems in the advancement of mackerel culture. Although not much has been studied about diseases of cultured mackerel, several parasites are recognised as potential threats. A brain myxozoan *Myxobolus acanthogobii* associated with scoliosis has been reported in cultured mackerel (Yokoyama et al., 2005). Another brain myxozoan, *Kudoa yasunagai*, has also been found in cultured mackerel though its pathology is still unclear (Shirakashi, personal observation). Among them, the most common and important parasite is a skin fluke (Okamoto et al., 2005; Yamamoto et al., 2006).

Capsalid skin flukes are considered as a serious and chronic problem in aquaculture (Ogawa, 2004; Ogawa and Yokoyama, 1998; Ogawa et al., 1995). The culturing conditions, high host density and parasite eggs entangling to the net, lead to epidemics of skin flukes in fish farms. Heavily infected fish often suffer from skin lesions and blindness resulting in growth reduction, secondary bacterial and viral infections and mortalities (Ogawa, 2004). Apparently, chub mackerel is highly susceptible to the skin fluke (Okamoto et al., 2005; Yamamoto et al., 2006). Mortalities associated with heavy skin fluke infection have been observed when water temperature is greater than 24 °C (Yamamoto et al., 2006). To date, the control of skin fluke infections of cultured mackerel is limited to freshwater. These treatments are commonly practiced for treating skin fluke infestations. However, they are extremely labour-intensive and time consuming. Moreover, bathing and handling can cause great stress to the fish and post-treatment mortalities often occur under high water temperature conditions (Stephens et al., 2003; Yamamoto et al., 2006). This is especially problematic for mackerel as they are

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relatively sensitive and highly susceptible to handling. For a successful mackerel aquaculture, frequent bathing treatments are required during the high water temperature season in order to control the skin fluke infestation. However, this is associated with negative consequences. In fact, we experienced a loss of more than 1000 adult mackerel after 6 min freshwater bathing in a summer month (water temperature 28.5 °C, Yamamoto personal observation). Therefore, the development of alternative control measures that are both effective and safe for the fish is vitally needed.

Praziquantel (PZQ) is an anthelmintic commonly used for human and animal parasites such as schistosomes. The drug is also used for treating fish parasites and approved in Japan for aquaculture usage against *Benedenia seriolae*, a skin fluke of seriola fish. Oral PZQ administration, normally mixed with commercial food pellets, is shown to be effective against several fish parasites, including common skin fluke *B. seriolae* and *Neobenedenia girellae* (Hirazawa et al., 2004; Williams et al., 2007). However, PZQ is extremely bitter and has low palatability for fish, which makes oral administration difficult (Sitja-Bobadilla et al., 2006; Williams et al., 2007; Yamamoto et al., 2006). Therefore, feeding technique must be re-evaluated for the practical use of PZQ in aquaculture. In addition, efficacy of PZQ may differ between parasite species and between host fish (Poynton et al., 1997), thus evaluation of the drug should be conducted using the exact target host/parasite system. The aims of the present study was to identify the skin fluke observed in cultured chub mackerel using morphological and molecular data and to evaluate the efficacy of PZQ against the parasite using an improved oral administration method.

2. Materials and methods

2.1. Fish and parasite

A total of 1600 artificially produced 5 month old juvenile Chub mackerel of average body weight (BW) approximately 45 ± 11 g were used for the study. Fish were reared from eggs in land-based tanks for 4 months and then transferred to the sea cages. Prior to the transfer, half of the fish were vaccinated for red sea bream iridovirus by abdominal injection. The rest was injected with PBS in the same manner. The vaccination was conducted to reduce the risk of mass mortalities by the virus disease and for another study not reported in this paper. Eight days after the vaccination, four hundred vaccinated or non-vaccinated fish were transferred to each of the four net cages on the sea (4×4 m). In the cages, fish were fed once a day with ad lib amount of commercial dry pellet and mortality was checked daily. Fish were naturally infected to the skin flukes in the sea cages. Two to three fish were sampled as needed for monitoring of the skin fluke infection. Fish were taken from the cage by a hook and line and individually placed in a bucket with ice cold freshwater (4–7 °C). The cold water was used as anaesthetic to reduce stress and to make a measurement easier. Fish were placed in the cold freshwater for more than 6 min and skin and fins were wiped by hand to dislodge the parasite. The water was then filtered through a mesh (opening 64 μm) and the worms collected from each fish were counted under a stereomicroscope. Every sampled fish was measured for fork length and body weight.

2.2. Parasite identification

Skin flukes of various sizes that were dislodged during the sampling were used for identification. The worms that were fixed in 70% ethanol or mounted on a glass slide and stained with aceto-carmine were sent to Dr. K. Ogawa (Meguro Parasitological Museum) for morphological identification. Additionally, molecular analyses of three frozen specimens of different size were conducted. Genomic DNA extraction was carried out using the QIAmp DNA Mini Kit (QIAGEN Inc., Germany), following manufacturer's instructions. Internal transcribed spacer (ITS) regions of rRNA genes were PCR amplified using the following primer pair; PD-ITS-450F (forward: 5′-AGGTGAACCTGCAGAAGGATC-3′) and PD-ITS-R (reverse: 5′-TAATGCTTAAATTCAGCGGGT-3′) and with the following cycling program; initial denaturation of 95 °C for 2 min followed by 30 cycles of 95 °C for 50 s, 55 °C for 50 s, 72 °C for 50s and a final extension at 72 °C for 4 min (Hayward et al., 2001). The PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN) and subjected to bi-directional sequencing on a BioRad DNA Engine Dyad PTC-220 Peltier Thermal Cycler using an ABI BigDye™ Terminator v3.1 Cycle Sequencing Kit with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems), following the manufacturer's instructions employing the same primers as used in PCR.

The sequence data was edited and aligned with the desired sequences obtained from GenBank and a previously obtained sequence of *N. girellae* (502 bp) sampled from Japanese flounder, *Paralichthys olivaceus,* using Genetix ver. 9.1 (Genetyx Corporation).

2.3. Praziquantel treatment

We have conducted series of preliminary experiments to improve palatability of PZQ treated commercial pellets. The base medicated pellets were prepared by mixing with PZQ formulation powder or soaked in PZQ solution (powder and 95% ethanol) and dried. The pellets were further coated with various feeding stimulants (fish oil, krill extracts, sugar, or commercial fish attractants) and/or coating agents (agar or carboxymethyl cellulose sodium salt). In addition to the dry pellet, frozen krill coated with PZQ formulation was prepared. Equal amounts of the drug were used for each feed. Treated feed was administered to the fish kept in a 500 L polycarbonate tank and their behaviour was monitored. The feed was given until the fish stop to eat and the feed left in the bottom of the tank after 30 min of feeding were recovered to measure the feed intake. When medicated dry pellets were given, Fish showed strong avoidance behaviour and 70–95% reduction of feed intake compared to untreated pellets was observed. Therefore, we were unable to administer the desired dose of PZQ using commercial pellet. On the other hand, the fish showed adequate intake (14–22% reduction) without apparent avoidance toward the medicated frozen krill, thus, the treatment experiment was conducted using frozen krill as a basal feed.

The PZQ feed was prepared by mixing 300 mg kg^{-1} BW of Benesaru (50% praziquantel formulation, Aska Pharmaceutical Co., LTD, Tokyo) and sodium alginate (0.5% weight of total feed, spreading and sticking agent) with 1.3 to 1.6 kg (approx. 8% BW) of semi-defrosted frozen krill. This dose was chosen to follow the manufacturer's recommendation dose for *Benedenia seriolae* (Okabe, 2000). The PZQ treatment trial was conducted after the mean skin fluke intensity of sampled fish reached 10. Two trials were conducted during 15th–19th (trial 1 with vaccinated fish) and 20th–30th (trial 2 with non-vaccinated fish) November, 2010. In each trial, two cages were used and fish in one cage were treated with PZQ and those in another cage were assigned as untreated control. The water temperature during trials 1 and 2 were 20.8–23.0 °C and 18.9–21.7 °C, respectively.

Fish were sampled 1 day (trial 2, $N = 20$) or 4 days (trial 1, $N = 6$) prior to the drug treatment to assess the initial parasite intensity and were treated with PZQ for 3 consecutive days. The medicated feed was given once a day in the morning. Feeding was performed in a way so that the majority of fish in a cage ingest the given feed. The same feed without PZQ (krill and sodium alginate) was given to the control fish. To compare the efficacy of PZQ to the traditional treatment, control fish were subjected to freshwater bathing. The bathing treatment was conducted on the day after the end of the drug administration for the treatment group. Fish in a control cage were first chased into a small nylon cage and transferred to a lined cage filled with 1500 l of freshwater. In this manner, no dip netting was required and handling was minimised. Following 5 minutes bathing, fish were returned back

to a normal cage. The cage-nets were not changed during the experiment.

Ten fish from treated and control group were sampled 4–5 h after the drug administration and 1 day after the freshwater bathing. In addition, fish were sampled at 7 days post PZQ treatment (dtp) in trial 2. Worm intensity of each fish was determined following the method described previously. After parasite collection and fish measuring, all sampled fish were transferred to separate tanks to avoid testing the same fish again. To assess whether efficacy of PZQ differs between the worms at different developmental stages, the size of worms was measured at each sampling. Twenty worms were randomly selected from each experimental group and their body lengths (from the anterior end of the body to the end of opisthaptor) were measured on digital photographs using an image analysis software.

2.4. Statistical analyses

For comparison of fish-survival between the groups, the Kaplan–Meier analysis with a log-rank test was used. Difference in worm intensities between the samples was compared using the Wilcoxon signed-ranks test. The non-parametric Steel's test was used to compare the worm intensity before and after drug administration or freshwater bathing treatments. Analyses involving the body size of worms used analysis of variance (ANOVA) followed by Tukey–Kramer post hoc test, when necessary. All the statistical analyses were performed with JMP ver. 9 (SAS Institute).

3. Results

3.1. Parasite identification

Morphologically, the worm possessed the characteristics of *N. girellae* described in Hargis (1955), Ogawa et al. (1995) and Kinami et al. (2005). From the molecular study, partial ITS1, complete 5.8S and ITS2, and partial 28S sequences (1284 bp, GenBank Accession No. JF934745) were obtained. The sequences from three skin fluke isolates were consistent and were 100% identical with the sequences of *N. girellae* from the Japanese flounder (Partial ITS1, 502 bp) and from *Pseudosciaena crocea* (GenBank Accession No. AY551326), but 1 nucleotide different from *N. melleni* (= possibly the synonym with *N. girellae*, GenBank Accession No. AY551323) (Li et al., 2005; Whittington and Horton, 1996). Based on these data, the skin fluke of chub mackerel is identified as *N. girellae.*

3.2. Praziquantel treatment

Minor mortality, probably due to handling, was observed immediately after the fish were transferred from land-based tanks to sea cages and also after freshwater bathing (Fig. 1). The mortalities of control groups tended to be higher than the PZQ treated groups, but no statistical difference was detected (Kaplan–Meier analysis, $p = 0.16$). During the PZQ administrations, fish ingested medicated krill without apparent avoidance or vomiting behaviours. All the given medicated feed was eaten and there was no decline in food consumption after the drug treatment.

Ninety and 60% reduction of the mean parasite intensity was observed after one PZQ administration in trials 1 and 2, respectively (Fig. 2). It was clearly demonstrated in both trials that oral PZQ treatment effectively removed the majority of skin flukes from the mackerel, 86.5% and 81.4% for trials 1 and 2, respectively (Steel's test; $p = 0.03$–0.04 for trial 1, $p < 0.001$ for trial 2). However, complete eradication of the parasite could not be achieved by the drug. The infection prevalence remained over 80% throughout the experiment and an average of about 16% of worms (about 4 worms per fish) retained even after the three times PZQ administrations (Fig. 2). The changes of post-treatment parasite intensity varied between the trials. In trial 1, the worm intensity remained relatively low at 2 dpt. (2.22 ± 1.39 worms/fish). In contrast, the worm number in trial 2 increased by 7-fold during 2 days after PZQ treatment (Fig. 2 bottom, Wilcoxon, $Z = -3.32$, $p < 0.001$), though no further significant increase was observed between 2 and 7 dpt (Fig. 2).

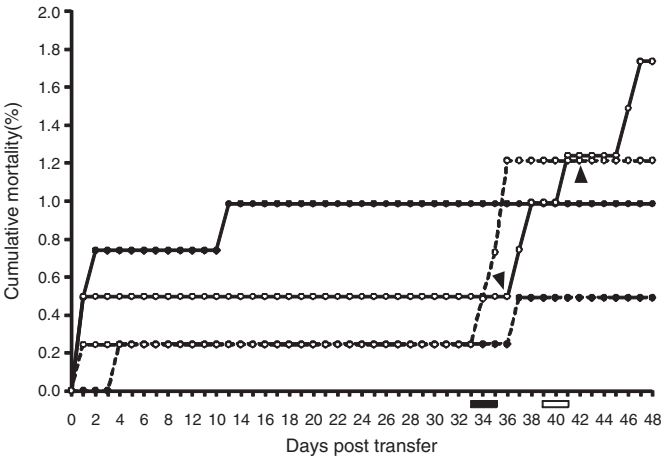

Fig. 1. Cumulative mortality of control (open circle) and treated (closed circle) groups after the fish transfer to the sea cages. Treatment trials 1 and 2 are plotted in solid and broken lines, respectively. Arrowheads indicate the freshwater bathing. Solid and open bars on *X* axis indicate drug administration period for trials 1 and 2, respectively.

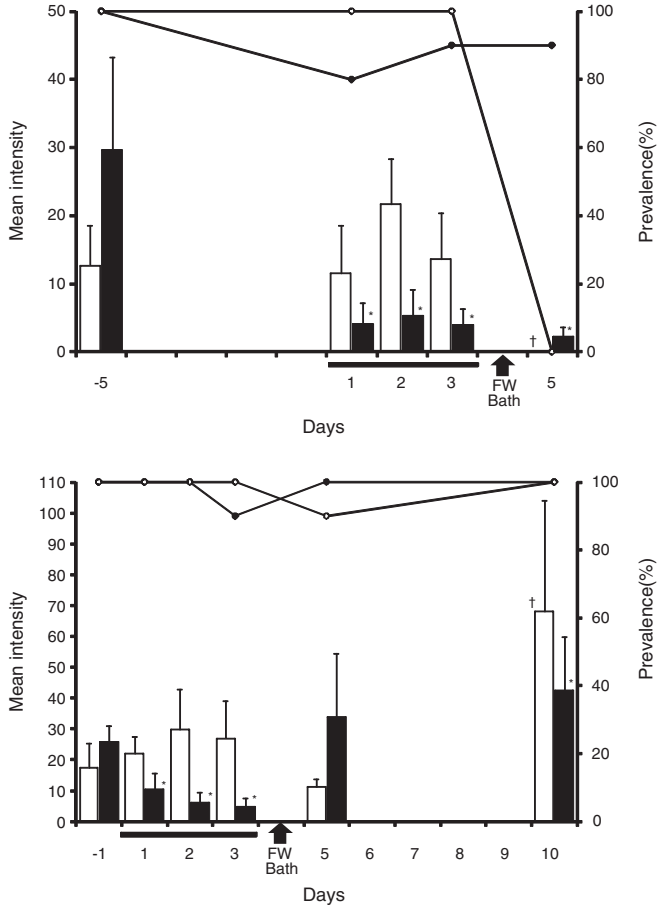

Fig. 2. Change of the mean (+SD) skin fluke intensity and prevalence in the treated groups (closed bar, closed circle) and the control groups (open bar, open circle) for trial 1 (top) and trial 2 (bottom). Bars on *X* axis indicate drug administration and arrows indicate freshwater bathing for the control groups. Crosses and asterisks indicate the significant difference from pre-treatment in control and treated group, respectively.

The parasite numbers on the controls were relatively constant until the freshwater bathing treatment (Fig. 2, Steel's test; $p>0.10$ for trial 1, $p=0.07$–0.20 for trial 2). Five minute freshwater bathing successfully eradicated the skin flukes in trial 1 (Fig. 1). However, in trial 2, on average 11.22 ± 2.54 worms were found a day after the freshwater treatment. Moreover, the worm number became 5 fold higher during the 5-day post-bathing period, while that of the non-bathed group (PZQ treated group) remained relatively unchanged (Wilcoxon, $Z=-3.75$, $p<0.001$ for control, $Z=-0.57$, $p=0.570$ for PZQ group). In trial 2, the worms found after the freshwater bathing were significantly smaller than those from pre-bathing, suggesting they are newly recruited ones (ANOVA, $F_{5,114}=17.42$, $p<0.0001$, Tukey–Kramer, $p<0.05$). These worms showed nearly 60% increase in the mean body length (0.26 ± 0.02 to 0.41 ± 0.11 mm) during the next 5 days. Interestingly, the worms on PZQ treated fish became significantly smaller during 5 and 10 dpt (2.25 ± 1.22 mm to 0.43 ± 0.13 mm, t-test, $t_{78}=-4.02$ $p<0.0001$) (Fig. 3).

3. Discussion

Since the introduction of *Neobenedenia girrellae* to Japan in early 1990s via importation of amberjack, the parasite has spread to various aquaculture species, including yellowtail *S. quinqueradiata,* tiger puffer *Takifugu rubripes*, and Japanese flounder *P. olivaceus* (Ogawa, 2004; Ogawa and Yokoyama, 1998). In recent years, *N. girellae* further expanded its host range and started to cause problems in new aquaculture fish species such as seven-band grouper *Epinephelus septemfasciatus* and cobia *Rachycentron canadum* (Habu et al., 2009; Ogawa et al., 2006). This study confirms that chub mackerel is highly susceptible to *N. girellae* and suggests that the control of the parasite is the key to successful mackerel culture.

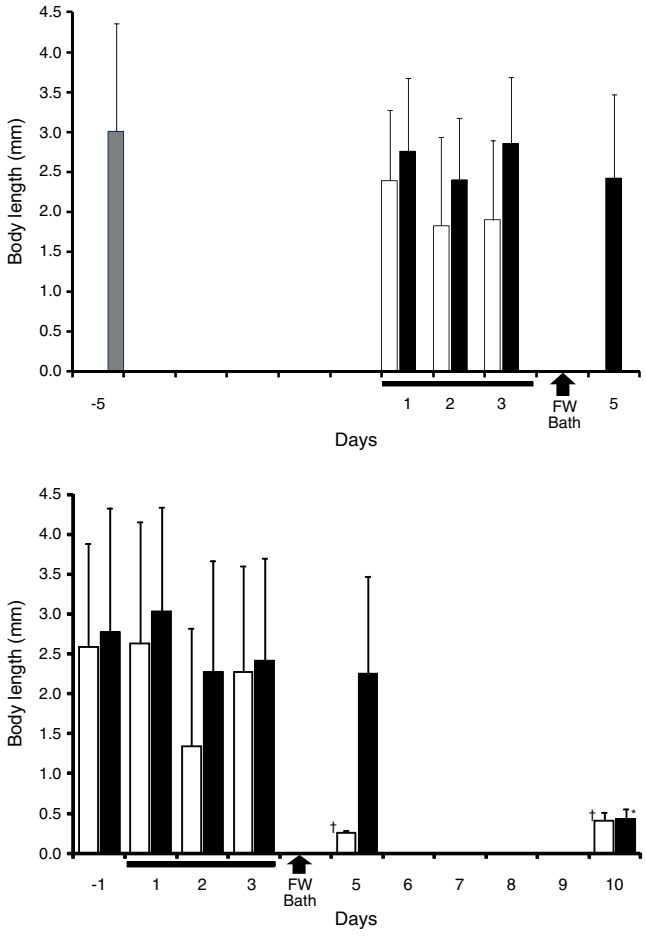

Fig. 3. Change of the mean (+SD) body length of skin flukes from control (open bar) and treated fish (closed bar) in trial 1 (top) and trial 2 (bottom). The pre-treatment samples of worms from two groups for trial 1 were mixed together, thus indicated by a gray bar. Bars on *X* axis indicate drug administration and arrows indicate freshwater bathing for the control groups. A cross and asterisk indicate the significant difference from pre-treatment in control and treated group, respectively.

The 3-day oral administration of 150 mg kg^{-1} BW PZQ effectively reduced the number of skin flukes. Hirazawa et al (2004) also reported the efficacy of oral PZQ treatment against *N. girellae* on spotted halibut, *Verasper variegatus*. However, they showed that the fish given 150 mg kg^{-1} BW PZQ for 3 days harbour more parasites (13.3 ± 5.5) than those given 40 mg kg^{-1} BW for 11 days (9.1 ± 8.5), because the fish rejected the pellet medicated with the high dose of PZQ. Similarly, yellowtail showed strong rejection for dry pellets coated with higher dose of PZQ (150 mg kg^{-1} BW day^{-1}) and only 21.6% reduction of skin fluke *B. seriolae* was achieved, while the fish given lower dose (50 mg kg^{-1} BW day^{-1}) showed 58.1% worm reduction (Williams et al., 2007). Yamamoto et al (2006) showed 3-day administrations of 150 mg kg^{-1} BW PZQ reduced the number of skin flukes of chub mackerel by 58%, but the fish showed a significant reduction in medicated feed intake. In the present study, PZQ treatment appeared to be more effective than in the past studies as over 80% reduction in worm intensity was observed and only about 4 worms per fish remained after drug treatment. This is probably due to the difference in palatability of medicated feed.

Low palatability of PZQ is the major shortcoming of this effective drug for application in aquaculture. The majority of past studies reported intake reduction of PZQ treated feed in various fish species and thus the efficacy of oral PZQ treatment could not be properly evaluated. For instance, giltheaed sea bream, *Sparus aurata*, showed 45% reduction in food intake when 116.3 mg kg^{-1} BW day^{-1} PZQ was given in dry pellets (Sitja-Bobadilla et al., 2006). Our preliminary trial also showed as much as 95% reduction in feed intake when PZQ is added to the pellet. Frozen krill used in the present study significantly increase the palatability of PZQ and overcame its shortcoming. Frozen krill has a lower cost per weight than commercial dry pellets and can be routinely used in aquaculture for the short period of drug treatment. However, storage of frozen feed may be problem in some fish farms, thus evaluation of alternative basal feeds or additive substances are also important to develop more effective PZQ administration methods.

Although it appears that the efficacy of oral PZQ treatment on skin flukes shown in the present study is higher compared to other reports (Hirazawa et al., 2004; Williams et al., 2007; Yamamoto et al., 2006), it is not as effective as the freshwater bathing as total worm eradication could not be achieved. Nearly 20% of the worms remained even after three days of PZQ administrations. This is not due to different developmental stages of the worms, because there was no significant change in worm size before and after the drug treatment. In other words, both immature and mature worms are equally resistant or susceptible to the PZQ. This was also shown in a previous study, where similar numbers of adult and newly infected *N. girellae* survived PZQ treatment (Hirazawa et al., 2004). Whether the survivor worms are a resistant strain or other factors, such as the infection site, caused susceptibility differences requires further study. The present study also showed that PZQ does not prevent re-infection of the fish. Significant increase of the skin fluke intensity was evident at 2 days post PZQ treatment in trial 2. The most logical explanation for this sudden increase of the post-treatment worm intensity is re-infection. However, the average size of the worm at this point was over 2.0 mm, suggesting the worms were not newly attached larvae. One possible explanation for this seemingly contradictory result is the selection procedure of worms for the measurement. Twenty worms were randomly selected for measurement from a container, but very small individuals were tended to be trapped with fish mucus and might have not been selected. Nevertheless, effects of PZQ did not last long.

Praziquantel is a highly metabolised drug and its concentration on fish skin falls below detection limit 24 h after oral administration (Okabe, 2000; Tubbs and Tingle, 2006). Therefore, for the control of skin flukes, repeated drug treatments are desirable with intervals of at least 6 to 8 days, which is the time it takes for attached larvae to mature at 25 °C and 30 °C, respectively (Hirazawa et al., 2010). In addition, cleaning or replacement of cage-nets is important to reduce numbers of available parasite eggs in the farming area and chance of post-treatment infection.

The traditional freshwater bathing is shown to be very effective to eradicate the skin flukes. No worms were found after the bathing treatment in trial 1. In trial 2, an average of about 11 worms was found after the bathing treatment, but their small size indicated they originated from reinfection. The freshwater bathing however, causes great stress to fish. In Atlantic salmon, handling, short period of confinement (10 min) and freshwater bathing are shown to cause a significant increase in plasma cortisol and IGF-I level (Wilkinson et al., 2006). Mackerel is a sensitive fish and extremely susceptible to handling. Post-bathing mortality in mackerel can be as high as 50% (Yamamoto personal observation), especially when the water temperature is high. Despite its effectiveness, freshwater bathing is not the practical control for mackerel since *N. girellae* outbreaks often occur in the season with water temperatures above 24 °C (Hirazawa et al., 2010; Yamamoto et al., 2006). Furthermore, bathing treatment may induce post-treatment infection as drastic increase of post-bathing infection was observed in trial 2 of the present study. Ohno et al (2009) reported a similar phenomenon that significantly higher numbers of *N. girellae* were found on amberjack, *Seriola dumerili*, and yellowtail, *S. quinqueradiata*, after 2 min of freshwater bathing compared to the fish bathed in the sea water. Removal of skin mucus during the freshwater bathing may make fish to become more susceptible to skin flukes. The cost of freshwater bathing may exceed its benefit under some circumstances, and the PZQ treatment may be a more beneficial method for the fish farmers.

The PZQ treatment is probably the most practical control method for skin fluke infections in mackerel aquaculture, today. However, it has to be noted that in Japan, the use of PZQ, without a prescription from a veterinarian, is approved only for *B. seriolae* infection at present. More empirical data on the efficacy of PZQ on *N. girellae* and development of more effective oral PZQ treatment techniques will help to establish this simple and effective control method in aquacultures of not only mackerel but of various species.

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