ニジマス(Oncorhynchus mykiss)のPolyinosinic-polycytidylic acid(PIC)投与による免疫賦活作用および同薬剤に対する耐性に及ぼす諸要因

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Factors affecting tolerance and immunoreactivity of rainbow trout (*Oncorhynchus mykiss*) to Polyinosinic-polycytidylic acid

Shotaro NISHIKAWA[1], Tetsuo NAKANO[2,3] and Hisae KASAI[1,*]

Abstract: Polyinosinic-polycytidylic acid (PIC), a synthetic double-stranded RNA, is a strong innate immune response inducer. Although it is an innate immune inducer, when administered to some fish species at low temperatures, PIC may be toxic and mortality may occur. Moreover, in mice, immunoreactivity is affected by nucleotide length of PIC. The aim of this study was to demonstrate the effects of body size, water temperature and length of PIC on tolerance and immunoreactivity of rainbow trout to PIC. Tolerance of fish to PIC was better for large fish (145 g) than for small fish (12 g). Furthermore, PIC-induced Mx gene expression when water temperature was high peaked at day 1 and then decreased sharply. When water temperature was low, Mx gene expression lasted longer and high mortality indicated toxicity of PIC. Observations on the effects of length of PIC revealed that Mx gene expression was not significantly related to the length of PIC.

Key words: Rainbow trout; Polyinosinic-polycytidylic acid; PIC; Innate immunity

Polyinosinic-polycytidylic acid (PIC) is a double-stranded RNA (dsRNA) consisting of two homopolyribonucleotides, polyinosinic acid (poly-I) and polycytidylic acid (poly-C), with intermolecular nucleotide-paired interactions, forming a stable double-helix (Chamberlin and Patterson 1965). PIC induces innate immune responses resulting in anti-viral effects in mammals (Field et al. 1967; Park and Baron 1968; Richmond and Hamilton 1969). According to some studies, PIC is a candidate adjuvant for human vaccines (Ichinohe et al. 2005, 2010; Phoolcharoen et al. 2011). In the case of fish, anti-viral responses after PIC administration were observed in pink salmon *Oncorhynchus gorbuscha*, sockeye salmon *Oncorhynchus nerka*, Atlantic salmon *Salmo salar* L. and in rainbow trout *Oncorhynchus mykiss* (Eaton 1990; Jensen and Robertsen 2002). PIC injection gave protection against RGNNV infection to grouper (Nishizawa et al. 2009; Thanasaksiri et al. 2014). It is also reported that a new method of vaccination with PIC protects fish against viral hemorrhagic septicemia (Nishizawa et al. 2011).

Some challenges to the use of PIC have been reported. It is reported that massive administration of PIC sometimes exacerbates pathological conditions in animal models (Zeleznick and Bhuyan 1969; Phillips et al. 1971). These negative pharmacological effects of PIC are related to both direct action and side effects associated with overproduction of type-I interferon and inflammatory cytokines by cells. In the case of fish, water temperature is thought to affect the immune response leading to mortality. Matsui et al. (2012) demonstrated that mortalities of PIC injected Japanese flounder *Paralichthys olivaceus* were higher when rearing was done at 13°C than when rearing was done at 17°C. Moreover, signs of toxicity such as redness or ulceration of the skin around the inoculation site were observed (Matsui et al. 2012).

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[1] Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato-cho, Hakodate, Hokkaido 041-8611, Japan.
[2] Technical research laboratories, Kyowa Hakko Bio Co., Ltd., Hofu, Yamaguchi 747-8522, Japan.
[3] Graduate school of Agriculture, Hokkaido University, Kita 9 Nishi 9, Kita-ku, Sapporo, Hokkaido 060-8589, Japan.
*Corresponding author: E-mail, hisae@fish.hokudai.ac.jp (H. Kasai).

High mortalities after PIC injection were also observed at 6℃ in Atlantic salmon parr. (Salinas et al. 2004).

Mx protein is an anti-viral protein induced by immune responses in organisms (Leong et al. 1998; Lindenmann 1962; Ko et al. 2002). Injection with PIC induces significant increases in the interferon-inducible genes including *Mx* in rainbow trout and this could be a useful marker of fish immune response to PIC (Purcell et al. 2004). Some reports describe Mx gene expression in fish after PIC injection, but the relationship between mortality and Mx gene expression after PIC injection is not well explained. In a study using zebrafish *Danio rerio*, Mx gene expression after PIC injection was related to the rearing water temperature, with higher water temperatures causing a higher immune response (Dios et al. 2010). But this does not explain the high mortality of fish in cold water. Another study using sevenband grouper *Epinephelus septemfasciatus* stated that Mx gene expression after PIC injection did not differ significantly with temperature (Thanasaksiri et al. 2014).

Focusing on the length of PIC, immunoreactivity and toxicity of PIC in mammals are known to be affected by the length of the double strand and its components; poly-I and poly-C (Morahan et al. 1972; Black et al. 1973; Machida et al. 1976). Longer strands cause higher immunoreactivity and toxicity. A recent study developed length-controlled PICs (Nakano et al. 2018). These can be used to study the effect of PIC length on immunoreactivity and toxicity.

The purpose of this study is to evaluate the relationship between fish immune response and the nucleotide length of PIC under high and low temperature in rainbow trout.

Materials and Methods

Fish

Rainbow trout were kindly provided by Hokkaido Research Organization, Fisheries Research Department, Salmon and Freshwater Fisheries Research Institute, Japan or hatched at Hokkaido University, Hakodate, Japan. The fish provided had mean body weights of 109 g (high temperature test), 145 g (low temperature test) and 65 g (comparison test for the length of PIC). The fish that hatched at Hokkaido Univ. had a mean body weight of 12 g. All the fish were reared using de-chlorinated tap water.

Preparation of PIC

Six PICs with different nucleotide length distributions were provided by Kyowa Hakko Bio Co., Ltd. (Tokyo, Japan). PIC showing the longest nucleotide length was purchased from GE Healthcare Japan (Tokyo, Japan). Lengths were determined in accordance with the method of Nakano et al. (2018). The nucleotide lengths of PIC used in this study are shown in Table 1. All PICs were dissolved in saline at 500–5000 μg/m*l* and stored at −30℃ until use.

Assessing the effect of fish size on mortality after PIC administration

Fish (mean body weight of 12 g and 145 g) were divided into 6 groups containing 12 fishes each. The fish were given 100 μg/fish, 200 μg/fish, 400 μg/fish, 800 μg/fish or 1000 μg/fish of PIC (uPIC70-400) by intraperitoneal injection. The control group was injected with 200 μ*l* of saline instead of PIC solution. The PIC-injected fish were reared using de-chlorinated water at 4.6℃ for 10 days. Mortality was monitored daily.

Assessing the effect of temperature on Mx gene expression after PIC administration

Fish (mean body weight of 109 g and 145 g) were divided into 10 groups containing

Table 1. Base pair length of PIC used in this study

	Length of double strand (kbp)	
Name	Lp	Lw
uPIC70-400	0.4	0.3
uPIC50-400	0.4	0.4
uPIC50-400L	0.7	0.6
uPIC100-400	0.5	0.4
uPIC100-400L	0.8	0.5
PIC400-400	1.1	0.5
PIC-HMW	4.5	NA

Lp: nucleotide length at the peak absorbance 260 nm
Lw: weight-average nucleotide length
NA: not available

12 fishes each. The fish were given 100 μg/fish, 200 μg/fish, 400 μg/fish or 800 μg/fish of PIC (PIC400-400) by intraperitoneal injection. The control group was injected with 200 μl of saline instead of PIC solution. The PIC-injected fish were reared using de-chlorinated water at 17.4℃ or 4.6℃ for 10 days. Three fish from each tank were sacrificed at 1, 3, 7 and 10 days post injection (dpi), and spleen samples were collected to analyze for expression profiles of Mx gene. Spleen was soaked in RNAlater (AM7020, Invitrogen, California, USA) and stored at −30℃ until RNA extraction.

Assessing the length of PIC on Mx gene expression

Fish (mean body weight of 65 g) were divided into 6 groups containing 12 fishes each. These fishes were injected with one of 6 types of PIC (each 3 μg/g fish) by intraperitoneal injection and the control group were injected with 200 μl of saline. The PIC-injected fish were reared in dechlorinating water at 8.7℃ for 10 days and spleens were collected as described above.

Expression profiles of Mx gene in fish

Total RNA was extracted using QuickGene SP kit RNA tissue (SP-RT, KURABO, Osaka, Japan) following manufacture's instruction. RNA was eluted in 50 μl of DEPC and stored at −80℃ until use. Reverse transcription was performed with reverse transcriptase XL (AMV) (2620A, TAKARA BIO, Shiga, Japan) and Random primer (3801, TAKARA BIO, Shiga, Japan) according to the manufacture's protocol. qPCR assays for Mx gene and housekeeping gene (Acidic ribosomal Phosphoprotein P0; ARP) were performed using the ABI 7300 Real time PCR System (4345240, Applied Biosystems, California, USA) according to a modified method of Purcell et al. (2004). Briefly, qPCR was carried out using Premix Ex Taq (Probe qPCR) (RR390A, TAKARA BIO, Shiga, Japan), the thermal profile for qPCR was 1 cycle of 95℃ for 1 min, 45 cycles of 95℃ for 15 sec, and 60℃ for 1 min. To assess PCR efficiency, 10-fold dilutions of the recombinant plasmid (Mx or ARP gene) were used to generate the standard curve for each assay plate. Gene expression values were calculated using the standard curve method provided by the ABI Sequence Detection System software. RNA loading differences were assessed with ARP gene, which was used to normalize the specific gene data. The saline injected group served as the calibrator group and fold increases were calculated relative to this group.

Statistical analysis

Statistical analysis was performed using SPSS Statistics version 25.0. (IB501XL, IBM, Tokyo, Japan) Comparisons between groups in each time course were calculated by Kruskal Wallis test. Differences were defined as statistically significant at $p < 0.05$.

Results

Tolerance of fish of different sizes to PIC

The tolerance of fish (mean body weights of 12 g) to different doses of PIC is shown in Fig. 1. The water temperature was 4.6℃ during the experiments. No mortalities were observed in rainbow trout that had a mean body weight of 145 g. However, the fish that had a mean body weight of 12 g showed mortalities of 25, 58, 85 and 73% after receiving PIC doses of 200, 400, 800 and 1000 μg/fish, respectively. Fish injected 100 μg/fish of PIC showed no mortality during the experimental period. Dead fish in the 12 g group exhibited symptoms such as the falling and standing of scales, bulging of eyes, abdominal dropsy, surface hemorrhage, and enlargement of spleen, which suggested abnormalities related to immunity (Fig. 2). For subsequent experiments, fish with a body weight of 109 g and 145 g were used.

Influence of water temperature on the expression of Mx gene by PIC administration

PIC administration induced a significant Mx gene expression in rainbow trout, but changes in expression over time differed depending on the water temperature (Fig. 3). Mx gene expression values when rearing water temperature was high peaked at 1 dpi and disappeared at 3 dpi. Mx gene expression levels at 1 dpi

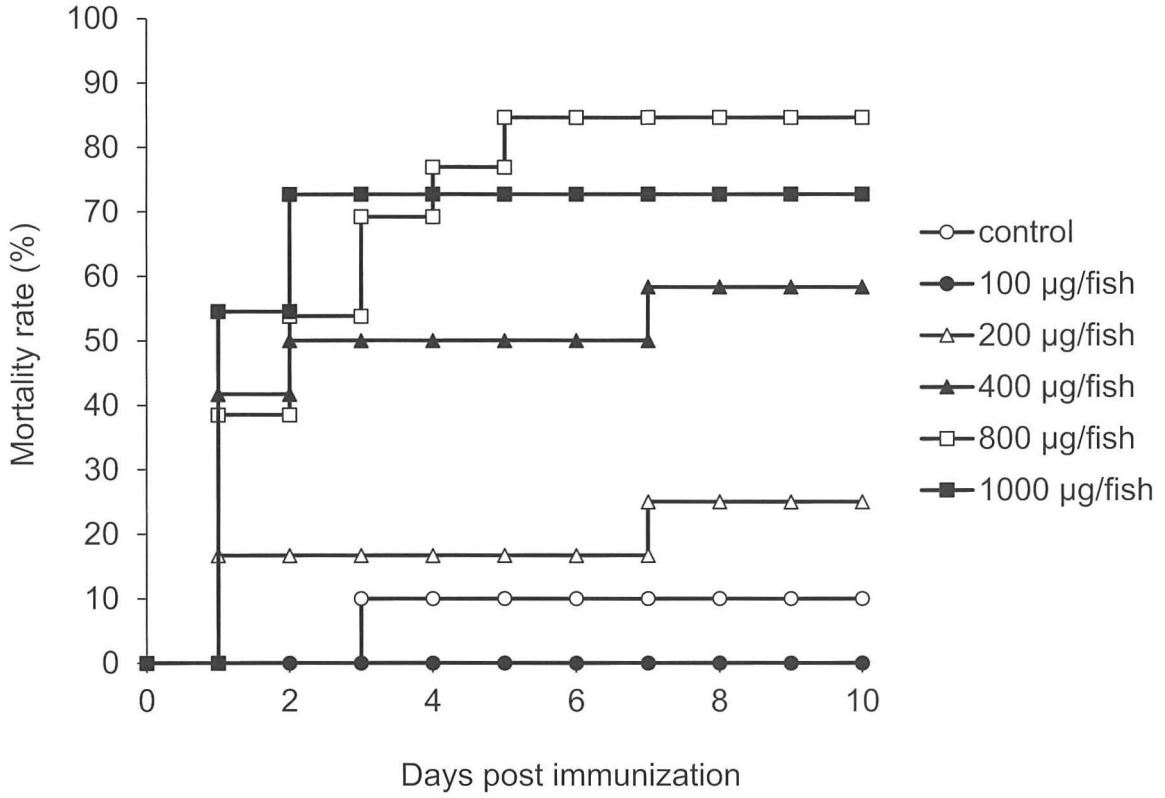

Fig. 1. Motality rate of rainbow trout following injection with PIC. The plot is a group with a mean weight of 12 g. The water temperature was 4.6℃ during the experiments.

were 15.7-fold, 18.8-fold, 23.5-fold, and 27-fold in the groups injected with 100, 200, 400 and 800 μg/fish of PIC, respectively (Fig. 3a). Mx gene expression increased with increases in the dose of PIC. On the other hand, variations in the levels of Mx gene expression for fish reared at 4.6℃ were moderate. The peak of Mx gene expression was observed at 3 dpi. Compared to the control group, Mx gene expression levels were 9-fold, 14.7-fold, 27.3-fold and 19.6-fold with doses of 100 μg/fish, 200 μg/fish, 400 μg/fish and 800 μg/fish of PIC, respectively. The Mx gene expressions at 10 dpi retained more than 30% intensity of each peak value (Fig. 3b). These results indicated that the effect of PIC administration on Mx gene expression levels was affected by the temperature of the rearing water. Lower temperatures caused high Mx gene expression to be maintained for longer periods.

Influence of PIC length on Mx gene expression

The Mx gene expression in six groups of rainbow trout injected with one of six types of PICs are shown Fig. 4. The water temperature for this experiment was 8.7℃. All of the PIC-injected fish showed significant induction of Mx gene expression at 1 dpi and 3 dpi, with a fading out at 7 dpi. These Mx gene expressions were not significantly related to the length of PIC.

Discussion

Salmonids such as rainbow trout and Atlantic salmon are usually cultured in temperate regions which may experience wide temperature variations. Depending on temperature and fish size, the innate immune activator PIC could be toxic for smaller fish (Salinas et al. 2004; Matsui et al. 2012). Growth stage and season must be taken into account. In this study, the effects of body size, water temperature and length of PIC on immunoreactivity and tolerance were examined. From our results, the tolerance level of rainbow trout to PIC was 100 μg/12 g (8.3 mg/kg fish). This result is low when compared with reports that mice, rats and guinea pigs tolerate doses of 20 mg/kg doses

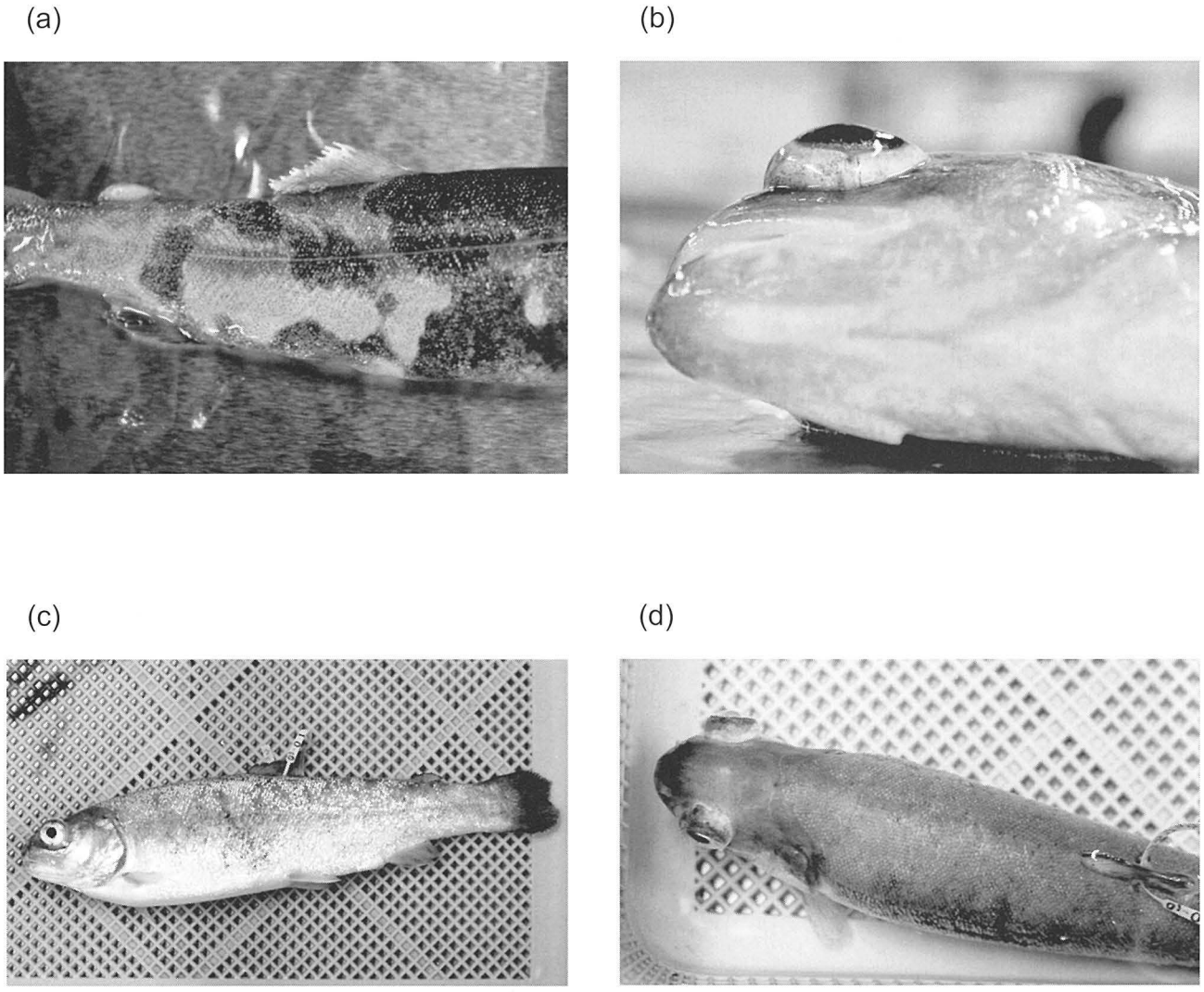

Fig. 2. Rainbow trout after injection with PIC in water at 4.6°C. (a) scale detachability, (b) bulging of eyes, (c) Irritation of body surface, (d) bulging of eyes, and abdominal dropsy.

following acute intraperitoneal administration (Phillips et al. 1971). Unlike mammals, fish are cold blooded and therefore more susceptible to environmental temperature change. It is postulated that tolerance level of rainbow trout to PIC was affected by water temperature as well as doses of PIC. On the effects of temperature, we evaluated gene expression of Mx as a marker of fish immune response to PIC. Our results showed that peaks of Mx gene expression in the spleen were observed at 1 dpi and depended on dose when water temperature was high. Moreover, Mx gene expressions declined rapidly by 3 dpi. On the other hand, when water temperature was low, peaking time varied and declines were less rapid. These results are consistent with reports by Thanasaksiri et al. (2014) that in PIC-injected sevenband grouper, at higher temperatures, highly up-regulated Mx transcripts occurred earlier while prolonged Mx gene expression was triggered at lower. Mx transcripts and proteins are induced by type I IFN in fish (Nygaard et al. 2000; Jensen et al. 2002). Prolonged Mx gene expression in low temperature was associated with long-term IFN induction, and that may have caused the negative pharmacological effects. Observations on the effects of the length of PIC revealed that Mx gene expression was not significantly related to the length of PIC. In mammal cells, dsRNA (or its analog PIC) acts as an inducer for type-I IFN. Toll-like receptors on the endosomal membrane and retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated

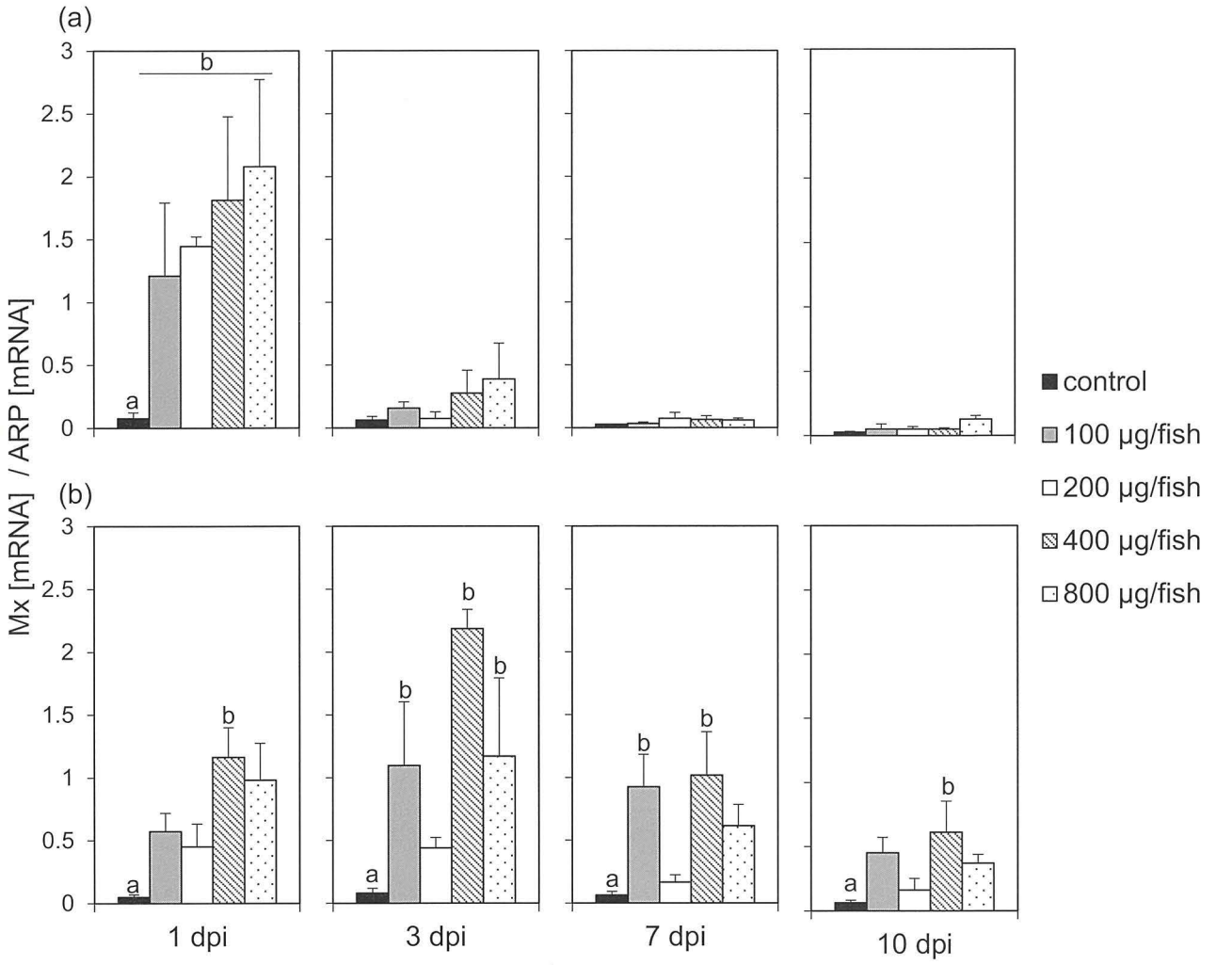

Fig. 3. Changes in Mx gene expression in spleen of PIC-injected rainbow trout followed by rearing at (a) high water temperature (17.4°C, mean body weight of 109 g) or (b) low temperature (4.6°C, mean body weight of 145 g). Bars represent standard deviation. Different letters represent statistical significance by Kruskal-Wallis test ($p < 0.05$).

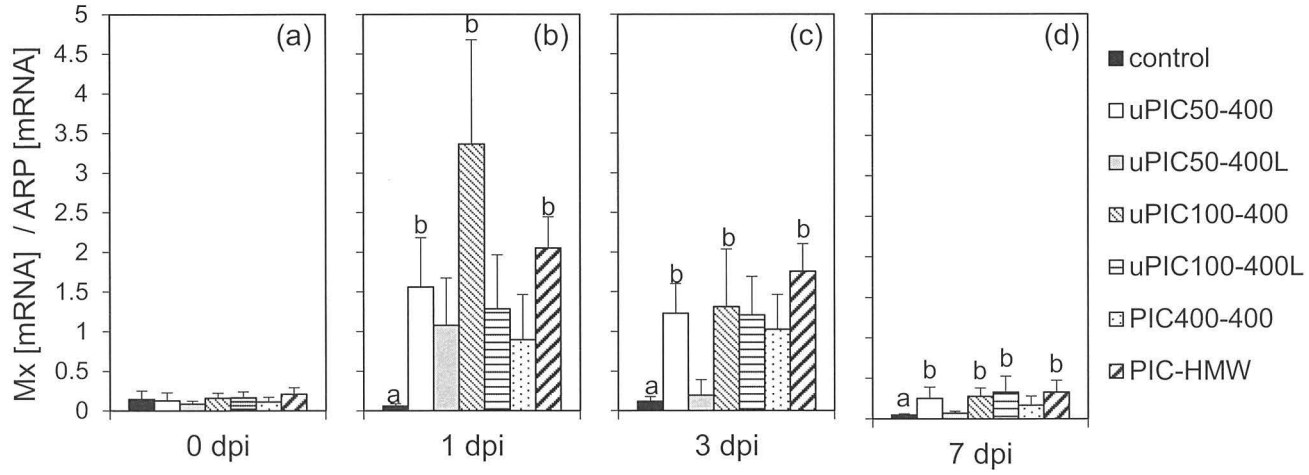

Fig. 4. Changes in Mx gene expression in spleen of rainbow trout immunized with different lengths of PIC. (a) 0 dpi, (b)1 dpi, (c) 3 dpi, (d) 7 dpi of each PIC. Bars represent standard deviation. Different letters represent statistical significance by Kruskal-Wallis test ($p < 0.05$).

gene 5 (MDA5) in the cytoplasm are identified as sensors for dsRNA. RIG-I recognizes dsRNA in addition to 5'-triphosphate end ssRNA, and the length of dsRNA determines the utilization of RIG-I and MDA5 for the recognition (Kato et al. 2008). Length of PIC is also important for inducing immune response (Lampson et al. 1970; Machida et al. 1976). Short PIC induced a strong innate immune response in myeloid cells (Mian et al. 2013). In teleost fish, detailed molecular pathway leading to IFN activation is not clear (Verrier et al. 2011), but our data showed that length of PIC did not affect innate immune response. Considering the results, sensing of dsRNA in teleost fish may not be developed to levels comparable with mammals. Further studies are needed to identify and characterize mediators in the IFN activation cascade in teleost fish.

In summary, PIC induced innate immune response in rainbow trout but doses of 16.7 mg/kg fish were lethal at 4.6℃. Low temperature was related with long-term IFN induction, with possible negative pharmacological effects. Furthermore, length of PIC did not affect innate immune response. These facts should be considered before PIC is used in cold water fish like Salmonids. If PIC is able to activate acquired immunity that has specificity against pathogens and immunological memory, it is exceedingly useful for preventing virus diseases.

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ニジマス（*Oncorhynchus mykiss*）の Polyinosinic-polycytidylic acid（PIC）投与による免疫賦活作用および同薬剤に対する耐性に及ぼす諸要因

西川翔太郎・中野哲郎・笠井久会

PIC はイノシンとシチジンからなる dsRNA であり，強い免疫賦活作用を有する。魚類に対しても免疫賦活剤としての利用が検討されているが，低水温下では魚に対し毒性を示す場合がある。また PIC は鎖長によって免疫賦活作用が異なることがマウスで報告されている。本研究では PIC のニジマスに対する毒性および免疫賦活作用に及ぼす水温ならびに構造について検討した。サイズの大きいニジマス（145 g）は小さいニジマス（12 g）よりも PIC に対する耐性が高かった。また，Mx 遺伝子発現量は高水温下（17℃）では PIC 投与 1 日後に最大値に達し，以後速やかに減少したのに対し，低水温下（4℃）では長期間発現し，死亡魚もみられた。鎖長の異なる 6 種類の PIC を比較したところ，PIC の鎖長と Mx 遺伝子発現量との間に有意な相関性が認められなかったことから，PIC のニジマスに対する免疫賦活作用には PIC の鎖長よりも水温が影響することが示された。