コイの新穴あき病の発症に対する温度と魚体サイズの関係

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Short communication

Susceptibility of Common Carp to the New Ulcer Disease-Causing Atypical *Aeromonas salmonicida* is Temperature-Dependent, but Not Body Size-Dependent

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ABSTRACT—In this study, we investigated the influence of body-size and temperature on the susceptibility of carp *Cyprinus carpio* to the new ulcer disease caused by atypical *Aeromonas salmonicida*. We observed that 4–7 cm long carp, weighing 1.6–8.6 g, exhibited similar susceptibility to the disease. Furthermore, *A. salmonicida* cultured at 25°C demonstrated significantly reduced virulence compared to the bacterium cultured at 20°C. Additionally, a higher disease occurrence rate was noted at a water temperature of 25°C during infection, with faster symptom recovery.

Key words: ulcer disease, susceptibility, temperature, body size, *Aeromonas salmonicida*

Introduction

A new ulcer disease caused by atypical *Aeromonas salmonicida* in the common carp, *Cyprinus carpio*, was first reported in Japan in 1996 (Kaku *et al.*, 1999; Matoyama *et al.*, 1999). It is a distinct infection from the ulcer disease caused by a different strain of atypical *A. salmonicida* in goldfish (Yamamoto, 2017). This new ulcer disease is reported to occur even in small juvenile carps (Kaku *et al.*, 1999); however, this has not been experimentally confirmed. Furthermore, it remains unclear whether susceptibility to this disease changes with host fish growth.

Our research group has been evaluating the immunotoxicity of chemicals with changes in susceptibility to infectious disease as an endpoint. Based on a series of studies, we chose carp as the host and *A. salmonicida* as the pathogen, since carp are a commonly used in both immunological and toxicological research. We have previously demonstrated that exposure to a high concentration of the synthetic steroid hormone dexamethasone significantly increased the mortality rate of *A. salmonicida*-infected carp (Nakayama *et al.*, 2017).

In the current study, as part of the aforementioned immunotoxicity assessment, we investigated potential variations in the disease occurrence rate and disease severity among carp based on factors such as body size, incubation temperature of *A. salmonicida*, and water temperature employed during the infection test.

Materials and Methods

Preparation of Aeromonas salmonicida

The pathogenic bacterium atypical *A. salmonicida* isolated from koi carp (strain T1031) was kindly provided by Dr. Tomonori Somamoto (Kyushu University). The bacterium was cultured on heart infusion (HI)-containing 1% agar plate (Nissui, Tokyo, Japan), and a single bacterial colony was then subcultured in HI broth at 20°C for 96 h. Cultured bacteria were dispensed and stocked at −80°C. Prior to the infection test, the bacterial stock was thawed and spread onto HI agar plates. A single colony was then pre-cultured in 1 mL HI broth at 20 or 25°C for 24 h, followed by main culture in 30 mL HI broth for 72 h. After incubation, the bacterial number was estimated by measuring the OD_{600} and was confirmed by enumerating colonies on HI agar plate after 144-h incubation.

Infection test-1

Carps of three different body-size (mean body weight; small: 1.4 g, medium: 4.4 g, and large: 8.0 g) were purchased from Chemical Evaluation and Research Institute (CERI; Kurume, Japan). To acclimate them to the laboratory environment, each body-size group was reared in a separate 60-L glass tank containing 40 L rearing water. Test fish were kept at 25°C under 14:10-h light:dark photoperiod. They were fed dry pellets (Koi No.2; NOSAN, Yokohama, Japan) at 2% (small and medium) or 3% (large) body weight per day, and half of the rearing water was changed daily.

The infection test was conducted in 30-L glass tanks filled with 20 L rearing water. Carp from each body-size group (14 small, 10 medium, and 7 large fish) were mixed in three different tanks three days prior to the start of the infection test; two tanks were used for the infected groups and one for the uninfected control. The water temperature in each tank was maintained at 19.8–20.9°C by a water bath system. Daily, 40 L of water was replaced in the test tanks using a flow-through system. Fish were fed dry pellets during the whole experimental period with a daily feeding rate equivalent to 1%

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of the total biomass in each tank. For bacterial infection, the test water of each tank was reduced to 3 L, and 3.4×10^6 colony forming unit (CFU)/mL of *A. salmonicida* cultured at 20°C was added to the two infected group tanks. Fish in the uninfected group received 30 mL HI broth. After 1 h, 17 L of water was added to each tank to adjust to 20 L. Mortality and appearance symptom in the fish was observed daily until 14 days post-infection (dpi). Dead fish were removed, and their body length and weight were measured. At 14 dpi, the surviving fish were anesthetized with 0.04% (v/v) phenoxyethanol, and their phenotypic symptoms were observed and standard body length and weight were measured. The disease occurrence rate was calculated by counting the number of individuals that presented typical symptoms such as ulcers, erosion, and redness (Fig. 1A).

Infection test-2

Carps of three different body-sizes (mean body weight; small: 1.7 g, medium: 4.3 g, and large: 8.4 g) were purchased from CERI. One week prior to the start of the infection experiment, each size of carp (14 small, 10 medium, and 7 large fish) was mixed and placed in a 30-L tank containing 20 L rearing water. Four such tanks were prepared: and two of which were set at 20°C and two at 25°C using a water bath system. The water change and feeding regimes were the same as those used in infection test-1.

Fish in tanks for each water temperature received 6.6×10^5 or 2.1×10^6 CFU/mL of *A. salmonicida* cultured at 20 or 25°C, respectively. The experimental infection methods and observations were the same as those used in test-1. Water temperature was maintained at 19.5–20.1 or 24.1–25.1°C. During the latter half of the experimental period, as shown in Fig. 1B and C, we noted individuals lacking redness and bleeding from each wound, displaying new tissue formation without scale regeneration. These were designated as "recovered" individuals, and the percentage of recovery was calculated among those individuals manifesting disease symptoms.

Statistical analyses

All statistical analyses were performed using the R software (ver. 4.2.2; R Foundation for Statistical Computing). Data on body length and weight in the same-sized groups were analyzed for differences between treatments by multiple comparisons using Tukey's test, followed by Levene's test to check the homogeneity of variance across treatments. Differences in onset and mortality rates between body-sizes within a tank in both infection tests and across treatments in infection test-2 were analyzed using Fisher's exact test. Bonferroni corrected *p*-values were used for multiple comparisons.

Results

In infection test-1, no differences were observed in disease occurrence rates among different body-sizes within the same tank (Table 1). The overall disease occurrence rates for all individuals were determined to be 39% for Tank 1 and 27% for Tank 2. In this trial, there was one small-sized fish mortality in Tank 1 (at 14 dpi) and two small-sized fish mortalities in Tank 2 (at 13 and 14 dpi), resulting in a low overall mortality rate. No

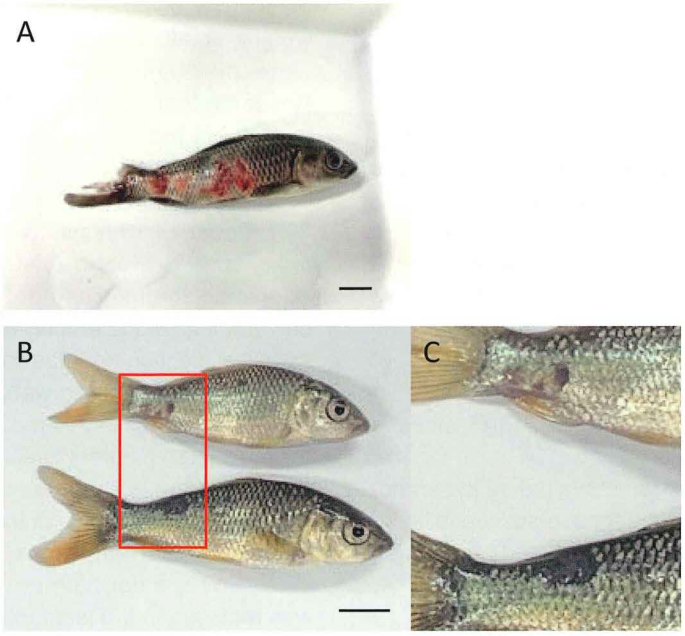

Fig. 1. (A) An individual that died showing typical symptoms of the new ulcer disease. (B) Individuals defined as "recovering individuals" in this study. (C) New tissues were formed, but scales were not regenerated. Scale bars indicate 1.0 cm.

Table 1. Body length, body weight, and diseased fish numbers and rates in different body-size groups of common carp two weeks after *Aeromonas salmonicida* infection

Body-size	Measured item	Control	Infected	
			Tank 1	Tank 2
Small	N	14	14	14
	Body length (cm)	4.2 ± 0.1	4.1 ± 0.2	4.1 ± 0.2
	Body weight (g)	1.7 ± 0.1	1.7 ± 0.2	1.7 ± 0.2
	Diseased	0 (0%)	3 (21%)	6 (43%)
Medium	N	10	10	10
	Body length (cm)	5.7 ± 0.3	5.7 ± 0.2	5.8 ± 0.2
	Body weight (g)	4.6 ± 0.7	4.7 ± 0.6	4.8 ± 0.6
	Diseased	0 (0%)	4 (40%)	1 (10%)
Large	N	7	7	6*
	Body length (cm)	7.0 ± 0.2	7.1 ± 0.3	7.1 ± 0.2
	Body weight (g)	8.6 ± 0.8	8.2 ± 0.7	8.6 ± 1.0
	Diseased	0 (0%)	5 (71%)	1 (17%)
All	N	31	31	30
	Diseased	0 (0%)	12 (39%)	8 (27%)

Body length and weight data are presented as mean ± standard deviation.
*: One large fish from Tank 2 accidentally jumped out, and thus, was excluded from the analysis.

statistically significant differences were detected when comparing the body length and weight among the different tanks for each size category (Table 1).

In infection test-2, we set the carp rearing temperature at either 20 or 25°C, and had corresponding bacterial cultivation temperature. The disease occurrence rate (81%) was significantly higher in carp reared at 25°C infected with *A. salmonicida* cultured at 20°C, compared to other three groups (Table 2). The next highest incidence rate (42%) was observed in the carp reared at 20°C with bacteria cultured at 20°C. In both groups, typical symptoms appeared at 3 dpi. Conversely, the two groups infected with *A. salmonicida* cultured at 25°C showed relatively low disease occurrence rates (16% and 32%). *A. salmonicida* cultured at 20°C caused mortality in two or seven individuals at 20 or 25°C infection temperatures, respectively, whereas the bacteria cultured at 25°C did not induce mortality in carp at all (Table 2). Furthermore, in the group where infection experiments were conducted at 25°C, signs of healing were observed at 14 dpi. These recovered individuals were not observed at around 7 dpi and had a distinctly different appearance from those with mild disease. No differences in disease incidence or mortality were observed based on body-size within the same tank (Table 2).

Discussion

Within the range of growth stages used in this study, there was no observed dependence of susceptibility to *A. salmonicida*-caused infectious diseases on carp body-size. The major Indian carp *Labeo rohita* (Ham.) were infected with *Aeromonas hydrophila* at 1, 2, and 3 wk after hatching, and no differences in mortality were observed, regardless of the timing of infection (Swain *et al.*, 2006). The response to bacterial infections is almost the same from the embryonic to juvenile stages (Castro *et al.*, 2015). Thus, there was probably no difference in the susceptibility to bacterial disease among the growth stages of the carp used in this study.

Table 2. Body length and weight of common carp reared at different temperatures and infected with *Aeromonas salmonicida* incubated at different temperatures

Carp reared at		20°C	20°C	25°C	25°C
A. salmonicida cultured at		20°C	25°C	20°C	25°C
Small	Body length (cm)	4.2 ± 0.2	4.1 ± 0.2	4.2 ± 0.2	4.1 ± 0.1
(N = 14)	Body weight (g)	1.8 ± 0.2^{a,b}	1.6 ± 0.2^{a}	2.0 ± 0.3^{b}	1.6 ± 0.2^{a}
	Diseased	4 (29%)^{a,b}	2 (14%)^{a}	10 (71%)^{b}	5 (36%)^{a,b}
	Mortality	0	0	3 (21%)	0
	Recovery	0	0	4 (40%)	3 (60%)
Medium	Body length (cm)	5.6 ± 0.3	5.6 ± 0.3	5.6 ± 0.3	5.5 ± 0.2
(N = 10)	Body weight (g)	4.3 ± 0.7	4.4 ± 0.4	4.3 ± 1.0	3.8 ± 0.7
	Diseased	6 (60%)^{a,b}	1 (10%)^{a}	8 (80%)^{b}	3 (30%)^{a,b}
	Mortality	1 (10%)	0	2 (20%)	0
	Recovery	0	0	4 (50%)	0
Large	Body length (cm)	6.8 ± 0.4	7.1 ± 0.3	6.9 ± 0.2	6.9 ± 0.3
(N = 7)	Body weight (g)	7.5 ± 1.0	8.0 ± 1.2	7.1 ± 0.8	7.2 ± 0.8
	Diseased	3 (43%)	2 (29%)	7 (100%)	2 (29%)
	Mortality	1 (14%)	0	2 (29%)	0
	Recovery	0	0	2 (29%)	1 (50%)
Total	Diseased	13 (42%)^{a}	5 (16%)^{a}	25 (81%)^{b}	10 (32%)^{a}
(N = 31)	Mortality	2 (6.5%)	0	7 (23%)	0
	Recovery	0	0	10 (40%)	4 (40%)

Numbers with different letters are significantly different across treatment groups (Bonferroni-adjusted p-value = 0.00833).

The incidence rate of ulcer disease was approximately two-times higher at the rearing temperature of 25°C compared to 20°C, regardless of whether the incubation temperature of *A. salmonicida*. Matoyama *et al.* (1999) observed faster mortality occurrence and lesion formation after infection with atypical *A. salmonicida* at 27°C as opposed to 21°C. These temperature-dependent changes in susceptibility to infectious diseases are likely linked to the strong influence of water temperature on bacterial growth rate or fish immune system (Bly and Clem, 1992; Bowden, 2008). The growth rate of *A. salmonicida* has been reported to increase rapidly with temperature (Groberg *et al.*, 1978). The ability of carp to produce antibodies is temperature dependent, and both humoral and cellular immunity are believed to be similarly affected (Rijkers *et al.*, 1980). Considering these data, we assumed that the bacterial growth rate and/or each innate immunity-related activity in the carp would be more pronounced at 25°C than at 20°C. Consequently, the bacterium may have produced more toxins or that these heightened immune activities may have inadvertently resulted in tissue damage, leading to more pronounced symptoms. Meanwhile, it is crucial to consider that incubation at temperatures beyond the optimal range leads to the loss of the A-layer, a recognized virulence factor of *A. salmonicida*, resulting in its attenuation (Ishiguro *et al.*, 1981; Long *et al.*, 2023).

Conversely, the observed healing of symptoms exclusively at 25°C can also be attributed to the temperature-dependent responses. Prior research has indicated that carp skin damage heals faster at 18–21°C, as compared to lower temperatures of 10–13°C (Ang *et al.*, 2021). The temperature-dependent disparities in disease occurrence rate and accelerated symptom recovery observed in this study may be attributed to this factor.

In this study, we have ascertained that the susceptibility of carp to atypical *A. salmonicida* disease remains consistent across various growth stages, at least within the body-weight range of 1.6–8.6 g. Additionally, our findings indicate that for optimal infection test results, *A. salmonicida* should be cultivated at temperatures below 20°C. Furthermore, the infection tests themselves can be conducted within 20–25°C, which is commonly employed in toxicity assessments in carp (20–24°C is recommended, OECD, 2019). Our proposed method for assessing immunotoxicity offers the advantage of using small-sized fish, which minimizes the required experimental space as well as the quantities of rearing water and chemicals involved.

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コイの新穴あき病の発症に対する温度と魚体サイズの関係

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本研究では，非定型 *Aeromonas salmonicida* によって引き起こされる新穴あき病に対するコイの感受性が，コイの体サイズや温度に依存して変化するかを検証した。その結果，体長が 4–7 cm，体重が 1.6–8.6 g の範囲のコイにおける同感染症に対する感受性はほぼ同等であることがわかった。また，25°C で培養した *A. salmonicida* は20°C で培養したものと比べて，顕著に病原性が低下した。さらに，コイの飼育水温は20°C よりも25°C で高い発症率が観察されたが，治癒も早かった。

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