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Effects of daily temperature fluctuation on the survival of carp infected with *Cyprinid herpesvirus 3*

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ABSTRACT

Fluctuating water temperatures can affect fitness in fish when the opportunity to select habitats with appropriate temperature is limited. Despite the importance of the relationships between water temperature and host–pathogen interactions, reports on the susceptibility of fish to infectious viruses under conditions of changing water temperature are limited. Here, we compared the survival rates of common carp (*Cyprinus carpio*) infected with *Cyprinid herpesvirus 3* (CyHV-3) in water in which the temperature varied from 22 °C ± 3 °C and in water with a constant temperature of 22 °C or 25 °C. We also examined changes in concentrations of CyHV-3 DNA and cortisol released from infected fish into ambient water as indicators of CyHV-3 transmission and stress response, respectively. The survival rates of fish infected with CyHV-3 were lower, and concentrations of CyHV-3 DNA and cortisol were higher, in the fluctuating-temperature treatments than in the constant-temperature treatments. Our findings provide direct evidence that carp are highly susceptible to CyHV-3 infection when water temperatures change diurnally. Moreover, such temperature fluctuations can promote transmission of CyHV-3 in the wild. Preserving a variety of aquatic environments including water temperature may help to prevent disease outbreaks and to conserve fish populations.

1. Introduction

The outbreak and rapid spread of emerging infectious diseases are of growing concern for human health, livestock, and biological conservation (Daszak et al., 2000; Jones et al., 2008; Smith et al., 2009). It would not be possible to completely eliminate infectious viruses that have been introduced into natural ecosystems (Smith et al., 2009), and infectious disease outbreaks are complicated by environmental variability (e.g., host–pathogen interactions can vary in relation to environmental conditions such as temperature and moisture).

Water temperature is one of the most important environmental variables influencing poikilothermic organisms and host–pathogen interactions (e.g., Ndong et al., 2007; Sano et al., 2009). Therefore, fish species may be required to select habitats with optimal temperature to maximize their immunity to disease (Perelberg et al., 2008), while avoiding suboptimal habitats (e.g., those that impose thermal stress). However, fish often experience large daily changes in water temperature. For example, in the littoral zone of Iba-naiko, a lagoon connected to Lake Biwa in Japan, the maximum daily change in water temperature was 3.3–4.3 °C (increase) and 2.7–3.2 °C (decrease) between spring and early summer (Takahara et al., 2011). Fluctuating temperatures can cause changes in behavioral and physiological responses (e.g., food intake, metabolism, growth rate, stress responses) in fish (Diana, 1984; Pushkar' et al., 2010; Takahara et al., 2011). Different responses to water temperature among fish species suggest that sensitivity to infectious diseases should also vary. Thus, evaluating the susceptibility of fish to pathogens under fluctuating temperature conditions is important for preventing outbreaks of infectious disease and for conserving fish populations.

Cyprinid herpesvirus 3 (CyHV-3), previously described as koi herpesvirus (KHV) (Hedrick et al., 2000) is a DNA virus recognized as the causal agent of a lethal disease of farmed and wild common carp (*Cyprinus carpio* Linnaeus, 1758). In Lake Biwa, Japan, approximately 100,000 common carp died between April and July 2004 following

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introduction of CyHV-3 (Matsui et al., 2008). Although additional major outbreaks have not been observed, some fish mortality has been attributed to CyHV-3 since 2004 in Japan (e.g., Minamoto et al., 2009b). Moreover, most natural environments across Japan are likely to have been contaminated by CyHV-3 (Honjo et al., 2010; Minamoto et al., 2012), indicating the potential for CyHV-3 outbreaks to occur in other watersheds. CyHV-3 infection in carp has been documented to occur in spring and autumn at water temperatures ranging from 18 °C to 26 °C (Gilad et al., 2003; Perelberg et al., 2003), and carp are highly susceptible to CyHV-3 at higher water temperatures (23 °C and 28 °C) than at lower temperatures (16 °C) (Yuasa et al., 2008). Despite the importance of the relationship between water temperature and mortality following CyHV-3 infection, the susceptibility of carp to CyHV-3 in response to changes in water temperature has not been well examined.

Here, we performed viral challenge experiments to evaluate the effects of fluctuating and constant water temperature on survival rates of *C. carpio* in the presence of CyHV-3. We first evaluated whether daily changes in temperature influenced survival rate after CyHV-3 exposure. Next, we examined changes in the ambient quantity of CyHV-3 DNA released by carp after CyHV-3 exposure, because CyHV-3 infection can be transmitted horizontally in water (Hedrick et al., 2000). We also examined cortisol concentrations released by the carp after CyHV-3 exposure, which provides an effective, non-invasive method for estimating stress levels in fish (Ruane and Komen, 2003; Scott and Ellis, 2007; Takahara et al., 2014). Finally, we discuss a potential approach for preventing CyHV-3 infection considering changes in water temperature.

2. Materials and methods

2.1. Experimental fish and maintenance

Juvenile common carp *C. carpio* were hatched in June 2006 and reared outdoors in an artificial pond for 4 years at Tamaki Station at the National Research Institute of Aquaculture, Fisheries Research Agency, Mie Prefecture, and transported to a laboratory at the Research Institute for Humanity and Nature (RIHN), Kyoto Prefecture. The carp were kept in 66-L plastic tanks, in which the recirculating water was continuously filtered and changed by halves every two weeks, for 2 months (approximately 25 individuals per tank). Carp were fed a commercial diet (Saki-Hikari®, Kyorin, Hyogo, Japan) three times each week and were maintained at 19 °C ± 1 °C under a 12:12 light–dark cycle. Fish health was checked by observing feeding response when food was given. Individuals with low appetite were moved to the other tanks in order to cure them and were never used in the below experiments.

2.2. Preparation of CyHV-3 solution

The virus was propagated using a common carp brain (CCB) cell line, originally developed from common carp brain (Neukirch et al., 1999) which was kindly provided by Professor Moshe Kotler of The Hebrew University, Israel. The CCB cells were cultured in medium containing 75% Dulbecco's modified Eagle's medium and 25% Leibovitz (L-15) medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics. CyHV-3 strain NRIA0301, isolated from infected carp in Japan in 2003, was stored at −80 °C. The CyHV-3 was propagated at 25 °C using CCB cells in L-15 medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, and antibiotics. After 10 days of incubation, complete cytopathic effects were observed and culture debris was removed by centrifugation a 2000 ×*g* for 5 min. CyHV-3 was collected in aliquoted supernatant at passage 3 and was stored at −80 °C until the experiment. Infectivity of the CyHV-3 stock, determined using CCB cells according to Reed and Muench (1938), was $10^{4.5}$ tissue culture infective dose ($TCID_{50}$) mL^{-1}. The stock was diluted 10 times with water and was used as the virus solution in the challenge experiments.

2.3. Viral challenge experiment

The aquarium experiment was performed according to a previously described method (Takahara et al., 2011) with slight modifications as follows. Four plastic beakers (diameter, 15 cm; height, 22 cm) in which water temperature was controlled were prepared, and one fish was introduced to each beaker. Before the start of each experiment, the beakers (i.e., experimental aquariums) were cleaned by soaking in bleach solution for half day and then were rinsed thoroughly with water. The beakers were placed on a shelf covered with an opaque vinyl curtain to minimize stress caused by external stimuli. Water was delivered (0.2 L h^{-1}) to each beaker through silicon tubes using a peristaltic pump placed outside the curtain. The beakers were allowed to overflow to maintain a volume of 1.4 L.

To evaluate the effects of changes in water temperatures on carp after CyHV-3 exposure, we established two systems, one with fluctuating temperature and one with constant temperature ($n = 4$ beakers each). The experiments were repeated two times with three different temperature conditions, each within the optimal range for infectivity of CyHV-3 (Gilad et al., 2003; Perelberg et al., 2003). In the first experiment, the constant temperature was 22 °C and the fluctuating conditions were 22 °C ± 3 °C, which are average daily temperature ranges frequently observed in natural carp habitats (Takahara et al., 2011). To compare the effects of higher temperatures, the second experiment used a constant temperature of 25 °C and fluctuating conditions of 22 °C ± 3 °C. The daily temperature changes were controlled as described in Takahara et al. (2011) with slight modifications as follows. Daylight period: 0900–1000 h, 22 °C; 1000–1500 h, 22–25 °C; 1500–1600 h, 25 °C; 1600–2100 h, 25–22 °C. Nighttime: 2100–2200 h, 22 °C; 2200–0300 h, 22–19 °C; 0300–0400 h, 19 °C; 0400–0900 h, 19–22 °C.

We divided 16 *C. carpio* into four treatment groups ($n = 4$ carp per group). Temperature conditions and average carp length ± S.D. were as follows: 22 °C, 10.8 ± 0.7 cm; 25 °C, 11.8 ± 1.6 cm; 22 °C ± 3 °C (first experiment), 10.0 ± 0.8 cm; 22 °C ± 3 °C (second experiment), 11.3 ± 1.6 cm. The fish were acclimated to the experimental conditions (12:12 light–dark cycle; lights on at 0900 h) for 5–10 days to allow for partial recovery from handling. During the acclimation period, temperature for the 22 °C and 22 °C ± 3 °C treatments was maintained at 22 °C, and temperature for the 25 °C treatment was maintained at 25 °C.

On experimental day 1, we stopped the peristaltic pump at 0830 h and added 1 mL virus stock solution (final concentration = 2.26 $TCID_{50}$ mL^{-1}) to each beaker using a micropipette to minimize disturbance. After 0.5 h of exposure to the virus, the pump was restarted. Carp were fed at 1500 h every other day; debris was automatically removed five times each day by suction using the pump. Carp were observed for mortality for 17 days post-exposure (dpe). Dead carp were removed from the beakers and immediately frozen at −30 °C. In the 22 °C experiment, surviving carp were observed for 27 dpe to confirm whether they acquired post-exposure immunity to CyHV-3.

To assess the concentrations of CyHV-3 DNA in ambient water during the experiment, we collected water samples from the bottom of each beaker through silicon tubes (flow rate; 0.2 L h^{-1}) into 50-mL plastic tubes on ice at 0950–1000 and 1350–1400 h, using a peristaltic pump. To examine cortisol levels, we collected water samples from each beaker into 200-mL plastic bottles on ice at 1000–1100 and 1400–1500 h. The collection of water samples was performed through filter paper (Whatman® No. 1; GE Healthcare, Tokyo, Japan) to remove suspended solids. In the first experiment, carp died at 10 dpe so water samples were collected until 9 dpe (see, Results). Samples were stored at −30 °C until analysis.

Carp surviving at the end of the experiment in the 22 °C group were anesthetized; abdominal dissection was performed and blood was extracted from the heart for antibody analysis. Blood and residual samples were frozen at −30 °C.