Thermal Stress Effects on Gene Expression and Phagocytosis in the Common Carp (Cyprinus Carpio): a Better Understanding of the Summer 2001 St. Lawrence River Fish Kill

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Abstract: In summer 2001, the St. Lawrence River (Québec, Canada) experienced perhaps the largest massive fish kill of its history, with more than 25 000 carp (Cyprinus carpio) found dead. This experiment therefore investigated the effects of heat stress on the gene transcription level of heat shock protein 70, cytochrome c oxidase and mitochondrial superoxide dismutase expression as well as on phagocytosis of kidney cell suspensions and animal behaviour. Our study suggests that in summer 2001, elevated water temperatures could have contributed to the fish kill through immunosuppression during an already stressful spawning period.

Keywords: Fish kill, Gene expression, Phagocytosis, St. lawrence river, Thermal stress.

1. INTRODUCTION

Water temperature is known to be a major factor affecting habitat suitability of fish [1-4]. Alterations in thermal regime of a river can have consequences on all aspects of fish life including reproduction, survival, feeding, growth, habitat selection, migration, intra- and inter-specific competition [1, 2, 5-9]. By affecting so strongly habitat suitability of fish, water temperature has the potential to be a direct or an indirect factor provoking important massive fish kill events.

In summer 2001, the St. Lawrence River experienced a massive fish kill event [8]. According to the literature, this event may have been the most important fish kill reported in the Great Lakes and St. Lawrence River history. Although the exact number of dead fish remains unknown, more than 25 000 common carp (Cyprinus carpio) were found dead, as well as some specimens of Catostomidae, Anguillidae, Acipluseraidae, Ictaluridae, Esocidae, Percidae, and Ctenarchidae. The mortality occurred in the St. Lawrence River between Montréal and Québec City (Fig. 1), from June 28\textsuperscript{th} until July 13\textsuperscript{th}. It has been reported that the ultimate cause of mortality was infections by Aeromonas hydrophila and Flavobacterium sp. [10]. Those bacteria are commonly found in freshwater ecosystems and are not harmful to healthy fish.

Exceptionally high water temperatures were measured in the period during which carp were spawning and were therefore already affected by energy expenses for reproduction. Water temperatures in the river reached 34˚C in some points [8]. Frequency analyses made on water temperature series showed that daily maximum water temperature around Montréal and Québec City was abnormally high (return period, i.e. the probability that the event will be equalled or exceeded, of 47 years) relative to other years [12]. Air temperatures were also abnormally elevated around Montréal, with a return period of 22 years. During the same period, the St. Lawrence River also experienced extremely low water levels, for which return periods in the Montréal area were around 30 years; with a maximum of 67 years in the Québec City area.

The main objective of this study was to test experimentally the hypothesis that elevated temperatures such as those measured during summer 2001 in the St. Lawrence River led to immunosupression triggering massive mortality of indigenous carp. We also examined whether heat stress affected the transcription level of genes encoding for proteins involved in protecting proteins against heat denaturation (heat shock protein 70, hsp70), in the fight against oxidative stress (mitochondrial superoxide dismutase, sod2) and in aerobic capacities (cytochrome c oxidase subunit 1, cco1). Fish behaviour was also monitored to investigate if the high temperatures used were stressful for the common carp.

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2. MATERIALS AND METHODS

2.1. Fish Sampling and Laboratory Exposure to Heat Stress

There are no official records of fish size, gender or stage of sexual maturity for the fish kill of 2001. For this experiment, carp were captured in June with gill nets in two different bays of the Lake St. Pierre archipelago (see Fig. (1) for the location of the lake) where they occur prior to the spawning period. Once deployed, nets were visited continuously to avoid wounding the fish. After capture, fish were kept in an oxygenated transportation container until the end of the day. An effort was made to capture female fish that weighed more than 11 Kg (around the same weight as those observed dead in 2001). Fish were transported by van using an oxygenated transportation container from Lake St. Pierre to Québec City to be kept in a controlled environment at the LARSA Laval University facilities, where all physical-chemical parameters could be monitored and adjusted as the experiment required. Photoperiod reproduced the natural cycle at the site of fish capture and the tanks were half-covered with a transparent lid, and half with an opaque lid to limit animal stress. Fish were kept into two separate 3000 L circular tanks. In the first one (Control Unit, CU), fish (n=5) were kept at the same temperature as when they were fished (18°C). In the second tank (Heat Treatment Unit, HU) (n=4), water temperature was gradually raised from 18°C to 34.8°C at the rate of one degree per day. Oxygen was kept at saturation and expected to decrease in concentration as water temperature increased. Before the experiment, a five-day acclimation period was necessary to let fish adapt to their new environment. There were several attempts to feed the fish during the acclimation period but since they were almost ready to spawn, there were not eating and the feeding was stopped. Once their behaviour was considered to have returned to normal, the experiment began for 17 days during which fish behaviour was observed daily. The maximal temperature was set to 34.8°C. At this temperature fish began to show different behavioural patterns (see Section 3.4).

At the end of the exposures, fish were sacrificed by a blow to the head. Gills were dissected and stored at -80°C for gene transcription analysis. Head kidneys were removed under sterile conditions and homogenised with stainless steel mesh in tissue culture medium RPMI 1640 (Sigma, On, Canada) supplemented with heparin (10 U/ml) (CDMV, Qc, Canada), HEPES (10mM) (Sigma, On, Canada), penicillin (100 U/ml)/Streptomycin (100 mg/ml) (Sigma, On, Canada), 10% heat-inactivated Foetal Bovine Serum (FBS) (Sigma, On, Canada), at pH 7.2. The cells were isolated by centrifugation on Lympholyte-M (density= 1.085) (Cedarlane, On, Canada) at 600g for 20 min at room temperature. Cells were collected from the Lympholyte-M interface and washed two times in PBS (Sigma, On, Canada) at 600g for 20 min at room temperature. Cells were collected from the Lympholyte-M interface and washed two times in PBS (Sigma, On, Canada) and resuspended in RPMI containing 10% FBS, 100U/ml penicillin, 100 mg/ml streptomycin and 10 mM HEPES. Viability of macrophages was assessed using trypan blue dye exclusion under a microscope with a hemacytometer.

2.2. Quantitative rt-PCR

For each gene, specific primers were determined (Table 1) using the Primer Express® software (Applied Biosystems). Total RNA was extracted from 60 mg of gills using the PureLink™ Micro-to-Midi™ Total RNA Purification System (Invitrogen). During this step, samples were submitted to DNAse I treatment, according to manufacturer’s instructions. For each sample, RNA quality was evaluated by elec-
pellets were then resuspended in 0.5 ml of 0.5% formaldehyde (Sigma, On, Canada) and 0.2% sodium azide (Sigma, On, Canada) diluted in BDFacsflow™ (Becton Dickinson, CA, USA). Cells were analysed by flow cytometry and 10 000 events were recorded. Analyses were done using two biomarkers defined as the percentage of phagocytes (macrophages containing one bead or more: M1) and then as the phagocytosis efficiency (macrophages containing three beads or more: M2).

2.4 Behavioural Observations

Fish holding tanks were located in an isolated room which was only accessed for behavioural observations, carried out in thirty-minute periods at three different times of the day (7h00, 12h45 and 20h00), and for five minutes of daily maintenance. Fish behaviour (swimming, air breathing and resting time) was observed and recorded every day of the experiment, as well as during acclimation.

2.5. Statistical Analyses

Statistical analyses were made using Kruskal-Wallis non-parametric analysis of variance, which is suitable for low sample size as in this study. Statistical significance was set at 0.05. Analyses were performed with Statistica V6.0.

3. RESULTS AND DISCUSSION

3.1. Capture and Husbandry of Large Common Carp

This study was intrinsically highly challenging to carry out, given the experimental context. In order to investigate the causes of the massive fish die off in the summer of 2001 during a heat spell in the St. Lawrence River, we attempted to reproduce in the laboratory the field situation that occurred in situ. To this end, we had to capture, transport and maintain in the laboratory very large fish. Largers fish are much more difficult to capture and handle without injury and stress than smaller fish and even the large holding tanks used represented a confined and stressful environment for these wild fish. Under such challenging experimental conditions added to the already stressful natural conditions faced by these fish during breeding, six attempts were required to control the outbreak of fungal and bacterial infections and successfully maintain these large fish for long enough to complete the heat exposures. The resulting low sample size in this study (n=5 or 4 for CU and HU, respectively) somewhat

<table>
<thead>
<tr>
<th>Table 1. Sequences of Specific Primer Pairs used in Quantitative Real-time PCR Analyses</th>
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<tbody>
<tr>
<td><strong>Gene</strong></td>
</tr>
<tr>
<td>β-actin</td>
</tr>
<tr>
<td>cco-1</td>
</tr>
<tr>
<td>hsp-70</td>
</tr>
<tr>
<td>sod-2</td>
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</tbody>
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^ forward primer, ^ reverse primer

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Specific Primers (5’–3’)</th>
</tr>
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<tbody>
<tr>
<td>β-actin</td>
<td>Internal standard</td>
<td>^GGGTATGGAGTCCTTGCGGT^</td>
</tr>
<tr>
<td>cco-1</td>
<td>Mitochondrial respiratory chain</td>
<td>^CAGCCAGAACATACAGTAG^</td>
</tr>
<tr>
<td>hsp-70</td>
<td>Protein folding and protection</td>
<td>^TCATGGGAGACACATGGA^</td>
</tr>
<tr>
<td>sod-2</td>
<td>Detoxification/oxidative stress</td>
<td>^TTATCGAGTTCCACACACG^</td>
</tr>
<tr>
<td>^ forward primer, ^ reverse primer</td>
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limits statistical analyses and interpretation, but was unavoidable under these exceptional experimental circumstances, which make this study unique.

3.2. Gene Transcription Levels

There was no difference in gene transcription level between the CU and the HU group for either hsp70, cco1 or mitochondrial sod2. This result is quite surprisingly since previous experimental investigations reported an up-regulation of HSP70 in gills of the goby (Gillichthys mirabilis) or the common carp in response to heat stress at both the genetic and protein levels (temperature range: 18 to 32°C for goby and 20 to 28°C for carp; [14, 15]). Heat shock proteins (HSPs) are molecular chaperone proteins that play a pivotal role in maintaining protein homeostasis during cellular exposure to proteotoxic stressors such as heat to prevent protein denaturation [15]. When protein homeostasis fails due to excessive stress, HSPs interact with stress denatured proteins, preventing their aggregation and/or degradation [16]. Thus, their expression levels are known to be up-regulated by heat stress [14, 17]. Examination of data distribution (Fig. 2A) shows that the lack of statistical significance could be due to a low sample size. There was little overlap of values for the transcription level of hsp70 between CU and HU fish, with HU fish generally expressing higher transcription levels (Fig. 2A). However, one individual in the HU group exhibited a very low transcription level of hsp70. While the three fish in the HU group with high values of hsp70 transcription level displayed a normal up-regulation, one individual, in contrast, expressed lower transcription level than all CU fish, suggesting that this fish had exceeded its thermal tolerance. Thus, in addition to low sample size, an alternative hypothesis could be that animals can no longer adapt effectively to heat stress at the highest temperature used (34.8°C). In contrast to hsp70, there was a negative trend in the transcription levels of genes encoding for enzymes involved in aerobic metabolism (cco1) and defense against oxidative stress (sod1). The transcription level of the two genes appears to be lower in the HU group compared to values in the CU group, as illustrated by data distribution (Fig. 2B and C). This trend of lower values in HU fish compared to the CU fish suggests a decrease in aerobic metabolism of heat-stressed fish. Interestingly, such a decrease in both cco-1 and sod2 transcription levels was first reported in gills of eels exposed to hypoxia during a 14 day period [18, 19]. Indeed, even though in our experiment we maintained oxygen levels to saturation, the increase in temperature decreased oxygen concentration from 9.4 to 6.9 mg/L. Thus, in the present study, the response of both cco-1 and sod2 in gills could be also associated to heat-induced hypoxia [18], suggesting that heat-stressed carp suffer from impaired aerobic metabolism. This
appears more consistent with the behaviour of fish (see section 3.4).

### 3.3. Phagocytosis

Phagocytic rate was significantly lower in fish submitted to high water temperature in comparison to those kept at the control temperature (Fig. 3). For cells from pronephros cellular suspensions that phagocytized one or more beads (p<0.05) and three or more beads (p<0.05), phagocytic activity was significantly lower (39.7%) for fish submitted to elevated water temperature. This data brings direct support to the hypothesis that immunosuppression was a main cause of mortality in common carp exposed to very high water temperatures during summer 2001 in the St. Lawrence River [12].

### 3.4. Fish Behaviour

At their arrival, fish demonstrated a high level of stress, as indicated by repeated hitting of the tank walls. After five days, they appeared to have acclimated to laboratory conditions and began to swim normally. Their general behaviour also appeared similar to that observed in their natural environment, reducing swimming in the afternoon and hiding in the shaded part of the tank (about 25 minutes hiding and 5 minutes swimming) and increasing their swimming activity around dawn and dusk (swimming almost continuously during the 30 minutes observation time) (Table 2). At the end of the acclimation period, there was no noticeable difference in behaviour between carp in the two tanks. In contrast, when water temperature in the heat treatment tank reached 33°C, carp spent more time near the surface compared to fish in the control unit (about 15 minutes versus 5 minutes over a 30 minutes observation period of swimming activity in HU and CU groups, respectively), although other behavioural end-
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The authors are indebted to Paul Messier, Jenny Guillemette, Geneviève Richard and Marc Létourneau for their hard work and skill for carp capture, as well as Philippe Brodeur, Nicolas Auclair and Remi Bacon for their field advice. Authors also want to give a special thanks to Marlène Fortier from Institute Armand Frappier (Michel Fournier’s lab) for technical help and phagocytosis analyses and to Jean-Christophe Therrien and Serge Higgins from Université Laval for their expert advice and assistance in the husbandry of the carp.

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