Vol. 22: 233–236, 1995 | DISEASES OF AQUATIC ORGANISMS
Dis aquat Org | Published July 13

NOTE

Effect of water temperature on infections with the microsporidian *Enterocytozoon salmonis* in chinook salmon

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ABSTRACT: The effect of water temperature on the progress of infections associated with *Enterocytozoon salmonis* Chilmonczyk, Cox, Hedrick 1991 was examined in chinook salmon *Oncorhynchus tshawytscha* after intraperitoneal injections of mononuclear leukocytes infected with the microsporidian parasite. Experimentally infected and control fish were held at water temperatures of 9, 12, 15, 18 and 21°C for 12 wk and then one half of the exposed and control groups of fish at 9 and 12°C were shifted to 15°C and held for an additional 8 wk. Among fish held at constant water temperatures, severe infections occurred among exposed fish at 15 and 18°C resulting in 90.0% cumulative mortality in both groups. Disease and significant mortality was also observed at 21°C (47.5%). The parasite and signs of the disease slowly developed over time at 12°C and the cumulative mortality reached 73.7% between 13 and 20 wk. Although the development of the microsporidian was not arrested at a water temperature of 9°C, infections in chinook salmon were not severe and cumulative mortalities were low (10.0%). However, parallel groups of exposed chinook salmon at 9°C which were shifted to 15°C showed a cumulative mortality of 60.0% by 8 wk after transfer to the higher water temperature. Shifting the exposed fish from 12 to 15°C did not increase the mortality rate from that of fish kept constantly at 12°C. The control fish (not exposed to *E. salmonis*) in all temperature groups did not show signs of the disease nor mortality throughout the study.

KEY WORDS: *Enterocytozoon salmonis* · Water temperature Chinook salmon · Microsporea

Enterocytozoon salmonis Chilmonczyk, Cox, Hedrick 1991 is an intranuclear microsporidian associated with a severe anemic condition in salmonid fish, particularly, chinook salmon *Oncorhynchus tshawytscha* (Hedrick et al. 1990, 1991, Baxa-Antonio et al. 1992). Spontaneous infections with the microsporidian have also been reported in steelhead trout *O. mykiss* (MacConnell et al. 1991) and among cultured populations of golden trout *O. aquabonita* and brook trout *Salvelinus fontinalis* (R. Hedrick unpubl. obs.).

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The progress of infection with the microsporidian has been described in chinook and kokanee salmon *Oncorhynchus nerka* (Hedrick et al. 1990, 1991). Earlier studies documented the occurrence of similar infections in chinook salmon raised in sea water or fresh water (Elston et al. 1987, Morrison et al. 1990). We have demonstrated transmission of the parasite and the progress of infections in chinook salmon held in fresh and sea water (Antonio & Hedrick unpubl.).

Enterocytozoon salmonis has been successfully propagated *in vitro* using a newly developed culture medium (Wongtavatchai et al. 1994). Using parasites from *in vitro* cultures of infected leucocytes as an inoculum, the effect of infections on chinook salmon at 5 water temperatures was examined.

Materials and methods. Juvenile chinook salmon (mean wt 20.0 g) were acclimated for 14 d at water temperatures of 9, 12, 15, 18 and 21°C in 132 l aquaria receiving flow through fish-pathogen-free well water. Fish at each temperature were divided equally into 3 groups of 20. Fish were anesthetized with 50 ppm tricaine methane sulfonate (Argent) and then 2 groups were inoculated intraperitoneally with *Enterocytozoon salmonis* (isolate CA-1)-infected leukocyte suspensions from *in vitro* cultures at approximately 0.2 ml fish^{-1}. One group was injected in the same manner but only with an equal volume of culture medium.

Fish which succumbed to infection were examined for signs of the disease and presence of the parasite. The few fish remaining by 12 wk post-injection (PI) at 15, 18 and 21°C were sacrificed and examined for the presence of the microsporidian. Also, at 12 wk one half of the exposed and control fish at 9 and 12°C were transferred to 15°C to evaluate the effects of higher water temperature on mortality for an additional 8 wk. The remaining groups at 9 and 12°C were not shifted and were also evaluated for parasite-specific mortality until termination of the study at 20 wk PI.

Parasites were detected by imprints made from the posterior kidney that were air dried, fixed with methanol, stained with May-Grunwald Giemsa (Sigma) and then examined with a light microscope. External pathology of infected salmon included occasional uni or bilateral exopthalmos or extreme gill pallor. Internal signs were moderate to severe swelling of the kidney, spleen and posterior intestine. Occasionally, ascites was observed in the peritoneal cavity of *Enterocytozoon salmonis* infected chinook salmon. Detection of typical signs combined with presence of the microsporidian within leukocyte nuclei in stained imprints were used as criteria for confirmations of parasite-specific mortality due to *E. salmonis*.

Differences in mortality between groups at 12 wk PI were evaluated by the chi-squared test (Sokal & Rohlf 1969). Mortality occurring at 15, 18, and 21°C after 12 wk were not included for statistical analysis because the few fish remaining in these groups were sacrificed.

The procedure for the culture of infected leucocytes is described in detail by Wongtavatchai et al. (1994). Briefly, the spleen and kidney of chinook salmon experimentally infected with *Enterocytozoon salmonis* were aseptically removed and the mononuclear cells separated by Ficoll Paque (Pharmacia) density gradient centrifugation. These mononuclear cells, in which *E. salmonis* develops, were propagated in a medium (SL-1) supplemented with human recombinant interleukin-2 (HrIL-2) and polyclonal mitogens. After 10 d in culture at 20°C, the leukocyte suspension contained 1×10^5 cells ml^{-1}. Chinook salmon were then injected with 0.2 ml of the cell suspension per 20 g body weight of fish. An examination of these cells in the suspension used for inoculation revealed a 25% level of infection (Wongtavatchai et al. 1994). Fish in control groups received an intraperitoneal injection of an equal volume of sterile culture medium.

Results. The effect of water temperature on mortality of chinook salmon following experimental infections with *Enterocytozoon salmonis* is shown in Table 1. There was no mortality among the control groups at any temperature during the study. There were significantly different effects of water temperature on the mortality among experimentally infected groups as analyzed at 12 wk PI. Both the 15 and 18°C groups were significantly different from the 9 and 12°C ($p < 0.0001$) and the 21°C ($p < 0.01$) groups. In comparisons of mortality between 13 and 20 wk there were no differences between the 9°C shifted to 15°C, the 12°C shifted to 15°C or the fish kept continuously at 12°C. A statistical comparison of the mortality data was not possible at 20 wk PI among 15°C, 18°C, and 21°C groups because the few fish that remained at 12 wk PI had been previously sacrificed.

By 12 wk PI, fish from both the 15 and 18°C exposed groups suffered high cumulative mortalities (90.0%). Mortalities occurred between 42 and 81 d at 15°C and between 33 and 60 d at 18°C. Moribund and dead fish in these groups showed severe exopthalmos and extreme gill pallor. Internal signs included severe swelling of the kidney, spleen and posterior intestine and ascites was present in the peritoneal cavity. Of the fish remaining at 12 wk that were sacrificed and exam-

Table 1. *Oncorhynchus tshawytscha*. Effect of water temperature on mortality of chinook salmon following intraperitoneal injections of *Enterocytozoon salmonis* (isolate CA-1)-infected mononuclear leukocytes (5×10^3 cells/fish). Fish were held at the same temperature for 12 wk and then half of the groups at 9 and 12°C were transferred to 15°C and examined for an additional 8 wk. Fish at 15, 18 and 21°C were terminated at 12 wk post infection

	Temperature (°C)	Replicate	No. dead fish/no. total 4 to 12 wk	13 to 20 wk	Mean % mortality[a] 4 to 12 wk	13 to 20 wk
Same temperature	9	1	0/20	2/10	0.0a*	10.0a
		2	0/20	0/10		
	12	1	0/20	7/10	5.0a	73.8b
		2	2/20	7/9		
	15	1	16/20	–	90.0b	–
		2	20/20	–		
	18	1	18/20	–	90.0b	–
		2	18/20	–		
	21	1	10/20	–	47.5c	–
		2	9/20	–		
Shifted temperature	9 to 15	1		5/10		60.0b
		2		7/10		
	12 to 15	1		8/10		67.8b
		2		5/9		

*Statistical differences between treatments within each time period are indicated by a different letter ($p < 0.01$)

ined for presence of the parasite 67 and 25% of the fish at 15 and 18°C, respectively, were infected with the parasite. Fish in the 21°C group that were inoculated with the parasite suffered a 47.5% mortality by 12 wk and of the fish sacrificed at this time only 5% were found to be infected by examining stained kidney imprints.

Exposed fish held continuously at 9°C did not succumb to infection until 110 d PI and cumulative mortality (10.0%) at 20 wk PI was low. External and internal pathology of infected fish were not as severe as those found in infected fish from the higher temperature groups (15, 18, 21°C). Affected fish showed mild gill pallor and slightly swollen kidneys and spleens. The replicate groups at 9°C which were transferred to 15°C at 12 wk PI, however, showed high cumulative mortalities (60.0%) which were significantly different ($p < 0.0001$) from the mortality in the fish held continuously at 9°C (10.0%).

Although cumulative mortalities reached 73.7% between 13 and 20 wk among fish held constantly at 12°C, the infections developed more slowly (duration of mortality: 81 to 132 d PI) when compared to infected fish at 15 and 18°C. Shifting a part of the 12°C group of exposed fish to 15°C during the course of infection (Table 1) resulted in a similar level of mortality (67.8%) when compared to those held continuously at 12°C (73.7%).

Discussion. The optimum water temperature for the progress of infections with *Enterocytozoon salmonis* in chinook salmon occurred at 15 and 18°C. Mortality was delayed at 12°C, but with time infections became severe resulting in a cumulative mortality of 73.7% by 20 wk PI. Moderate to severe infections and significant mortality occurred among exposed chinook salmon at 21°C between 4 and 12 wk. At the lowest water temperature tested (9°C), mortality due to *E. salmonis* was delayed, infections were mild, and only low grade mortality occurred.

Earlier studies in our laboratory have shown that experimental transmission of *Enterocytozoon salmonis* to chinook salmon by injection, feeding and cohabitation results in severe infections and mortality at water temperatures of 15 to 18°C (Hedrick et al. 1991, Baxa-Antonio et al. 1992). In those studies, chinook salmon began dying at 53 d following intraperitoneal injection of cell suspensions from the kidney of infected fish (Hedrick et al. 1991). Mortality occurred among chinook salmon from 68 to 118 d after feeding infected tissues and at 120 d after cohabitation of noninfected fish and experimentally infected fish (Baxa-Antonio et al. 1992). In the current study, injections with infected mononuclear leukocytes from *in vitro* cultures induced signs of the disease and mortality in chinook salmon by 81 d PI at water temperatures of 12°C. Further comparison of the dose of the inoculum in experimentally induced infections, while needed, is complicated by the crude enumeration of parasites. The concentration of parasites is based solely on the percent of infected cells in the inoculum, which may be considered an underestimate because it excludes extracellular spores which are known to be infectious (unpubl. obs.).

The effect of water temperature can be directly on the development of the parasite, on the immune response of the fish, or both. In infections with the flagellate *Ichthyobodo necator* in Atlantic salmon *Salmo salar*, Robertson (1979) suggested that environmental variables, including temperature, were of secondary importance to the immunocompetence and susceptibility of the host to the parasite. Immunodepression in winter flounder *Pseudopleuronectes americanus* injected with the microsporidian *Glugea stephani* was not affected by temperature or dosage of spores but was due, in part, to prostaglandins or leukotrienes secreted by the host (Laudan 1986a, b). Although the direct effects of water temperature on the parasite or immune response of the fish are unknown in our current study, a depression of both B and T lymphocyte-like activities occurs in chinook salmon following infection with *Enterocytozoon salmonis* (Wongtavatchai et al. 1994). This may in part explain the susceptibility of salmon and trout with *E. salmonis* infections to secondary pathogens and mortality as proposed for other parasitic infections (Woo 1992). Water temperatures between 8 and 13°C are considered ideal for rearing Pacific salmon (Wood 1968). These temperatures which overlap with the 2 lower temperatures examined in our study were suboptimal for *E. salmonis* infections when compared to 15 and 18°C.

The effect of temperature directly on parasite development was stressed by Lom (1979), who proposed that in *Trypanosoma* spp. infections in fish the seasonal occurrence of the disease was the direct result of temperature requirements of the parasite. Studies are currently under way to determine the direct effects of temper-ature on *Enterocytozoon salmonis* as propagated in *in vitro* cultures.

Although the development of *Enterocytozoon salmonis* was slowed and mortality was low at 9°C in our study, infections and mortality among chinook salmon became severe after transfer of the host to a higher temperature (15°C). Shifting a part of the 12°C group to a higher temperature (15°C) failed to significantly increase mortality. This result suggests that, although a lag in the onset of mortality occurs at the lower temperature (12°C), once infections have had time to progress a small upward shift in temperature (to 15°C) has less influence on the final mortality.

Our experiments clearly show that even at relatively non-permissive temperatures (9°C), infection occurs

and once established can rapidly progress at more optimal temperatures. A similar resumption of development on the microsporidan *Glugea stephani* was observed at 28 d after the host, English sole *Parophrys vetulus*, was returned to a temperature of 19 to 20°C after a period of 42 d at 10°C (Olson 1981).

The development of the parasite and the presence of infections among exposed fish held in all the temperatures examined in our study suggest that *Enterocytozoon salmonis* can tolerate a wide range of water temperatures. Severe and lethal infections may occur in fish when water temperatures become optimal for development of the parasite (15 to 18°C) and may in part explain the greater frequency of more severe epizootics among trout and salmon in the warmer rearing conditions and conversely, the more prolonged chronic losses associated with salmon held at colder water temperatures.

Acknowledgements. This research was funded in part by the California Department of Fish and Game and the U.S. Department of Agriculture 93-37204-9342. Dr J. Wongtavatchai kindly provided the infected mononuclear leukocytes used in the exposure studies.

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Responsible Subject Editor W. Körting, Hannover, Germany

Manuscript first received: October 13, 1994
Revised version accepted: February 20, 1995