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Effect of experimental *Diphyllobothrium dendriticum* infection on the blood leucocyte pattern of brown trout at two temperature levels

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Leucocyte composition in the peripheral blood of brown trout *Salmo trutta* m. *lacustris* (L.) aged 1+ was studied in relation to *Diphyllobothrium dendriticum* (Cestoda) infection at two temperature levels, heated about 15 °C and non-heated which decreased from 11 °C to 7 °C. Blood samples were taken from control and infected fish 12 weeks post infection. The *D. dendriticum* infection (1–6 per fish) had a significant increasing impact on lymphocyte and neutrophil counts at both temperature levels while thrombocytes were more numerous in control fish, particularly in non-heated water. The total leucocyte counts were not influenced by the *D. dendriticum* infection. The present water temperatures created differences only in thrombocyte and total leucocyte counts, which were increased in non-heated aquaria.

Introduction

Diphyllobothrium dendriticum (Nitzsch) (syn. *D. norvegicum* Vik) (Cestoda) is a common tapeworm species in salmonids and coregonids in Europe and North America (e.g. Halvorsen 1970, Kennedy 1978, Curtis 1983, Ching 1988, Valtonen *et al.* 1988, Andersen and Valtonen 1992). The procercoid stage of *D. dendriticum* species occurs in copepods, the plerocercoid stage in fish while the final hosts are gulls, *Larus* spp. (e.g. Vik 1957). *D. dendriticum* larvae penetrate the stomach wall of fish and encapsulate on the stomach and/or visceral organs.

In the early 1990s, an indication of the temperature-related pathogenicity of *D. dendriticum* was obtained at a fish farm in northern Finland where the mortality of sea trout (*Salmo trutta* m. *trutta* (L.)) and brown trout (*Salmo trutta* m. *lacustris* (L.)) caused by *D. dendriticum* heart infection started at 12 °C (Rahkonen *et al.* 1996a). The warm summer in 1991 increased *D. dendriticum* induced mortality compared to the following, cooler summer in 1992 (Rahkonen *et*

al. 1996a). In experimental infections with *D. dendriticum* in juvenile brown trout, the size and migration activity of the worms increased with increasing temperature, but *D. dendriticum* (1–15 per infected fish) induced mortality did not occur even though the temperature was raised gradually to 24–26 °C (Rahkonen and Valtonen 1998).

The cellular response against *D. dendriticum* including the migration of different leucocyte types to the affected fish tissues is well documented (Sharp *et al*. 1989, Sharp *et al*. 1992, Rahkonen and Koski 1997). In addition, the fish hosts are shown to produce specific antibodies against *D. dendriticum* (Sharp *et al*. 1989, 1992). However, to our knowledge there are no reports on the effect of temperature on the immune response of fish induced by *D. dendriticum*. Information about the defence reactions of fish in different temperatures could help us in defining circumstances where *D. dendriticum* might be fatal to fish.

The objectives of this study were to examine the effect of (1) *D. dendriticum* infection, and (2) temperature on the blood leucocyte composition in 1+ brown trout. The water temperature levels, about 10 °C and 15 °C, were chosen based on the observations at a farm in northern Finland where mortality started at 12 °C.

It has been demonstrated that the peripheral lymphocyte number coincides with the increase in the antibody level of sea trout vaccinated against furunculosis (Rahkonen *et al*. 1996b). In addition, the blood leucocytes of rainbow trout are shown to respond to internal injury caused by injected adjuvant (Finn and Nielsen 1971a). Thus, we expected that *D. dendriticum* infection would be reflected in the blood leucocyte counts and composition, since it has been shown to elicit both humoral and cellular responses in fish (e.g. Sharp *et al*. 1992). We also predicted that lymphocyte counts will increase in warmer water due to an overall activation of metabolism and immunological defence reactions to temperature (Ellis *et al*. 1989, Finn and Nielsen 1971b).

Materials and methods

The blood samples for the present study were collected from an experiment with 6 heated aquaria (4 exposed to *D. dendriticum* and 2 control) and 6 non-heated aquaria (4 exposed and 2 control), each containing 17 brown trout aged 1+ (mean weight around 8 g). The experiment was conducted between 16 October 1996 and 8 January 1997. The fish in exposed aquaria were intubated with a copepod dose of about 8 procercoids (in a pepsin solution) at a water temperature of 11.5 °C. The control fish received only a drop of pepsin solution. Temperature was raised to approximately 15 °C in every second aquaria on the following day and varied between 14–15 °C during the 11-week maintenance. In the last week, 12 weeks post infection (PI), it was necessary to drop the temperature to about 13.5 °C because of problems with oversaturation of oxygen. Water temperature in the non-heated aquaria decreased slowly from 11.5 °C to about 7.5 °C at the end of the experiment (12 weeks PI) because of the natural decrease in the temperature of tap water in winter.

The method concerning intubation of the fish with *D. dendriticum* procercoids is described in more detail by Rahkonen and Valtonen (1997, 1998) and the present experimental design by Rahkonen and Valtonen (1998).

After twelve weeks of maintenance, a total of 107 fish were sampled for blood. The experimental room was kept dark and the fish were netted individually with the aid of a torch to avoid stress which is known to have an influence on the blood picture (Pickering *et al*. 1982, Ellsaesser and Clem 1986). The fish were stunned by a blow to the head. Blood samples were taken from the caudal vessels using heparinised syringes.

Infected fish were collected from two non-heated and three heated aquaria to acquire an adequate sample, and control fish from one heated and one non-heated aquarium. Leucocyte counts of 16 infected fish at both temperature levels and of 13 control fish in non-heated and 12 control fish in heated water were determined. For total leucocyte counts blood was diluted 1:50 (volume/volume) in Shaw's solution (Shaw 1930) and counted using a Neubauer haemocytometer. Differential counts (150 leucocytes/smear) were made from air-dried, methanol-fixed and stained (May-Grünwald-Giemsa) blood smears. Absolute lymphocyte, neutrophil and thrombocyte concentrations were calculated from the total and differential blood cell counts.

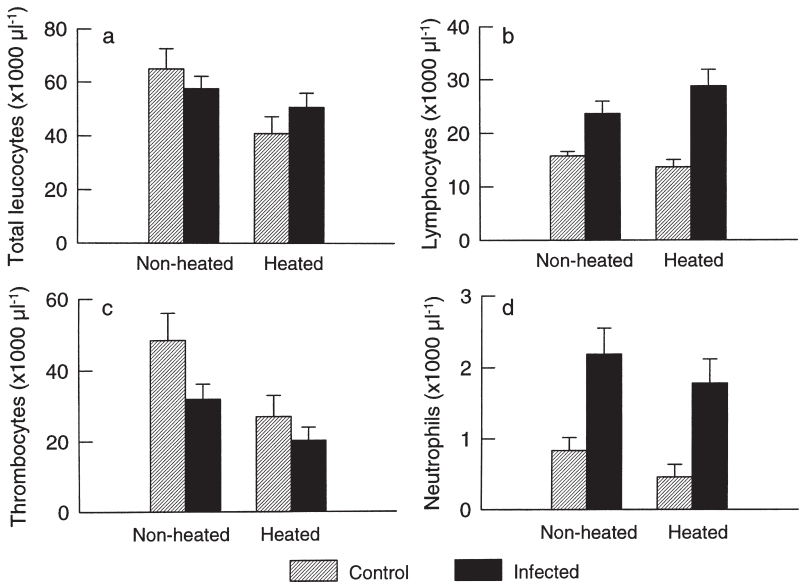

Fig. 1. The mean number of total and differential (lymphocytes, neutrophils and thrombocytes) leucocyte counts (+SE) in the peripheral blood of 1+ brown trout relative to *D. dendriticum* infection at two temperature levels (heated 14–15 °C, non-heated 11.5→7.5 °C).

After taking the blood sample the fish were measured, weighed, dissected and examined for *D. dendriticum* (method detailed in the Rahkonen and Valtonen 1998).

Computations were carried out with SYSTAT statistical software (SYSTAT 1996). Two-way ANOVA and MANOVA were used for leucocyte data. For the ANOVA model the normality of the error residuals was tested with Lilliefors' test, and the homogeneity of variances with Cochran's test (Day and Quinn 1989).

Results

The mean number of plerocercoids in the infected fish was 2.4 (SD = 1.5, range 1–6) and 2.3 (SD = 1.6, range 1–6) taken from heated and non-heated aquaria, respectively.

There was no significant difference in the total leucocyte counts between infected and control fish (two-way ANOVA, $F_{(1,53)} = 0.382$, $p > 0.05$, log-transformed data) (Fig. 1a). Temperature had an effect on leucocyte numbers (two-way ANOVA, $F_{(1,53)} = 9.689$, $p = 0.003$, log-transformed data) as the total number was greater for fish kept in non-heated water, especially for the control fish (Fig. 1a). The interaction term (temperature × infection) was not statistically significant.

Infection had a strong influence on differential (lymphocyte, neutrophil and thrombocyte) leucocyte counts (two-way MANOVA, Wilks' $\lambda = 0.482$, $F_{(3,51)} = 18.24$, $p < 0.001$, log-transformed data). The univariate *F*-tests revealed that the infection had an effect on all leucocyte types (Table 1) which was greater for lymphocytes and neutrophils in infected fish while lower for thrombocytes (Fig. 1b–d).

Temperature also had a statistically significant effect on differential leucocyte counts in general (two-way MANOVA, Wilks' $\lambda = 0.774$, $F_{(3,51)} = 4.966$, $p = 0.004$, log-transformed data). However, the impact was significant at the 5% risk level only for thrombocytes, which were clearly more numerous in non-heated water (Table 1, Fig. 1b–d).

Table 1. Results from the univariate *F*-tests for the effect of *D. dendriticum* infection and temperature on the lymphocyte, neutrophil and thrombocyte counts in 1+ brown trout. Two temperature levels were used: heated 14–15 °C, non-heated 11.5→7.5 °C.

	F	df	*p*
Factor: infection			
Lymphocytes	32.833	1, 53	< 0.001
Neutrophils	25.231	1, 53	< 0.001
Thrombocytes	5.427	1, 53	= 0.024
Factor: temperature			
Lymphocytes	0.016	1, 53	> 0.05
Neutrophils	2.972	1, 53	> 0.05
Thrombocytes	12.704	1, 53	= 0.001

The interaction term (temperature × infection) was not statistically significant at the 5% risk level for any of the dependent variables.

No clear correlation between the intensity of infection and the lymphocyte, neutrophil or thrombocyte counts was found in either heated (Spearman's rank correlation coefficient 0.06, 0.21 and 0.04, respectively) or non-heated water (Spearman's rank correlation coefficient –0.02, 0.25 and –0.18, respectively).

Discussion

In this experiment we studied circulatory leucocytes to determine the effect of *D. dendriticum* infection and water temperature on defence reactions of brown trout. Circulatory leucocytes were selected to represent defence reactions since it is known that they reflect both tissue damage (Finn and Nielsen 1971a) and antibody synthesis in fish (e.g. Rahkonen *et al.* 1996b).

In the present study a significantly higher number of circulatory neutrophils and lymphocytes were observed in infected fish as compared to the controls. The increase is evidently a consequence of *D. dendriticum* infection, but the number of larvae (1–6) do not seem to affect the counts. Small standard errors in the samples of the infected fish indicate that the aquarium effect was not notable.

Neutrophils possessing phagocytic capability are often the first leucocyte type to migrate to the site of infecting parasite. Within *in vitro* experiments, Sharp *et al.* (1991) demonstrated that cells adhering to the procercoid tegument of *D. dendriticum* were mostly neutrophils with some macrophages. Sharp *et al.* (1992) observed that during weeks 3–6 PI with *D. dendriticum* the number of neutrophils and macrophages had increased at the site of infection in rainbow trout (*Oncorhynchus mykiss* Walbaum). It can be suggested that at the same time in peripheral blood the level of neutrophils will increase. The present study shows that the elevation of the blood neutrophil level could still be seen 12 weeks PI. However, Sharp *et al.* (1992) monitored the encapsulation of *D. dendriticum* plerocercoids for 20 weeks and found it a continuing process. At the peritoneal surface of the stomach they were completely encapsulated at week six PI, but free worms still occurred within the stomach wall (Sharp *et al.* 1992).

Sharp *et al.* (1992) reported that at 14 °C specific antibodies against *D. dendriticum* in rainbow trout first appeared at week five PI. At the same time and later on the predominating cells at the site of infection were lymphocytes instead of neutrophils. The level of specific antibodies was not determined in the present study, but high blood lymphocyte counts in the infected fish (double in heated water compared to control fish) 12 weeks PI were most probably an indication of antibody synthesis in brown trout. Lymphocyte numbers in the peripheral blood of brown trout have been demonstrated to increase concominant with specific antibodies after furunculosis vaccination (Rahkonen *et al.* 1996b).

On the other hand, the present two temperature levels did not create differences in the number of lymphocytes and neutrophils. Both temperature levels fall between the "optimum temperature range" for brown trout (4–19 °C) (Elliot 1981). In addition, the immune response (antibodies and lymphocyte counts in blood) against *Aeromonas salmonicida* -bacteria, for example, has been shown to develop in brown trout at low temperatures (≤ 7 °C) as well (e.g. Rahkonen *et al.* 1996b). Thus it is likely that the present temperatures were too close to each other to create real differences in lymphocyte and neutrophil numbers. The samples taken 12 weeks PI did not reveal whether there were differences in the rapidity of responses between the two temperature levels, however.

The increase of lymphocytes and neutrophils in infected fish did not lead to an increase in total leucocyte level since the thrombocyte counts were lower in the infected fish, especially in non-heated water. There are only a few reports on the effect of infection on the blood thrombocyte level of fish. Lester and Budd (1979) observed that in coho salmon (*Oncorhynchus kisutch* Walbaum) experimentally infected with *Vibrio anguillarum* or *Renibacterium salmoninarum* bacteria the number of circulating thrombocytes was markedly reduced. Thrombocytopenia have also been found as a result of stress. Pickering and Pottinger (1987) showed that crowding stress caused a marked and prolonged reduction in the blood thrombocytes of brown trout and rainbow trout. The observed decrease in the thrombocyte levels of infected brown

trout in the present study could be a response to *D. dendriticum* infection, but a stress response caused by handling, for instance, cannot be excluded. This thrombocytopenic response might serve to release clotting factors in preparation for tissue damage as proposed by McLeay (1973). In contrast to lymphocytes and neutrophils, water temperature had an effect on thrombocyte counts showing they were clearly more numerous in non-heated water. This was reflected in the total leucocyte count as well which increased in non-heated water.

On the whole, our results show that *D. dendriticum* infection had an impact on the differential leucocyte counts. Contrary to our expectations, however, the present warmer temperature level did not increase the leucocyte response compared to cooler water, although the size of plerocercoids doubled compared to non-heated water 12 weeks PI and, as well, they migrated more actively outside the body cavity (in the heart region and muscles) (Rahkonen and Valtonen 1998). Accordingly, there might exist certain occasions where the prevailing temperature increases the pathogenicity of plerocercoids through rapid growth and migration by more than defence reactions are able to respond. This was obviously the case at a fish farm in northern Finland in the early 1990 (Rahkonen *et al.* 1996a) although we were not able to generate similar circumstances in later experiments (Rahkonen and Valtonen 1998).

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