

# Indications of non-specific protective mechanisms in rainbow trout *Oncorhynchus mykiss* with diplostomosis

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**ABSTRACT:** To study effects on protective immunity in fish exposed to cercariae of the digenean *Diplostomum spathaceum*, rainbow trout (*Oncorhynchus mykiss*) were repeatedly infected with cercariae for a 12 wk period at a water temperature of 15 °C. Intensity of infection, expressed as the relative density of metacercariae per fish, increased during the first 8 wk and then reached a plateau in experimentally infected fish. However, the recovery of the parasite, i.e. the percentage of the total amount of cercariae that managed to establish themselves as metacercariae in the lens, decreased throughout the experimental period. Compared with control fish, the infected fish showed a marked increase in the proportions of neutrophils and monocytes in the peripheral blood and the level of total immunoglobulins was significantly higher. Specific antibody levels to antigens from 3 developmental stages of the parasites were determined in an ELISA. A specific antibody response in the infected fish was, however, not recorded. The continuous decrease in recovery of the parasite in this study indicate that repeated exposure to *D. spathaceum* induced some degree of protective immunity in the fish. As specific antibody production was not recorded in the infected fish, this protection is more likely to be related to cell mediated immunity or to non-specific mechanisms of protection than to a humoral antibody response.

## INTRODUCTION

Diplostomosis is a parasitic disease of fish that in the chronic stage can result in blindness of the host. It is caused by metacercariae of the digenean *Diplostomum spathaceum*, which establish themselves in the eye lens of fish, which is an immunologically privileged site (Shariff et al. 1980). In this respect, *D. spathaceum* differs from many of the endoparasitic helminths, which evoke an antibody response in infected fish (Molnar & Berzi 1965, Harris 1970, 1972, Harris & Cottrell 1976, Cottrell 1977, McArthur 1978, Wood & Matthews 1987, Szalai et al. 1988). However, recent studies of fish injected intraperitoneally (i.p.) with antigen preparations from *D. spathaceum* or with whole parasites have clearly shown that *D. spathaceum* can evoke a specific antibody response in the fish (Bortz et al. 1984, Whyte et al. 1987). Further, protective immunity to the parasite has been demonstrated in fish injected with dead parasites (Speed & Pauley 1986, Stables & Chappell 1986a).

Although injection of parasites obviously evokes both an antibody response and protective immunity in fish, there is a need for a long-term study on the development of protection in fish exposed to *Diplostomum spathaceum* by the natural route. The ability of migrating diplostomulae to induce a non-specific cellular response in fish infected with *D. spathaceum* has been reported from histological studies (Erasmus 1959, Ratanarat-Brockelmann 1974). Further, Bortz et al. (1984, 1988) detected antibodies to *Diplostomum* spp. in infected wild fish. In contrast, Stables & Chappell (1986a) were unable to show a humoral antibody response in rainbow trout experimentally exposed to cercariae of *D. spathaceum*. However, the infection rate differed between experiments performed at the same temperature during summer and winter and was interpreted as an induction of protective mechanisms in their study.

The present experiment was designed to study if fish repeatedly exposed to cercariae develop protective mechanisms and if so, whether the protection is related to specific immunity. For this purpose rainbow trout

were kept in water at 15 °C and were repeatedly exposed to *Diplostomum spathaceum* during a 12 wk period. The infection rate of the parasite as well as haematological and immunological parameters in the fish were followed.

## MATERIALS AND METHODS

**Fish.** One-year old rainbow trout (45 ± 2 g) from Färnäs hatchery (Sweden) were kept in an artificial light regime simulating outdoor conditions at a temperature of 15 ± 1 °C in 400 l tanks. The water was recirculated through a sandfilter and continuously aerated. The fish were fed daily with commercial trout pellets (EWOS) ad libitum.

**Production of antigen.** Infective larvae (cercariae) and post-penetration larvae (diplostomulae) were produced according to Whyte et al. (1988). Parasite eggs obtained from the faeces of experimentally infected herring gulls (*Larus argentatus*) were incubated for 9 to 11 d at 28 °C. Hatched miracidia were collected and snails of a laboratory strain of *Lymnaea palustris* were exposed to infection with 4 miracidia for 6 h at room temperature. Snails were tagged with nail polish and housed in 100 l glass tanks in a constant temperature room at 18 °C under 12 h light:12 h dark regime. They were fed ad libitum on fresh lettuce supplemented by commercial fish food (Tetra Werke, F. R. Germany). The water in the tanks was continuously recirculated through a bottomfilter (Eheim) and aerated. Within 7 to 10 wk post-exposure to miracidia, shedding of cercariae commenced and cercariae were collected by isolating infected snails in 50 ml beakers for 12 h under warm, light conditions. The cercariae suspensions from the individual snails were pooled and the density of cercariae was estimated in six 0.2 ml aliquots stained with Lugol's iodine.

The post-penetration larvae, the diplostomulae, were collected after penetration of isolated pieces of rainbow trout skin, using the technique described by Whyte et al. (1988). The upper compartments of the skin-penetration tubes were filled with cercarial suspension while the lower compartments were filled with RPMI cell culture medium (Gibco). Tubes were incubated overnight in bright light conditions at room temperature.

Metacercariae were obtained from the lens of rainbow trout at least 6 wk post-infection with cercariae.

**Infection of fish with cercariae.** Two hours prior to infection, the fish were placed in an infection box with individual compartments, each inhabited by 1 fish and filled with 1.2 l of aerated water. Each fish was exposed for 15 min to a weekly dose of ca 200 cercariae. Five groups of fish were exposed for 1, 3, 5, 8 or 12 wk,

respectively. Corresponding groups of control fish were sham exposed under identical conditions. The experiment was performed between June and August.

**Sampling.** Each group was sampled at 1, 3, 5, 8, and 12 wk, respectively; 15 to 17 fish were anaesthetized with MS 222 (Sandoz) and blood samples were collected from the caudal vein in heparinized vacutainer tubes. The blood was then centrifuged at 300 × g for 10 min. Plasma was stored at -20 °C before screening in the ELISAs. The total length and weight of each fish was measured. In addition, the eyes were dissected and the number of metacercariae was determined using a stereo-microscope (× 50).

**Differential counts of blood leukocytes.** Blood smears were fixed in methanol and stained with Hemacolor (Merck), and differential counts of 100 to 200 leukocytes were made.

**Preparation of positive and negative control plasma samples.** To obtain positive plasma, rainbow trout were injected i.p. with whole frozen parasites. Each fish received ca 200 cercariae, 300 diplostomulae, or 50 metacercariae. Two weeks later, a booster dose was given and after another 2 wk, fish were bled. The plasma samples obtained in this manner served as positive control samples in the ELISA. Negative control samples were obtained from non-infected rainbow trout.

**Antigen preparation.** Antigens from 3 developmental stages (cercariae, diplostomulae and metacercariae) were prepared by alternately freezing and thawing as described by Ingram & Al-Yaman (1988). Briefly, the parasites were diluted to ca 100 organisms ml<sup>-1</sup> in 0.05 M carbonate buffer (pH 9.6; coating buffer). Next, they were alternately frozen (-20 °C) and thawed 5 times. The antigen preparations were then centrifuged for 10 min at 200 × g. The resulting supernatants were then used as coating antigens in the ELISA measuring antibodies to *Diplostomum spathaceum*.

**ELISA-antibodies to *Diplostomum spathaceum*.** A modification of the indirect ELISA system described by Whyte et al. (1987) was used to determine plasma antibody levels to *Diplostomum spathaceum*. Microtitre plates (Nunc II Immunoplate, Nunc, Denmark) were coated at room temperature for 24 h with 200 µl well<sup>-1</sup> of one of the 3 different antigen preparations diluted 1:20 (cercariae) or 1:50 (diplostomulae and metacercariae) in coating buffer. After removal of the antigen solution, the plates were blocked with 200 µl well<sup>-1</sup> of 2% milk powder in phosphate buffered saline (PBS) for 30 min. After this, plates were washed 3 times with 0.9% NaCl with 0.5% Tween 20, and 100 µl of fish plasma, diluted 1:100 in PBS with 0.5% Tween 20 (PBS-Tween), was added to each well. Duplicate wells were used for each sample. Following overnight incubation at 4 °C the plates were again washed as above and then incubated with 100 µl well<sup>-1</sup> of a mouse

monoclonal antibody (clone 4C10 in ascites fluid) to rainbow trout IgM (Thuvander et al. unpubl.) diluted 1:10 000. The plates were incubated for 1.5 h at 37 °C. After washing, plates were incubated with 100  $\mu\text{l}$  well<sup>-1</sup> of horseradish peroxidase conjugated rabbit anti-mouse antibodies (Dakopatt, Denmark) diluted 1:1500 in PBS-Tween. Finally, after washing, 200  $\mu\text{l}$  well<sup>-1</sup> of the substrate tetramethylbenzidine (TMB, KEBO, Stockholm) at a concentration of 1 mg ml<sup>-1</sup> in 0.1 M acetate buffer, pH 6.0, containing 2  $\mu\text{l}$  3% H<sub>2</sub>O<sub>2</sub> ml<sup>-1</sup> was added. The reaction was stopped after 10 min with 50  $\mu\text{l}$  well<sup>-1</sup> 2M H<sub>2</sub>SO<sub>4</sub>, and the optical density (OD) at 450 nm determined. Results are expressed as the mean OD values for duplicate wells.

**ELISA-total immunoglobulin.** Microtitre plates were coated for 24 h at room temperature with 200  $\mu\text{l}$  well<sup>-1</sup> of rabbit anti-trout IgM sera diluted 1:1000 in coating buffer. Subsequently the plates were washed 3 times. After this, fish plasma serially diluted in PBS-Tween was added and the plates were incubated overnight at 4 °C. Following this step the procedure described above, for detection of bound fish antibodies, was employed. Results are expressed as OD value for plasma diluted 1:1600.

**Statistical analysis and definitions.** The numbers of parasites were compared with Kruskal-Wallis test and Mann-Whitney U-test. Haematological and immunological parameters were tested with Student's t-test and analysis of variance (ANOVA). All of the statistical tests were 2-tailed and considered significant at the 0.05 level.

Relative density of metacercariae refers to the number of larvae per fish at autopsy. Recovery refers to the percentage of the total amount of cercariae that managed to establish themselves as metacercariae in the lens.

## RESULTS

### Experimental infections

As can be seen in Fig. 1a, the relative density of metacercariae in the lens increased gradually in the

exposed fish for the first 8 wk of exposure (Kruskall Wallis test, DF = 4, H = 56.87,  $p < 0.001$ ). The mean number of metacercariae per fish was  $149 \pm 48$ . After 8 wk of exposure the relative density of metacercariae reached a plateau (Mann Whitney U-test, N = 33, Z = 0.11, NS).

The percentage of the total amount of cercariae that managed to establish themselves as metacercariae in the lens, i.e. the recovery, decreased continuously throughout the experiment (Fig. 1b). The lens was the only site in the eye found to be infected. All of the fish in the control groups remained uninfected throughout the experiment.

### Haematology

Results from the differential leucocyte counts are shown in Fig. 2. Four clearly distinct cell-populations, namely lymphocytes, thrombocytes, neutrophilic granulocytes (neutrophils) and monocytes were identified in the blood smears (Thuvander et al. 1987).

A general increase in the proportion of neutrophils was recorded for both groups of fish during the experiment. The proportions of neutrophils were, however, significantly higher in the experimentally infected fish than in the controls (2-way ANOVA: weeks of exposure,  $F = 18.19$ ,  $p < 0.0001$ ; treatment,  $F = 11.43$ ,  $p < 0.0001$ ; interaction between weeks of exposure and treatment,  $F = 3.54$ ,  $p < 0.018$ ).

The proportion of monocytes increased in the infected fish but not in the control fish throughout the experimental period. As for the neutrophils, this cell population was also higher in the infected fish than in the controls (2-way ANOVA: weeks of exposure,  $F = 10.74$ ,  $p < 0.0015$ ; treatment,  $F = 5.39$ ,  $p < 0.0018$ ; interaction between weeks of exposure and treatment,  $F = 1.94$ ,  $p < 0.12$ ).

The proportion of lymphocytes decreased significantly in both groups of fish throughout the experimental period. The relative lymphocyte counts were, how-

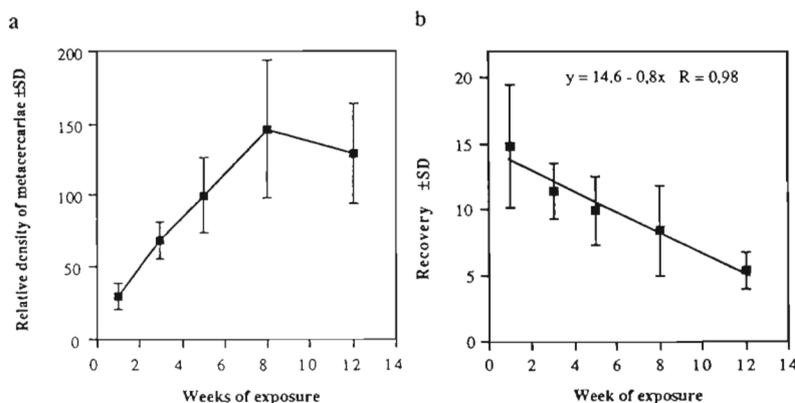


Fig. 1. Infection rate of *Diplostomum spathaceum* in rainbow trout exposed to a weekly dose of 200 cercariae as described in detail in 'Material and methods'; shown as (a) the relative density of metacercariae, and (b) mean recovery, i.e. the percentage of the total amount of cercariae that managed to establish themselves as metacercariae in the lens of fish. Results are expressed as mean  $\pm$  SD for 15 to 17 fish

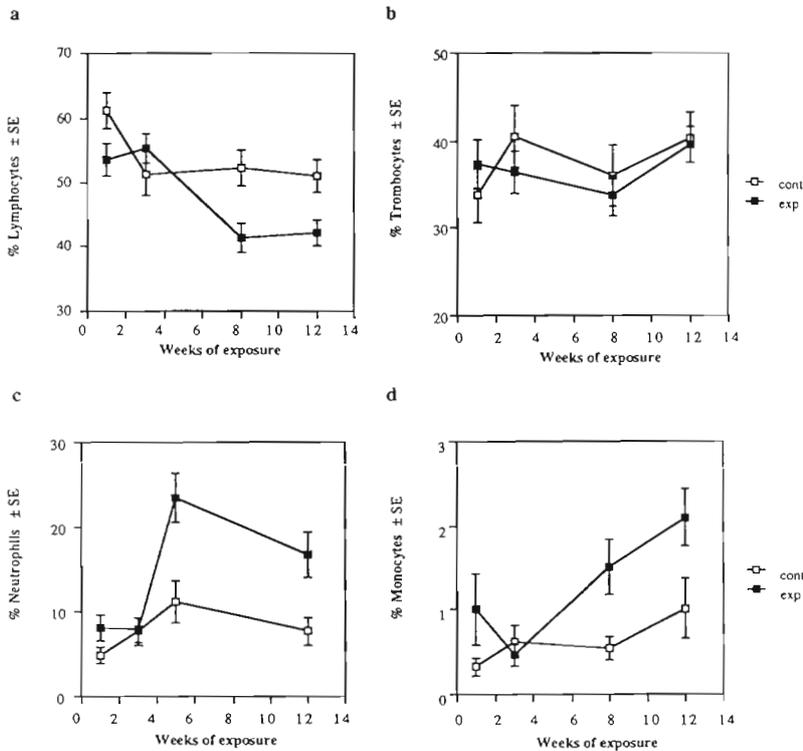


Fig. 2. Differential leucocyte counts in control rainbow trout and in fish experimentally infected with live *Diplostomum spathaceum* cercariae; (a) lymphocytes, (b) thrombocytes, (c) neutrophils, (d) monocytes. Results are expressed as mean  $\pm$  SE for 10 to 15 fish

ever, significantly lower in the infected fish as compared to the controls (2-way ANOVA: weeks of exposure,  $F = 8.55$ ,  $p < 0.0001$ ; treatment,  $F = 10.50$ ,  $p < 0.0016$ ; interaction between weeks of exposure and treatment,  $F = 3.40$ ,  $p < 0.021$ ).

The proportions of thrombocytes remained unaltered throughout the experiment in both groups of fish (ANOVA:  $F = 0.88$ ,  $p < 0.52$ ).

#### Total Ig levels

OD values for fish in the ELISA measuring total Ig levels are shown in Fig. 3a. Although the total Ig levels were significantly higher in infected fish than in controls, a general increase in OD values was also observed during the experimental period, i.e. total Ig levels increased in both infected and control fish over the summer (2-way ANOVA: weeks of exposure,  $F = 8.36$ ,  $p < 0.0001$ ; treatment,  $F = 5.84$ ,  $p < 0.017$ ; interaction between weeks of exposure and treatment,  $F = 3.20$ ,  $p < 0.015$ ).

#### Specific antibody levels

As can be seen in Fig. 3b to d, the ELISA measuring specific antibodies to cercariae, diplostomulae and metacercariae revealed no differences in OD values

between infected and control fish. Instead, a general increase in OD values that correlated with the observed increase in total antibodies, was observed in both groups of fish throughout the experiment (2-way ANOVA, cercariae: weeks of exposure,  $F = 3.96$ ,  $p < 0.005$ ; treatment,  $F = 1.31$ ,  $p < 0.25$ ; interaction between weeks of exposure and treatment,  $F = 0.135$ ,  $p < 0.97$ ; diplostomulae: weeks of exposure,  $F = 6.30$ ,  $p < 0.0001$ ; treatment,  $F = 0.085$ ,  $p < 0.77$ ; interaction between weeks of exposure and treatment,  $F = 0.73$ ,  $p < 0.58$ ; metacercariae: weeks of exposure,  $F = 3.83$ ,  $p < 0.006$ ; treatment,  $F = 0.33$ ,  $p < 0.5$ ; interaction between weeks of exposure and treatment,  $F = 1.18$ ,  $p < 0.32$ ).

The results of the estimations of antibodies in plasma from fish injected i.p. with parasitic antigens are shown in Fig. 4. OD values were significantly higher for fish immunized with cercariae (Students' t-test,  $t = 3.69$ ,  $p < 0.0027$ ), diplostomulae (Students' t-test,  $t = 3.26$ ,  $p < 0.0099$ ) or metacercariae (Students' t-test,  $t = 3.37$ ,  $p < 0.0055$ ) than for control fish.

#### DISCUSSION

In this study, the relative density of metacercariae in fish increased up to Week 8 and then reached a plateau. As the timing of the plateau did not coincide with

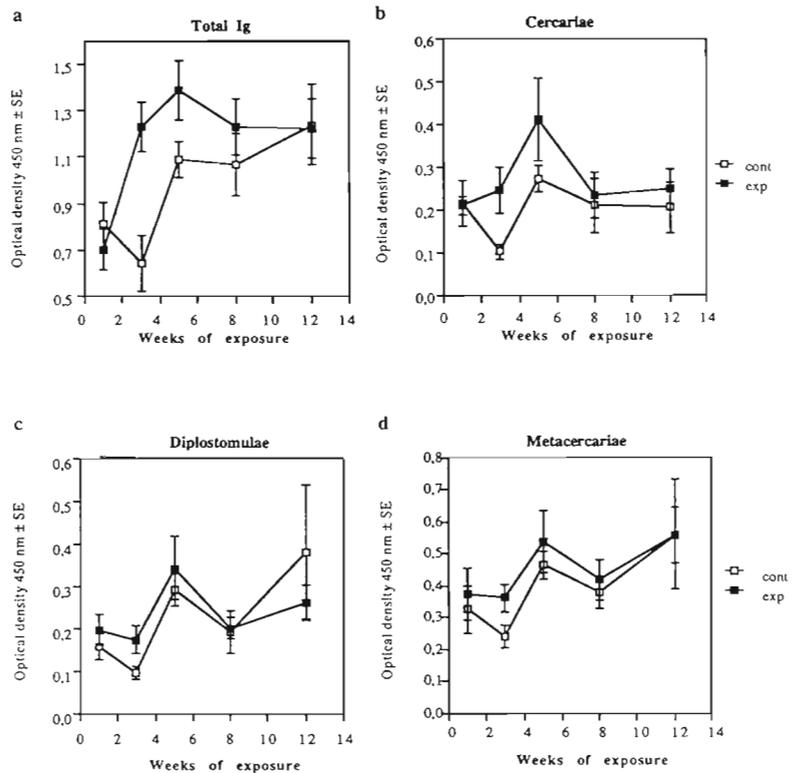


Fig. 3. Humoral antibody levels expressed as OD<sub>450</sub>-values to antigens from *Diplostomum spathaceum* in experimentally infected and control rainbow trout – as described in detail in 'Material and methods'. Results are expressed as mean ± SE for 8 to 16 fish. (a) Total immunoglobulin levels; (b) antibodies to cercariae; (c) antibodies to diplostomulae; (d) antibodies to metacercariae

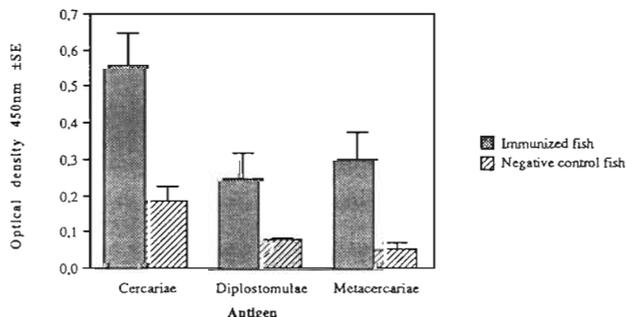


Fig. 4. Humoral antibody levels expressed as OD<sub>450</sub>-values to antigens from *Diplostomum spathaceum* in rainbow trout injected i.p. with the different developmental stages of the parasite and in control fish – as described in detail in 'Material and methods'. Results are expressed as mean ± SE for 4 to 8 fish

observed changes in haematological or immunological parameters, the saturation was probably due to space limitations in the lens. More importantly, the recovery of the parasite decreased continuously throughout the 12 wk experimental period. Since we used laboratory-maintained cercariae bred under identical conditions throughout the experiment, it is not likely that the decrease in infection rate was due to a decreased ability of the cercariae to infect the fish. Further, the life span of the metacercariae of *Diplostomum spathaceum* is several years, and losses due to natural mortalities of

metacercariae can thus be neglected (Stables & Chappell 1986b). A possible explanation for the decreased recovery of the parasite would be related to structural changes in the fish skin or gill epithelium associated with the growth of the fish during the experiment. A more probable explanation is, however, that the decreased recovery of the parasite was due to the induction of mechanisms in the fish protective against the migrating diplostomula. The infection pattern observed in this experiment suggests that protective mechanisms are induced in fish following repeated exposure of the fish to live cercariae of *D. spathaceum*. This finding agrees with several reports on induction of protective mechanisms in fish exposed to endoparasitic helminths (Kennedy & Walker 1969, Orr et al. 1969, McVicar & Fletcher 1970, Wood & Matthews 1987).

We were unable to detect a specific humoral antibody response in rainbow trout repeatedly infected with cercariae of *Diplostomum spathaceum* by the natural route. This result is similar to observations reported by Stables & Chappell (1986a) but contrasts with studies of wild fish (Bortz et al. 1984, 1988) in which antibodies to *D. spathaceum* were detected. However, in agreement with previous reports (Bortz et al. 1984, Whyte et al. 1987), a specific antibody response was observed in the present study in fish following i.p. immunization with 3 different developmental stages of the parasite. The absence of a specific antibody response to *D. spathaceum* in this experiment

is therefore, not likely to be due to the use of an inappropriate immunological assay, although the present method might not be sensitive enough to reveal a weak antibody response.

Even though no specific antibody response to *Diplostomum spathaceum* was observed among the experimentally infected fish in this study, the total immunoglobulin levels were significantly higher in the experimentally infected fish when compared with controls. This finding might be the result of a weak antibody response to several antigens derived from the infecting parasite. Further, total immunoglobulin levels increased throughout the experiment in both groups of fish. This observation could be related to seasonal changes in the production of immunoglobulins as has been reported by Yamaguchi et al. (1980). It is of importance to note that the increase in total immunoglobulin correlated with a general increase in the OD values in the ELISA measuring antibodies specific to different larval stages of *D. spathaceum* in plasma. This increase is probably caused by non-specific interactions between fish antibodies and parasite antigens.

In mammals, induction of cellular immunity is required to give protection to helminth infections. In schistosomiasis, for example, damage of the schistosomula is mainly caused by antibody and/or complement-dependent adherence of eosinophilic granulocytes (eosinophils), although macrophages and neutrophils also take part in damaging and killing the parasites (for reviews see McLaren 1980, Joseph 1982, Butterworth 1984). Similarly, complement-mediated leucocyte adherence resulting in damage to plerocercoid larva of the cestode *Ligula intestinalis* have been demonstrated in roach *Rutilus rutilus* (Hoole & Arme 1986). Eosinophilic granulocytes have been reported to occur in rainbow trout (Ellis 1977, Bielek 1981). No eosinophils were, however, observed in the blood smears from fish in the present study. Instead, infected fish showed a marked increase in the proportions of neutrophils and monocytes. These findings are related to observations from histological sections of infected fish, in which neutrophils and monocytes/macrophages were found to be associated with the migrating diplostomulae (Erasmus 1959, Ratanarat-Brockelman 1974). It remains to be seen whether or not these cells take part in a cytotoxic reaction against *Diplostomum spathaceum*.

In conclusion, *Diplostomum spathaceum* evoked a humoral antibody response in fish injected with parasite antigens but specific antibodies were not detected when fish were infected by the natural route. The recovery of the parasite, however, decreased throughout the experimental period, which indicates that an induction of protective immunity occurred. This protection could be related to the increased proportions of neutrophils and monocytes in the infected fish.

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