Humoral response of roach (*Rutilus rutilus*) to digenean *Rhipidocotyle fennica* infection

T. M. AALTONEN,* E. T. VALTONEN *and* E. I. JOKINEN

Department of Biological and Environmental Science, University of Jyväskylä, Vapaudenkatu 4, FIN-40100 Jyväskylä, Finland

(*Received 28 May 1996 ; revised 30 September 1996 ; accepted 1 October 1996*)

SUMMARY

The humoral immune response of roach (*Rutilus rutilus*) to cercariae of the digenean trematode, *Rhipidocotyle fennica*, was studied. Antibodies against *R. fennica* were found in wild roach in lakes where fish are infected by the parasite. Antibody levels were higher in sera collected in September than in sera collected in June, due to infection of *R. fennica* during the late summer. In experimental aquarium studies, roach immunized with homogenized cercariae produced antibodies against *R. fennica*. An especially strong response was elicited by infecting fish with living cercariae emerging from infected clams. The specificity of the antibodies, as shown in Western blots, was different between fish immunized with homogenized cercariae and those fish infected with living cercariae. The specificity and amount of antibodies depended on the route of immunization. The challenge experiment with *R. fennica* indicated that previous infection of fish gives some protection against *R. fennica*.

Key words: roach, *Rhipidocotyle fennica*, anti-parasite antibody.

INTRODUCTION

A freshwater fish, *Rutilus rutilus*, has been found to harbour 15 protozoan and 36 metazoan parasite species in a set of 4 lakes in Central Finland (Brummer-Korvenkontio, Valtonen & Pugachev, 1991; Koskivaara, Valtonen & Prost, 1991*a*, *b*; Valtonen, Holmes & Koskivaara, 1997). The digenean trematode *Rhipidocotyle fennica* was among the most common species, often dominating the infracommunities of the fish (Valtonen *et al*. 1997). It composed approximately half of all parasites counted in roach from 2 eutrophic lakes located downstream from a pulp and paper mill. The high number of parasites was interpreted as being due to impaired immunity in the fish. However, the sizes of the populations of *Anodonta piscinalis*, the first intermediate host of *R. fennica*, differ in the 4 lakes and this may have had an effect on the abundance of *R. fennica* (Valtonen *et al*. 1997). Our experimental studies in the same area support the assumption of impaired immunity. Roach caged in a lake polluted by bleached kraft mill effluents show significantly lower levels of IgM and weakened responsiveness against antigens than fish caged in a reference lake (Jokinen, Aaltonen & Valtonen, 1995).

R. fennica needs 3 distinct host species and possesses 2 aquatic free stages. Worms mature in pike (*Esox lucius*) and asexual development takes place in the first intermediate host clam *Anodonta piscinalis*. (Taskinen, Valtonen & Gibson, 1991). The cercariae from the clam emerge mainly between mid-July and mid-September. After attaching to roach cercariae encyst mainly in the skin and fins. Metacercariae can infect pike, the final host, not earlier than 3 weeks after infection of roach (Taskinen *et al*. 1991; Gibson, Taskinen & Valtonen, 1992; Taskinen, Valtonen & Mäkelä, 1994; Taskinen & Valtonen, 1995).

Information on the immune response of teleosts against metazoan parasites is limited. It has been suggested that immunological responses, both cellular and humoral, are important in regulating the parasite burden of fishes and in influencing the changes in parasite populations (Thomas & Woo, 1995). The immunological response of fish against only some of the digenean parasites has been studied. The best documented example of this is the response against *Diplostomum spathaceum* (reviewed by Chappell, Hardie & Secombes, 1994). Antibodies to *Rhipidocotyle johnstonei* have been found in fish (Cottrell, 1977) but there is no immunological work done on the immunity against *R. fennica*. *R. johnstonei* and *R. fennica* differ significantly in the way of infection. *R. johnstonei* occurs in the muscle and connective tissue of the host and does not make a cyst.

In this study we examined (1) the development of anti-*R. fennica* specific antibodies in roach and (2) the role of antibodies in protecting the roach against the parasite. Roach from lakes differing in water quality and trophic levels were sampled and ex-

* Corresponding author. Tel: +358 14 602 224. Fax: +358 14 602 221. E-mail tumiaa@jyu.fi.

https://doi.org/10.1017/S0031182096008499 Published online by Cambridge University Press

perimental aquaria studies were performed by exposing roach to naturally emerged cercariae and by immunizing the fish with homogenized *R. fennica* cercariae.

MATERIALS AND METHODS

Fish

Roach (*Rutilus rutilus*) from 6 lakes belonging to the same water system in central Finland were caught on 10–16 June and again on 15–16 September, 1992. The mean lengths and weights of the fish ($n = 229$) from different lakes, the trophic status of the lakes, and the occurrence of the clam *Anodonta piscinalis* are given in Table 1. Antibodies against *Rhipidocotyle fennica* in the serum of the fish, as well as the number of parasites in the fish, were studied.

For experimental studies 99 roach were angled from the oligotrophic and unpolluted Lake Peurunka 14–22 May 1992. The average weight and length of these fish were 23 g (S.D. = 7·9) and 144 mm (S.D. = 14), respectively. These fish were free from *R. fennica* because no *A. piscinalis*, the first intermediate host of *R. fennica*, occur in lake Peurunka. To remove protozoan ectoparasites the fish were treated after catching using a commercial drug, Ichide, at 100 μl/10 l (N.T. Laboratories Ltd, England). The fish were kept in a 250 l aquarium filled with aerated tap water at a constant temperature of $17{\cdot}5 \pm 0{\cdot}5$ °C, and fed daily with commercial pelleted dry food (TESS, Raisio, Finland). The roach, adapted to aquaria for 2 months, were divided into 3 groups. Group A ($n = 32$) was infected naturally by exposing the roach for 5 days to cercariae produced by infected *A. piscinalis*. Simultaneously with the infection of group A fish in group B ($n = 30$) were injected intraperitoneally with homogenized *R. fennica* cercariae (150 μg protein/fish) in saline emulsified with an equal volume of Freund's complete adjuvant (FCA, Difco Laboratories, Detroit, USA). Group C ($n = 37$) served as a control group without primary infection or immunization. Otherwise the fish in all experimental tanks were handled identically. Thirty days after the immunization the fish in the 3 groups were marked intradermally with alcian blue and moved into a single aquarium. The fish were given a challenge infection by exposing them for 24 h to cercariae of *R. fennica* shed from 40 infected clams placed in the aquarium. Blood samples were taken, and the number of parasites on the fins were counted, on day 1 ($n = 48$) and on day 14 ($n = 51$) after the challenge.

Blood samples

The blood was collected from the caudal vein for serum separation. After collecting, the blood was allowed to clot for 3 h at room temperature (22 °C) and overnight at 4 °C. Next day, the blood was centrifuged at 4000 ***g*** for 5 min and the sera were stored at −20 °C.

Homogenization of R. fennica

Thousands of cercariae were collected using a disposable Pasteur pipette from water after their release from *A. piscinalis*. Cercariae were filtered onto a nitrocellulose membrane (1·2 μm, Millipore, USA), scraped from the filter and stored at −20 °C. Cercariae were homogenized on ice, in saline, with an ultrasonic disintegrator (High Intensity Ultrasonic Processor, Sonics & Materials, USA). Homogenization was for 2 min, with a cycle of 1 s on and 1 s off. Whole homogenate was used as immunogen. The protein concentration (1·5 mg/ml) of the homogenate was determined by the Bradford assay (Bradford, 1976) using commercial reagents (Bio-Rad Laboratories, Richmond, USA).

R. fennica *antigen*

The sonicated material was centrifuged at 5500 ***g*** for 15 min and the supernatant was separated. The protein content of the supernatant was determined. The extract of sonicated cercariae was used as the trapping antigen in ELISA for the anti-parasite antibody. The antigenic protein components of the supernatant were assayed by Western blotting.

Quantification of serum anti-parasite antibodies

The amount of specific anti-*R. fennica* antibody in roach serum was determined using ELISA (enzyme-linked immunosorbent assay). The plates were coated with a soluble protein extract from whole cercariae 10 μg/ml 50 mM carbonate buffer (pH 9·6). After masking with bovine serum albumin, samples of diluted roach sera (dilutions $1:10^2$, $1:10^3$ and $1:10^4$) were incubated in the wells for 30 min at 37 °C. The bound antibodies were detected with biotin-conjugated anti-roach IgM (Aaltonen, Jokinen & Valtonen, 1994). Next, alkaline phosphatase-conjugated avidin (Biomakor, Rehovat, Israel) was added. Washing with phosphate-buffered saline containing Tween 20 (0·05 %), pH 7·4 was performed between each step. P-nitrophenylphosphate in 1 M diethanolamine buffer (pH 9·8) was used as a substrate. The optical density was read with a Titertek plate reader (Flow Laboratories) at 405 nm. The calibration curve was constructed using a pool of high titre sera obtained from another experiment in which roach were infected by *R. fennica* cercariae shed from *A. piscinalis* (Aaltonen *et al.* unpublished observations). The concentration of anti-*R. fennica* antibodies of the pooled serum was defined to be 10000 artificial units per ml (U/ml). Fish with antibody levels of 0–500 U/ml were considered

https://doi.org/10.1017/S0031182096008499 Published online by Cambridge University Press

Table 1. Fish studied from 6 lakes in Central Finland at 2 sampling times

Lake	Type of lake	Occurrence of *Anodonta piscinalis**	Fish (June/September)		
			n	Mean weight (g)	Mean length (mm)
Saravesi	Eutrophic	+++	21/18	27/31	148/147
Kuusvesi	Oligotrophic	+	18/20	27/34	140/149
Vatia	Eutrophic, polluted	+	19/20	36/34	162/219
Leppävesi	Eutrophic	++	21/21	23/25	143/141
Ahveninen	Eutrophic	+	14/16	29/36	149/157
Peurunka	Oligotrophic	−	21/20	34/61	156/195

* Occurrence of *A. piscinalis*: many (+++), moderate (++), few (+), none (−). The occurrence of *A. piscinalis* was monitored during several years using a bottom dradge, by diving or by enumeration of glochidia on fish.

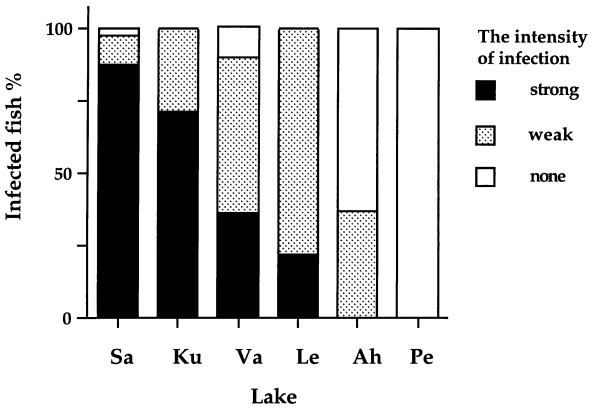

Fig. 1. The proportions of roach infected by *Rhipidocotyle fennica* in the lakes studied: Lake Saravesi (Sa), Lake Kuusvesi (Ku), Lake Vatia (Va), Lake Leppävesi (Le), Lake Ahveninen (Ah) and Lake Peurunka (Pe). Classification of infection: strong infection was > 30 cercariae/tail, weak infection was ⩽ 30 cercariae/tail and not infected: no cercariae in the tail. Numbers of fish and descriptions of the lakes are given in Table 1.

negative because the mean + 2 S.D. of the controls, on day 1 post-infection (p.i.) was less than 500 U/ml. The fish with antibody concentrations exceeding 3000 U/ml were considered strongly positive.

Enumeration of parasites

The fins of roach were examined microscopically on a glass plate by dissecting the tail and dividing each fin ray. The numbers of metacercariae were counted. Newly penetrated parasites were not easily seen until the formation of cysts had proceeded for 2–3 days p.i. Fish with fewer than 30 cysts were considered to have a weak infection and those with more than 30 were classified as strongly infected.

Western blotting analysis

Proteins extracted from sonicated cercariae with saline were separated by SDS–PAGE according to the method of Laemmli (1970) using a Mini Protean II dual slab gell system (Bio-Rad). The samples were mixed with an equal volume of reducing buffer and heated at 96 °C for 4 min to denature the proteins. Molecular weight standards and samples (100 μl, 1·7 mg protein/ml) were loaded onto the stacking gel and electrophoresed into a 12·5% polyacrylamide running gel. Electrophoretic transfer of polypeptide bands from the gel to a nitrocellulose membrane (Hoefer Scientific Instruments, USA) was done using a Mini-Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). After transfer, the blot was blocked with 5% non-fat milk powder in Tris-buffered saline (TBS) for 3 h. The immunostaining was performed in a Mini Protean II Multiscreen Apparatus (Bio-Rad). Three sera from fish infected by *R. fennica* and control serum from an uninfected fish were used as primary antibody. Blots were incubated with primary antisera for 1 h, washed, incubated with rabbit anti-roach IgM, diluted 1:600 (Aaltonen *et al.* 1994) as the secondary antibody, washed and incubated for 1 h with goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma, Chemical Co, St Louis, USA) diluted 1:3000. The blot was then washed and the colour developed using nitro-blue tetrazolium (NBT) and bromo-chloro-indolyl-phosphate (BCIP).

Statistical analysis

The Mann-Whitney U-test at a 5% level of confidence was used to assess the significance of differences.

RESULTS

Wild fish

The fish from Lake Peurunka were free from *R. fennica*. The prevalence and the intensity of infection varied in fish from different lakes (Fig. 1). All fish from Lake Leppävesi and Lake Kuusvesi and nearly all from Lake Saravesi and Lake Vatia were infected by *R. fennica*. The strongest infection was found in the fish from Lake Saravesi, where 87% of fish

https://doi.org/10.1017/S0031182096008499 Published online by Cambridge University Press

Table 2. Anti-*Rhipidocotyle fennica* antibodies in wild roach caught in June (VI) and September (IX) in 1992 from 6 lakes in Central Finland

	Antibody positive (> 500 U/ml) (% of fish)		Strong responders (> 3000 U/ml) (% of fish)		Serum antibody concentration (U/ml)	
Lake	VI/IX	Mean	VI/IX	Mean	VI/IX	Mean
Saravesi	81/66	69	24/44	33	2055/3298	2629
Kuusvesi	22/65	45	11/30	21	639/2068	1391
Vatia	37/45	41	0/20	10	607/1324	975
Leppävesi	52/38	44	0/5	2	792/843	817
Ahveninen	36/62	50	7/6	7	1399/1311	1351
Peurunka	5/15	10	0/0	0	53/280	164

Table 3. Development of anti-*Rhipidocotyle fennica* antibodies in roach after experimental immunization in aquaria

		Antibody response (% of fish)		
Group	*n*	No antibodies (< 500 U/ml)	Weak response (500–3000 U/ml)	Strong response (> 3000 U/ml)
A: Exposed to living cercariae	33	37·5	37·5	25·0
B: Immunized with homogenized cercariae	30	73·3	26·7	0·0
C: Control	36	94·6	5·4	0·0

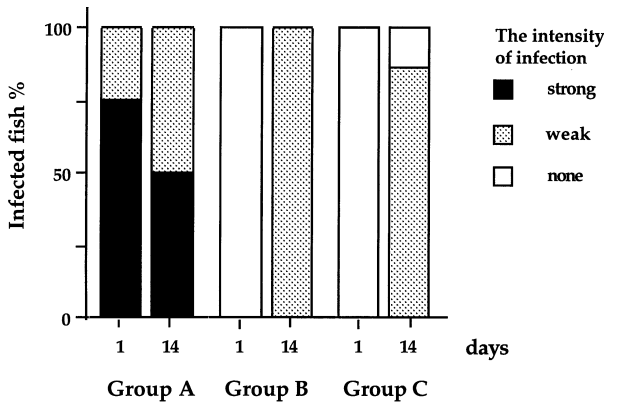

Fig. 2. The proportions of strongly, weakly or non-infected roach in experimental *Rhipidocotyle fennica* infections. The numbers of parasites were counted on days 1 and 14 after the challenge infection. Group A was infected by exposing to cercariae 30 days earlier, group B was immunized at the same time with homogenized cercariae and control group C received no treatment.

showed strong infection (> 30 metacercariae/tail) and only 3 % were uninfected. Only weakly infected roach (⩽ 30 metacercariae/tail) were found in Lake Ahveninen.

Antibodies against *R. fennica* were present in fish from all lakes where the parasites exist (Table 2). For example, 69 % of the fish in Lake Saravesi (June and September) were seropositive and almost half of them had high antibody concentrations (> 3000 U/ml). Four out of 41 roach from Lake Peurunka showed low antibody levels. In general, high antibody levels were found in fish from the lakes in which the roach were strongly infected. The sera collected in September more often had higher anti-*R. fennica* antibody levels than those collected in June, although the increase was statistically significant only in the case of Lake Kuusvesi.

Immunization experiments

The roach in aquaria responded to the experimental infection and produced antibodies against *R. fennica* (Table 3). In group A, where fish were exposed to naturally emerged cercariae for 5 days, 63 % of the fish produced antibodies and 25 % produced high levels (> 3000 unit/ml). The roach immunized with homogenized cercariae (group B) responded weakly and only 27 % of fish produced antibodies against the parasite. In the control group (C), no antibodies against *R. fennica* were found except on day 14 p.i., when low antibody levels were detected in 2 roach (5 % of groups).

Challenge with R. fennica

In the challenge experiment, there were no statistically significant differences in the number of parasites on the fins of control fish (group C) and immunized fish (group B) on day 14 p.i. (Fig. 2). The fish infected earlier by living cercariae (group

https://doi.org/10.1017/S0031182096008499 Published online by Cambridge University Press

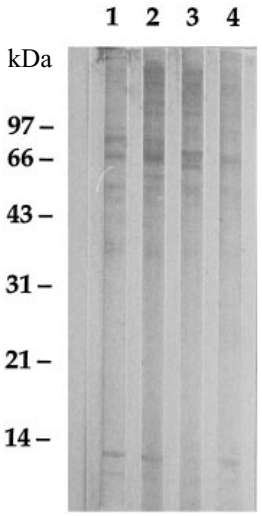

Fig. 3. Western blot analysis of homogenized *Rhipidocotyle fennica* cercariae. The proteins of the homogenate were resolved by SDS–PAGE, electroblotted onto nitrocellulose and detected with sera of roach from the aquarium experiment against *R. fennica* proteins. Lanes 1 and 2 were probed with sera from 2 fish in group A, lane 3 with serum from 1 fish in group B, and lane 4 with non-immune serum from 1 fish in group C.

A), had mature metacercariae on their fins already at the time of the challenge. Fourteen days after the challenge, the number of parasites in group A was lower than at day 1 p.i.

Western blotting of antigenic components of R. fennica

The results of Western blotting showed that immunization by injection of homogenized parasites and natural infection by cercariae, produced antibodies with different binding properties to the compounds of the parasite (Fig. 3). The antisera of fish from group A (lanes 1 and 2) detected 2 major bands of molecular weights between 66 and 97 kDa besides many minor ones. The antibodies of fish from group B (lane 3) probed with two different components; one between 66 and 97 kDa and another between 43 and 66 kDa. No reaction was evident with control serum except 1 faint band of approximately 66 kDa.

DISCUSSION

Rhipidocotyle fennica – specific antibodies were found in wild roach in lakes where the fish were infected by the parasite. Previously uninfected roach produced antibodies when immunized with homogenized cercariae or infected with cercariae emerging from infected clams also in aquaria. These findings are parallel to previous studies on humoral antibody response against metazoan parasites. Antibodies have been reported in rainbow trout (*Salmo gairdneri*) against a digenean trematode *Diplostomum spathaceum* (Bortz *et al*. 1984; Whyte *et al*. 1987), in plaice (*Pleuronectes platessa* L) against *Rhipidocotyle johnstonei* and in plaice and grey mullet (*Chelon labrosus*) against *Cryptocotyle lingua* (Cottrell, 1977; Wood & Matthews, 1987).

Roach infected by *R. fennica* were found in 5 of 6 lakes studied and also anti-*R. fennica* antibody-positive fish were found in these lakes. The emergence of *R. fennica* cercariae begins in mid-July in Lake Saravesi (Taskinen *et al*. 1994). Thus, the *R. fennica*-specific antibodies in fish sera already present in early June must originate from infections in the course of the previous year. This is in accordance with other studies, where antibodies have been demonstrated to persist for a long period. For example Thuvander *et al*. (1987) found antibodies as long as 46 weeks after *Vibrio anguillarum* vaccination. The increased antibody levels in September are most probably due to fresh infections during the late summer.

Low levels of antibody against *R. fennica* were detected in the sera of 4 wild roach (total $n = 41$) from Lake Peurunka, where *A. piscinalis* does not occur. This was an unexpected finding that probably results from cross-reactions with serum antibodies against other parasites or microbes. The antibody concentrations in fish from Lake Peurunka were an order of magnitude lower than those of fish in the other lakes and they were the background level of the assay.

Anti-*R. fennica* antibodies were found in both experimental groups A and B. Strong antibody response was noted in group A which was infected with living cercariae. Antibody levels found in this group were similar to those measured in wild roach from lakes containing clams infected with *R. fennica*. In contrast to group A, the response in group B was very weak. The percentage of roach responding to immunization with homogenized parasite was only 27% compared to 63% in group A. In addition, serum antibodies were present only in low concentrations.

The weak response in immunization can not be explained by a small amount of antigen. Williams & Hoole (1992) induced antibody response in roach by using 100 μg of protein from homogenized worm *Ligula intestinalis* and in our experiment fish were given 150 μg of protein from cercariae. The timing of sampling (30 days post-immunization) can not be the reason for low level response in immunization because our earlier studies showed that high levels of specific antibodies can be determined in serum of roach 28 days after i.p. immunization (Aaltonen *et al*. 1994). Further, a weak response is not likely to be due to the poor immunogenicity of the antigen because there was a strong antibody response after the infection with living cercariae (group A). The route of immunization, i.p. injection compared with

infection by living cercariae, is the most probable explanation for the difference in the antibody development.

The banding patterns from Western blotting of the sera from immunized fish and those infected by being exposed to living cercariae suggest differences in the specificity of antibodies. Serum from fish immunized with homogenized cercariae probed with a band below 97 kDa. This band, not probing with serum from fish infected by living cercariae or from control fish, may represent antigenic material from the furcae of cercaria, because in natural infection only the head of the cercariae, not the furcae, penetrates the host (Taskinen *et al*. 1991). Another band just below 66 kDa binds only with antibodies in the serum of fish infected with living cercariae. These antibodies may be directed against compounds secreted by cercariae. The trematode cercariae have penetration glands from which hydrolytic enzymes are excreted (Dawes, 1956). Proteins secreted by *R. fennica* are not known but various developmental stages of another fluke, *Paragonimus* sp. in mammalian hosts secrete enzymes, for instance cathepsin-like cysteine proteinases. These enzymes are suggested to be involved in penetration and lysis of the tissues (Song & Dresden, 1990).

An interesting question arises: does previous infection or immunization give enhanced resistance to *R. fennica*? Protection against *D. spathaceum* by the injection of sonicated metacercariae in rainbow trout was demonstrated by Speed & Pauley (1985) and also acquired immunity was reported after consecutive exposures (Höglund & Thuvander, 1990). Our results also suggest that previous infection can confer protection against the parasite. This is supported by 2 observations. First, the roach reacted against *R. fennica* by developing antibodies capable of recognizing cercarial structures. Secondly when challenged with cercariae, the number of metacercarial cysts on fins did not increase. If the fish were not able to prevent new parasites from attaching to fish the number of cysts should have increased. We found that a smaller percentage of fish were strongly infected after the challenge (14 days later) than at the time of the challenge infection (day 1). However, the proportion of strongly infected fish should not decrease markedly in 2 weeks, because metacercarial cysts are stable structures. Parasites, including digeneans, are generally aggregated in their fish host (see Anderson, Whitfield & Dobson, 1978) and the decreased numbers of metacercariae noted after the challenge infection may be explained by an uneven distribution of cercariae already in the primary infection.

When infecting a host, cercariae must penetrate the skin. During penetration the cercariae contact with immune cells in the blood and in the skin resulting in the synthesis of antibodies. Antibodies against pathogenic organisms are found in serum and also in the mucus of fish (Ingram, 1980; Peleteiro & Richards, 1985). It is not possible to say whether the protection against *R. fennica* is due only to antibodies. Other defence mechanisms, such as cell-mediated immunity or non-specific humoral factors, may have been activated. For example activation of trout macrophages increase larvicidal activity for diplostomules *in vitro* if the larvae are opsonized with immune serum (Whyte, Chappell & Secombes, 1989). Also, other serum components like complement or lysozyme have been found to have effects against the protection of *D. spathaceum* (Whyte, Chappell & Secombes, 1990). The role of these other factors should not be ignored as potential resistance mechanisms against *R. fennica* and they need to be studied.

In conclusion, we found that antibodies against *R. fennica* do develop in roach. Specific antibodies were detected in blood of wild, naturally infected fish and this finding was verified by performing experimental infections in aquaria. The specificity of the antibodies depended on the route of immunization i.e. via intraperitoneal injection or natural infection by living cercariae. Our results suggest that previous infection may confer some protection against the parasite.

We greatly appreciate receiving help from M.Sc. Iva Králova in collecting the cercariae of *R. fennica* and processing the homogenate. We also wish to thank Dr Berne Jones for checking the English of the paper. This research was financed by the Research Council for the Environmental Sciences of the Finnish Academy, by the University of Jyväskylä and by the Graduate School on Fish Biology and Fisheries in Finland.

REFERENCES

AALTONEN, T. M., JOKINEN, E. I. & VALTONEN, E. T. (1994). Antibody synthesis in roach (*Rutilus rutilus*); analysis of antibody secreting cells in lymphoid organs with ELISPOT-assay. *Fish and Shellfish Immunology* **4**, 129–140.

ANDERSON, R. M., WHITFIELD, P. J. & DOBSON, A. P. (1978). Experimental studies of infection dynamics: infection of the definitive host by the cercariae of *Transversotrema patialense*. *Parasitology* **77**, 189–200.

BORTZ, B. M., KENNY, G. E., PAULEY, G. B., GARCIA-ORTIGOZA, E. & ANDERSON, D. P. (1984). The immune response in immunized and naturally infected rainbow trout (*Salmo gairdneri*) to *Diplostomum spathaceum* as detected by enzyme-linked immunosorbent assay (ELISA). *Developmental and Comparative Immunology* **8**, 813–822.

BRADFORD, M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.

BRUMMER-KORVENKONTIO, H., VALTONEN, E. T. & PUGACHEV, O. N. (1991). Myxosporea parasites in roach, *Rutilus rutilus* (Linnaeus), from four lakes in central Finland. *Journal of Fish Biology* **38**, 573–586.

CHAPPELL, L. H., HARDIE, L. J. & SECOMBES, C. J. (1994). Diplostomiasis: the disease and host parasite interactions. In *Parasitic Diseases of Fish* (ed. Pike, A. W. & Lewis, J. W.), pp. 59–86. Tresaith, Dyfed: Samara Publishing Ltd.

COTTRELL, B. (1977). The immune response of plaice (*Pleuronectes platessa* L.) to the metacercariae of *Cryptocotyle lingua* and *Rhipidocotyle johnstonei*. *Parasitology* **74**, 93–107.

DAWES, B. (1956). *The Trematoda*, 2nd edn. Cambridge University Press, Cambridge.

GIBSON, D. I., TASKINEN, J. & VALTONEN, E. T. (1992). Studies on bucephalid digeneans parasitising molluscs and fishes in Finland. II The description of *Rhipidocotyle fennica* n.sp. and its discrimination by principal components analysis. *Systematic Parasitology* **23**, 67–79.

HÖGLUND, J. & THUVANDER, A. (1990). Indications of non-specific protective mechanisms in rainbow trout *Oncorhynchus mykiss* with diplostomosis. *Diseases of Aquatic Organisms* **8**, 91–97.

INGRAM, G. A. (1980). Substances involved in the natural resistance of fish to infection – A review. *Journal of Fish Biology* **16**, 23–60.

JOKINEN, E. I., AALTONEN, T. M. & VALTONEN, E. T. (1995). Subchronic effects of pulp and paper mill effluents on the immunoglobulin synthesis of roach, *Rutilus rutilus*. *Ecotoxicology and Environmental Safety* **32**, 219–225.

KOSKIVAARA, M., VALTONEN, E. T. & PROST, M. (1991*a*). Dactylogyrids on the gills of roach in Central Finland: features of infection and species composition. *International Journal for Parasitology* **21**, 565–572.

KOSKIVAARA, M., VALTONEN, E. T. & PROST, M. (1991*b*). Seasonal occurrence of gyrodactylid monogeneans on the roach (*Rutilus rutilus*) and variations between four lakes of differing water quality in Finland. *Aqua Fennica* **21**, 47–55.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.

PELETEIRO, M. C. & RICHARDS, R. H. (1985). Indentification of lymphocytes in the epidermis of the rainbow trout, *Salmo gairdneri* Richardson. *Journal of Fish Diseases* **8**, 161–172.

SONG, C. Y. & DRESDEN, M. H. (1990). Partial purification and characterization of cysteine proteinases from various developmental stages of *Paragonimus westermani*. *Comparative Biochemistry and Physiology* **95**, 473–476.

SPEED, P. & PAULEY, G. B. (1985). Feasibility of protecting rainbow trout, *Salmo gairdneri* Richardson, by immunizing against the eye fluke, *Diplostomum spathaceum*. *Journal of Fish Biology* **26**, 739–744.

TASKINEN, J. & VALTONEN, E. T. (1995). Age-, size- and sex-specific infection of *Anodonta piscinalis* (Mollusca: Unionidae) with *Rhipidocotyle fennica* (Digenea: Bucephalidae) and its influence on host reproduction. *Canadian Journal of Zoology* **73**, 887–897.

TASKINEN, J., VALTONEN, E. T. & GIBSON, D. I. (1991). Studies on bucephalid digeneans parasitising molluscs and fishes in Finland. I Ecological data and experimental studies. *Systematic Parasitology* **19**, 81–94.

TASKINEN, J., VALTONEN, E. T. & MÄKELÄ, T. (1994). Quantity of sporocysts and seasonality of two *Rhipidocotyle* species (Digenea, Bucephalidae) in *Anodonta piscinalis* (Mollusca: Bivalvia). *International Journal for Parasitology* **24**, 877–886.

THOMAS, P. T. & WOO, P. T. K. (1995). Immunological approaches and techniques. In *Fish Diseases and Disorders*, *Vol. 1 Protozoan and Metazoan Infections* (ed. Wood, P. T. K.), pp. 751–771. Cambridge University Press, Cambridge.

THUVANDER, A., HONGSLO, T., JANSSON, E. & SUNDQUIST, B. (1987). Duration of protective immunity and antibody titres measured by ELISA after vaccination of rainbow trout, *Salmo gairdneri* Richardson, against vibriosis. *Journal of Fish Biology* **10**, 479–486.

VALTONEN, E. T., HOLMES, J. C. & KOSKIVAARA, M. (1997). Eutrophication, pollution and fragmentation: effects on the parasite communities in roach (*Rutilus rutilus*) and perch (*Perca fluviatilis*) in four lakes in Central Finland. *Canadian Journal of Fisheries and Aquatic Sciences* (in the Press).

WHYTE, S. K., ALLAN, J. C., SECOMBES, C. J. & CHAPPELL, L. H. (1987). Cercariae and diplostomules of *Diplostomum spathaceum* (Digenea) elicit an immune response in rainbow trout, *Salmo gairdneri* Richardson. *Journal of Fish Biology* **31** (Suppl. A), 185–190.

WHYTE, S. K., CHAPPELL, L. H. & SECOMBES, C. J. (1989). Cytotoxic reactions of rainbow trout, *Salmo gairdneri* Richardson, macrophages for larvae of the eye fluke *Diplostomum spathaceum* (Digenea). *Journal of Fish Biology* **35**, 333–345.

WHYTE, S. K., CHAPPELL, L. H. & SECOMBES, C. J. (1990). Protection of rainbow trout, *Oncorhynchus mykiss* (Richardson), against *Diplostomum spathaceum* (Digenea): the role of specific antibody and activated macrophages. *Journal of Fish Diseases* **13**, 281–291.

WILLIAMS, M. A. & HOOLE, D. (1992). Antibody response of the fish *Rutilus rutilus* to the metacestoda of *Ligula intestinalis*. *Diseases of Aquatic Organisms* **12**, 83–89.

WOOD, B. P. & MATTHEWS, R. A. (1987). The immune response of the thick-lipped grey mullet, *Chelon labrosus* (Risso, 1826), to metacercarial infections of *Cryptocotyle lingua* (Creplin, 1825). *Journal of Fish Biology* **31** (Suppl. A), 175–183.