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Rainbow trout leucocyte activity: influence on the ectoparasitic monogenean *Gyrodactylus derjavini*

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ABSTRACT: The ectoparasitic monogenean *Gyrodactylus derjavini* from rainbow trout *Oncorhynchus mykiss* was exposed *in vitro* to macrophages isolated as peritoneal exudate cells or as pronephros cells from the host. Cells colonized the parasite especially in the mannose-rich regions in the cephalic ducts where ciliated structures were abundant. Opsonization with fresh serum, in contrast to heat-inactivated serum, enhanced colonization also on other body parts. The adverse effect of the activated macrophages towards *G. derjavini* was associated with a heat-labile component released from these cells to the culture medium. Analysis of substances released from the cells showed reactivity for a number of enzymes, complement factor C3, interleukin (Il-1) and reactive oxygen metabolites. Chemotaxis assays with pronephric leucocytes showed chemoattractants in *G. derjavini*, and the respiratory burst level of macrophages was slightly elevated due to parasite exposure. It is suggested that skin leucocytes contribute to an increased level of complement factors in the trout skin during the host response, whereby a hostile microenvironment for the parasites is created. In addition, the IL-1 production could affect mucous cell secretion and hyperplasia and add to the antiparasitic action of the epithelium. Likewise, reactive oxygen metabolites and various enzymes are likely to be involved in the skin response.

KEY WORDS: Leucocyte · Complement · Interleukin · Respiratory burst · Rainbow trout · Parasite · Monogenean · *Gyrodactylus*

INTRODUCTION

It is well documented that fish are able to mount immune responses against various parasitic pathogens (Secombes & Chappell 1996), but effector mechanisms responsible for the host response of teleosts to infections with ectoparasitic monogeneans are still in dispute. It has been suggested that both cellular and humoral factors are involved in immunity against monogeneans infecting fragile gill tissue with superficial capillaries (Vladimirov 1971, Buchmann 1988, 1993). Correspondingly, studies have detected a number of immunologically important substances in the mucus and epithelium of rainbow trout infected with *Gyrodactylus derjavini* (Buchmann & Bresciani 1998). Although antibodies specific to monogenean antigens have been detected in infected fish (Buchmann 1993, Wang et al. 1997), these molecules are considered to be of minor importance in the response to gyrodactylid monogeneans (Buchmann 1998). Of particular importance is the presence of complement in the infected skin. Complement has been shown to possess a devastating effect on gyrodactylids (Harris et al. 1997, Buchmann 1998) and is known to be produced by macrophages at least in mammals (Lappin & Whaley 1993). As gyrodactylids are seen to escape surface areas rich in mucous cells and macrophages during the response phase (Buchmann 1997, Buchmann & Bresciani 1998, Lindenstrøn & Buchmann 1998), it could be suggested that macrophages in the skin have some influence on the amount of complement and mucous cells in the fish skin and thereby affect the microenvironment of these ectoparasites. Some studies have shown that fish leucocytes are able to interact with both protozoans

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(Cross & Matthews 1993, Steinhagen & Hespe 1997) and metazoan parasites as cestodes (Sharp et al. 1991, Hoole 1994, Taylor & Hoole 1995, Morley & Hoole 1997), digeneans (Wood & Matthews 1987, Whyte et al. 1989), nematodes (Ramakrishna et al. 1993) and acanthocephalans (Hamers et al. 1992). The relationship between monogeneans and fish leucocytes is nonetheless still unknown. The present study was therefore conducted to elucidate the effect of macrophages and their products on the survival of *G. derjavini* infecting the rainbow trout skin.

MATERIALS AND METHODS

Parasites. A laboratory strain of *Gyrodactylus derjavini* kept on rainbow trout (11 to 12 cm body length) in freshwater at 11°C was used. Following anaesthetization of fish in tricaine methanesulphonate (MS 222) (80 mg l^{-1}), fish were killed and their fins were dissected and transferred to water-filled glass beakers (4 ml) and gently scraped with a needle. Parasites were allowed to move to the bottom of the beaker, whereafter fin debris and mucus were removed with a pipette. After several exchanges of water during 1 h, dead or inactive parasites were removed. Only actively moving parasites were used for experimentation.

Parasite extract. Living parasites were transferred to Leibovitz-15 (L-15, Gibco) medium or water in Eppendorf tubes and subjected to at least 10 freezing/thawing cycles followed by mechanical disruption with the aid of a pipette. The supernatant was used for chemotaxy and/or respiratory burst studies.

Determination of optimal temperature for experimentation. In order to determine the optimal experimental temperature for *in vitro* exposure of *Gyrodactylus derjavini* duplicate glass beakers containing 4 to 7 parasites were placed at 3, 11, 17, 22, 25 and 30°C. Parasite activity was then recorded with 2 to 6 h interval for 5 d.

Serum. Blood samples were drawn from the caudal veins in 6 uninfected rainbow trout (17 to 20 cm total body length). Following clotting (1 h at 11°C), blood was centrifuged and serum recovered. In order to produce zymosan-activated serum (ZAS), zymosan (Sigma Z-4250) was added to pooled serum (10 mg ml^{-1}) and incubated at room temperature (RT) for 60 min followed by centrifugation at 700 × *g* (5 min). The supernatant was recovered and used immediately for chemotaxis assays.

Isolation of macrophages for parasite incubation. Five uninfected rainbow trout (18 to 20 cm body length) were injected intraperitoneally with 0.2 ml squalene (Sigma S-3626) (Klesius & Sealey 1996) 3 to 4 wk prior to cell harvest. Fish were then injected intraperitoneally with 5 ml Eagle's Minimal Essential Medium (MEM) (Sigma M-0643), whereafter the medium containing peritoneal exudate cells was harvested with a Pasteur pipette through a body wall incision. The recovered cells were pooled, whereafter the suspension was layered on a discontinous Percoll (Pharmacia) gradient (density 1.06 to 1.07) and subjected to centrifugation (300 × *g*) for 15 min. The cells in the interface band were removed and transferred to MEM, centrifuged (300 × *g*) for 15 min and then resuspended in MEM for use in parasite exposure assays (see Table 1). Cells to be tested for enzyme activity were washed and resuspended in Hank's Balanced Salt Solution (HBSS) containing penicillin (100 U ml^{-1}) and streptomycin (100 µg ml^{-1}). The cell concentration was determined with a Neubauer haemocytometer and adjusted to 70 000 cells ml^{-1} both for parasite exposure and enzyme tests. Samples of cells were spread on slides, air dried, fixed in ethanol and stained with Giemsa for subsequent study of cell morphology. Unfixed cells on slides were tested for non-specific esterase activity with the α-naphthyl acetate assay (Sigma kit 91-A).

Incubation of cells. Cells were used immediately for parasite exposure or incubated in sterile glass beakers (4 ml) for 8 h, whereafter the supernatant was recovered for parasite exposure or analysis. Cover slips were incubated with cells in 3 beakers for 3 to 8 h, whereafter they were fixed for scanning electron microscopy (SEM) as described below.

Exposure of parasites. Parasites were exposed (at 11°C) in duplicate groups each comprising 4 to 7 parasites in 4 ml glass beakers containing 0.5 ml medium according to Table 1. Thus, *Gyrodactylus derjavini* (opsonized in undiluted serum or untreated) were incubated with 70 000 cells, undiluted supernatant from cells (untreated or heat inactivated), with tissue culture medium alone or tap water. Heat inactivation of serum and supernatants was performed by incubating at 44°C for 20 min (Sakai 1992). Opsonization of parasites in serum from uninfected fish (untreated or heat-inactivated) was conducted for 5 min.

Chemotaxis assays. In order to obtain a sufficient number of macrophages for cell migration studies the head kidneys from 4 rainbow trout were dissected and transferred to Leibovitz-15 (L-15, Gibco) medium with 100 U penicillin ml^{-1}, 100 µg streptomycin ml^{-1}, 10 U heparin ml^{-1} and 0.1% inactivated (56°C, 30 min) foetal calf serum (FCS). Following maceration of the pronephros through a stainless steel mesh, the cell suspension was layered on a discontinous Percoll gradient (34%/51%) and centrifuged (300 × *g*) for 15 min (Secombes 1990). The macrophages were collected from the interface, resuspended and washed in L-15, whereafter the cell concentration was adjusted to

10^6 cells ml^{-1}. Migration of macrophages towards chemoattractants was investigated in a modified Boyden chamber containing 48 wells. In the lower compartment 29 µl chemoattractant was added. A transparent polycarbonate membrane (Poretics Products no. 10469, CA, USA) with 5 µm pores was placed between the lower and upper compartment. In the latter 50 µl cell suspension (50 000 cells) was applied and the chamber was incubated in a humid chamber for 90 min at 17°C. Thereafter, the upper solution was removed with a pipette. Filters were scraped on the shiny upper side (Jang et al. 1995) to remove adhering cells from the upper chamber. They were then inverted and mounted on a slide with colourless nailpolish, stained with Giemsa and studied under a microscope (400× magnification). The number of cells were counted in 3 fields from each compartment. As chemoattractants (performed in triplicate), the following were used: fresh trout serum, ZAS, *Gyrodactylus derjavini* (130 emaciated parasites ml^{-1} L-15 medium), *G. derjavini* (21 parasites ml^{-1} water), L-15 medium alone (control) and distilled water.

Respiratory burst activity. Pronephros leucocytes isolated as described above were added to wells in a Nunclon 96 microwell plate (Nunc) (2.5×10^5 cells $well^{-1}$). Following 2 h incubation at 17°C unattached cells were removed in 2 washes with L-15 medium, whereafter parasite extracts in L-15, zymosan (Sigma Z-4250) in L-15 or cell culture medium alone were added according to Table 3. After 1 h incubation at 17°C cells layers were washed once with phenol red-free HBSS, whereafter nitro blue tetrazolium (NBT) (Sigma N-6876) (1 mg ml^{-1}) in HBSS (100 µl $well^{-1}$) was added. For chemical induction of respiratory burst in some wells phorbol myristate acetate (PMA) (Sigma P-8139) was added to a final concentration of 1 µg ml^{-1}. Following 30 min incubation the absorbance was read at 690 nm (Secombes 1990).

Analysis of enzyme activity in peritoneal macrophages. The enzymatic profile was determined for 19 enzymes (see Table 4) with API-ZYM (BioMerieux, France) by adding 65 µl cell suspension to each well, incubating for 12 h at RT, and developing according to the manufacturers instructions. Controls were conducted for all enzymes with HBSS.

Proteins in supernatant. The presence of various proteins (see Table 5) was detected with a dot-blot method at RT. Supernatant (3 µl) was applied to nitrocellulose membranes (Sartorius, Göttingen, Germany) (1 × 1 cm) and allowed to air-dry. Membranes were then blocked with phosphate-buffered saline (PBS) pH 7.1 containing 2% bovine serum albumin (BSA) for 30 min. Incubations with primary antibodies diluted in blocking buffer (see Table 4) were conducted for 2 h, followed by 2 × 10 min wash in PBS with 0.05% Tween 20. Peroxidase-conjugated swine anti rabbit antibody (DAKO P217) (1:2000) was then applied for 1 h, whereafter the membranes were rinsed 2 × 10 min in PBS/Tween 20 before development with DAB (DAKO 3000) and hydrogen peroxide. Positive dots were brown.

SEM. Parasites recovered from the different exposure groups were rinsed shortly in PBS and fixed in 2.5% cacodylate buffered glutaraldehyde for at least 7 d. They were then postfixed in osmium tetroxide, critically point dried, sputtered with gold and studied in a JEOL scanning electron microscope.

Statistics. Differences between mean numbers of cells (chemotaxis) and between mean absorbance values (respiratory burst assays) were evaluated with the Mann-Whitney *U*-test with a probability level of 0.05.

RESULTS

Experimental temperature

In vitro survival of parasites in freshwater was a few hours at 30 and 25°C, 24 h at 22°C, 48 h at 17°C, and 112 h at 11 and 3°C. The number of living parasites increased transiently in most groups due the release of offspring by the viviparous gyrodactylids (Fig. 1).

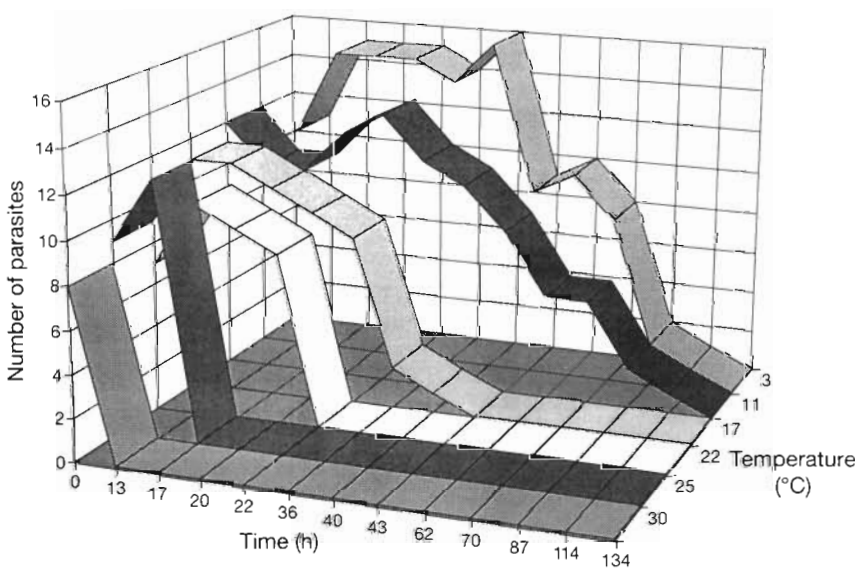

Fig. 1. *Gyrodactylus derjavini*. Survival (*in vitro*) in water at various temperatures

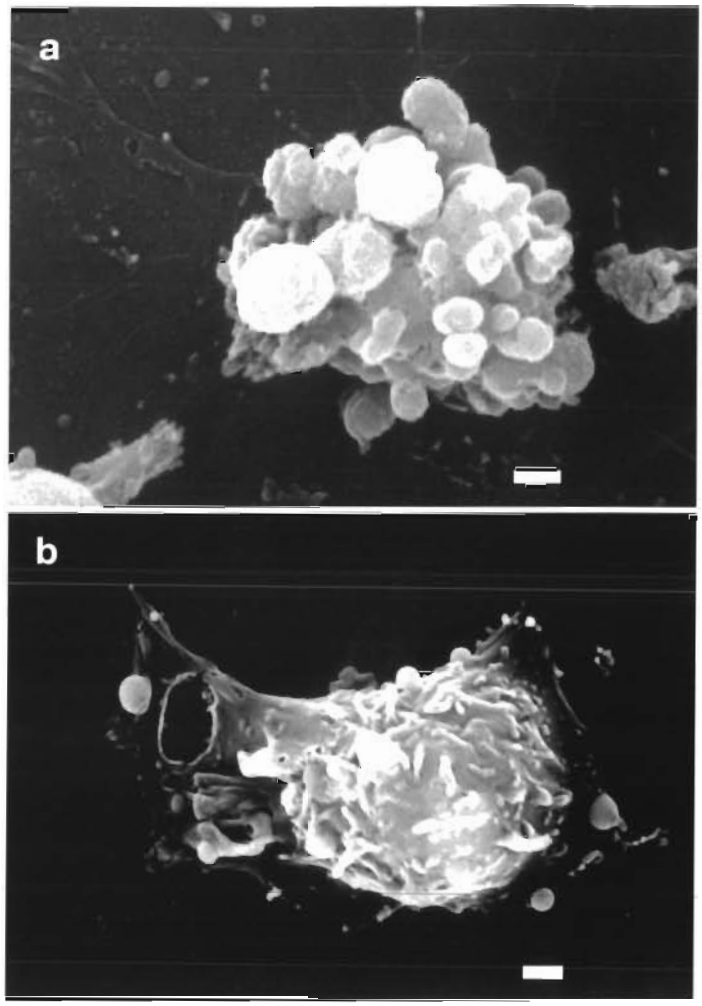

Fig. 2. Macrophages from *Oncorhynchus mykiss*. Different shapes of macrophages adhering to coverslips (3 h incubation). Scale bars = 1 µm. (a) Cell with numerous blebs. (b) Cell showing spreading and pseudopodia

Cells

The majority of the isolated peritoneal exudate cells fixed on slides contained a round to elongated nucleus with a varying but significant amount of cytoplasma and stained positive for non-specific esterases. The cell diameter varied between 6 and 18 µm. Few peritoneal cells (1 to 2%) showed the characteristic multilobulated nucleus of neutrophil leucocytes, whereas these cells comprised at least 10% of the pronephric leucocytes. When colonizing untreated coverslips, the peritoneal cells were seen adhering to the substrate and exhibiting pseudopodia and surface blebs, probably resulting from vacuoles (Fig. 2).

Exposure to macrophages

Control parasites showed the characteristic and intact monogenean surface without adhering cells (Fig. 3a). The cephalic duct openings exhibited an inner surface rich in microvilli (Fig. 3b). Parasites survived less than 3 h when exposed to peritoneal

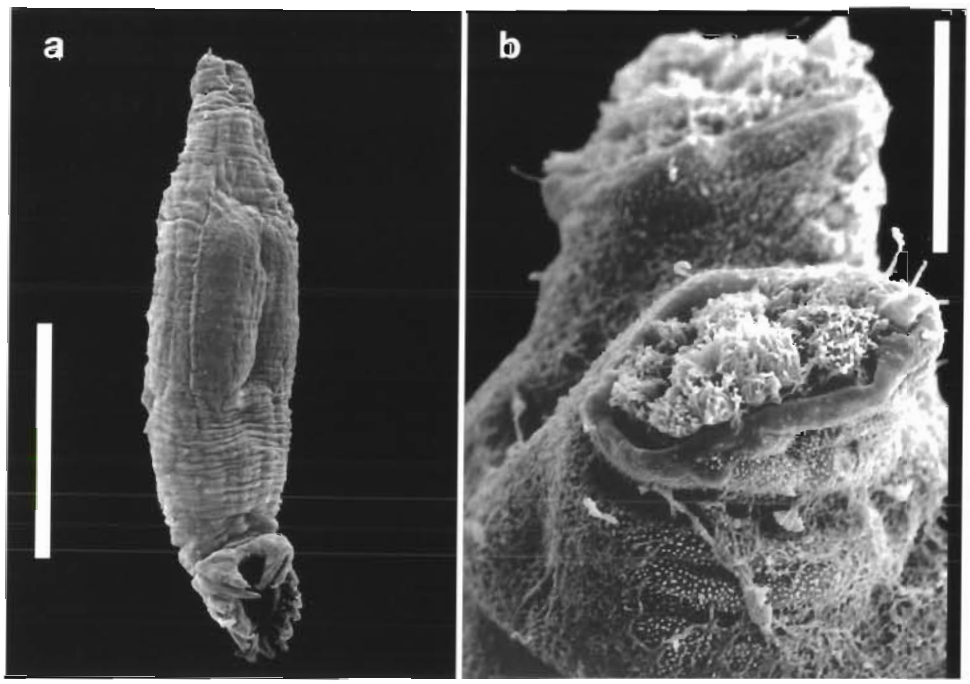

Fig. 3. *Gyrodactylus derjavini*. Intact specimen incubated in water 3 h. (a) Total body. Scale bar = 100 µm. (b) Cephalic gland openings showing numerous cilia and microvilli. Scale bar = 10 µm

Table 1. *Gyrodactylus derjavini*. *In vitro* exposure of parasites. iC: heat-inactivated serum, C: untreated serum, iS: heat-inactivated supernatant, S: untreated supernatant from activated macrophage-suspension, nac: non-activated pronephric cells

Group	No. of parasites	Exposure time	Survival time	Macrophage colonization
Parasites in water	5/6	8 h	>8 h	–
Parasites + medium	5/6	8 h	<7 h	–
Parasites + cells	6/7	8 h	<3 h	+
Parasites + cells	5/6	3 h	<3 h	+
Parasites + iC + cells	5/6	3 h	<3 h	+/–
Parasites + C + cells	4/5	10 min	<5 min	–
Parasites + C + cells	7/7	3 h	<5 min	+
Parasites + iS	4/6	3 h	>3 h	–
Parasites + S	5/6	3 h	<3 h	–
Parasites + iS nac	6/7	3 h	>3 h	–
Parasites + S nac	6	3 h	>3 h	–

macrophages or untreated supernatant from peritoneal macrophages at 11°C, whereas heat-inactivated supernatant or culture medium alone resulted in longer survival (Table 1). This adverse effect was not seen when parasites were incubated with cells or supernatant (untreated or heat-inactivated) isolated from the pronephros. Incubation of parasites in serum (for opsonization) with intact complement was lethal for the parasite and had a devastating effect on the surface structure within a few minutes. The tegument appeared disorganized, blebbed, damaged by perforations, and a severe constriction of the forebody was seen (Fig. 4). In contrast, heat-inactivated serum had no adverse effects. Macrophages colonized both opsonized and untreated parasites within 3 to 8 h. Thus, untreated parasites were attacked by macrophages particularly in the cephalic duct openings (Fig. 5a) and to a lesser extent on the opisthaptor within 3 h. Extended exposure resulted in colonization of the parasite body as well (Fig. 5b). Following opsonization of *Gyrodactylus derjavini* in untreated serum, macrophages were seen to attach to the total body of some but not all parasites (Fig. 5c). Parasites incubated with inactivated serum were colonized to a lesser extent. Only few cells attached to these and only in the anterior part of the monogenean. Parasites exposed to cell culture medium or water alone were not colonized.

The behaviour of parasites in the presence of macrophages was different compared to parasites in water or cell-free systems. In the last 2 cases *Gyrodactylus derjavini* readily attached (by the opisthaptor or the cephalic openings) to the glass surface in the incubation beaker and moved in a leech-like manner. When macrophages were present, parasites were often observed to avoid contact (i.e. attachment by use of opisthaptor and cephalic openings) with the substrate, whereby *G. derjavini* mostly was seen lying in the beaker.

Chemotaxis assay

The highest number of macrophages on the Boyden chamber membrane was counted in the ZAS assays. Both fresh serum and *Gyrodactylus derjavini* in L-15 affected macrophages to a lesser but still significant ($p < 0.05$) extent. The aqueous solution of parasite substances had a negligible effect (Table 2).

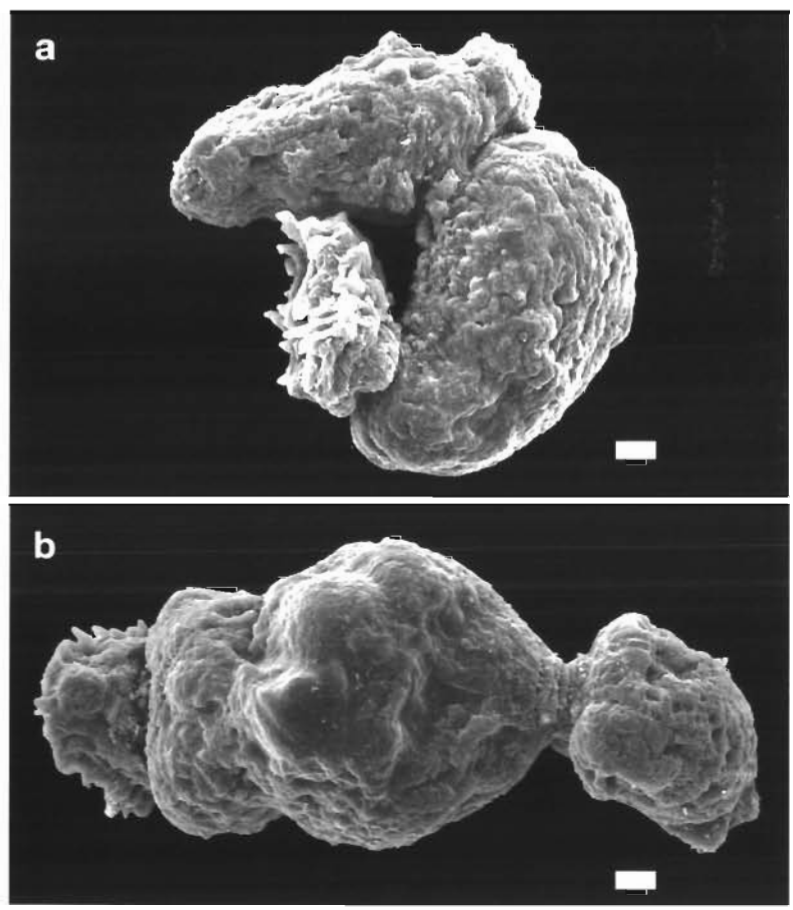

Fig. 4. *Gyrodactylus derjavini*. Parasite treated with complement-containing serum for 5 min showing constriction in the forebody. Scale bars = 10 µm. (a) Lateral view. (b) Dorsal view

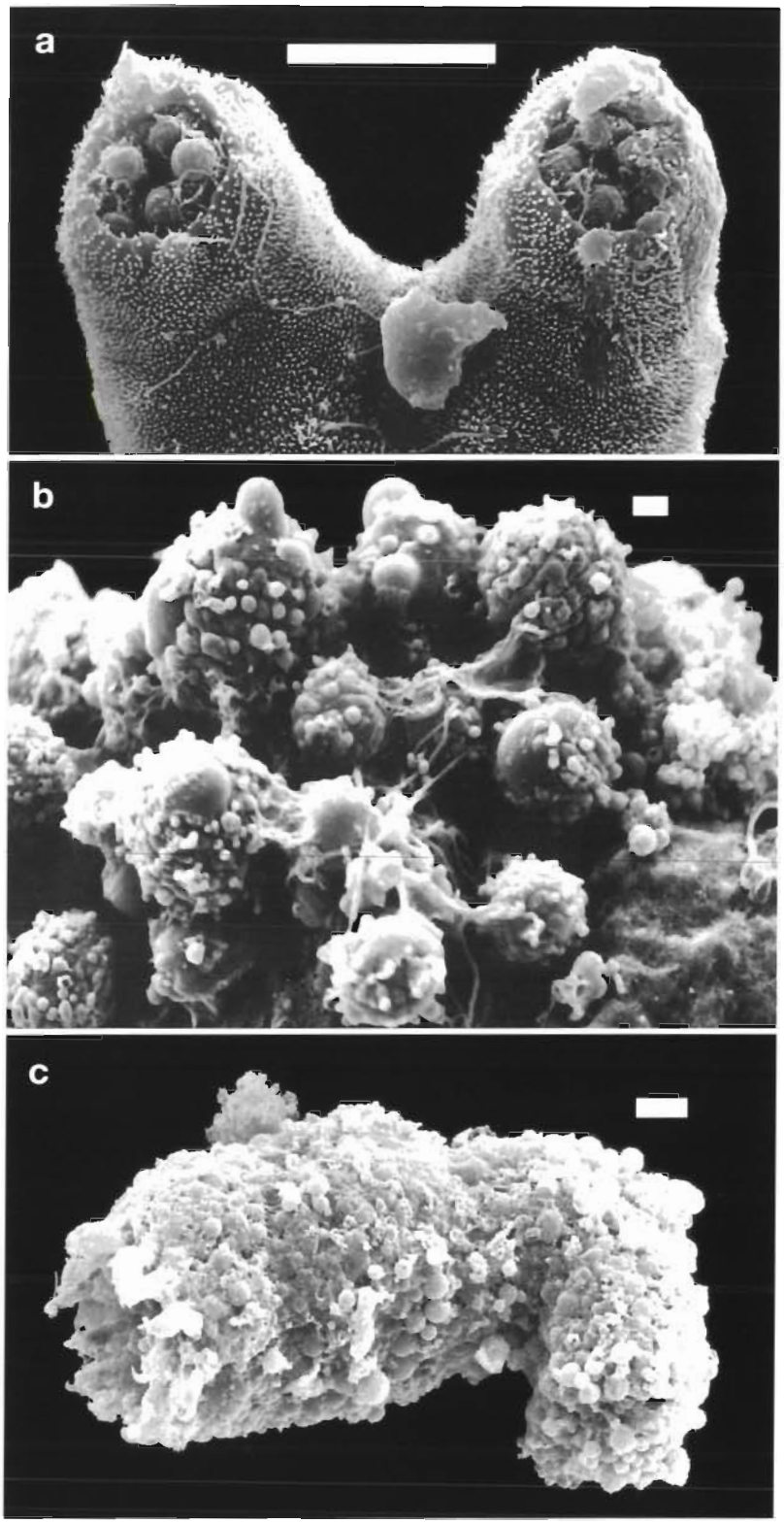

Fig. 5. *Gyrodactylus derjavini*. (a) Macrophages adhering to the cephalic duct openings of untreated parasite (3 h incubation). Scale bar = 10 µm. (b) Forebody of untreated *G. derjavini* partly covered by cells (8 h incubation). Scale bar = 1 µm. (c) Macrophages covering the total body of parasite opsonized in untreated serum (3 h cell incubation). Scale bar = 10 µm

Respiratory burst assay

The respiratory burst assay showed that zymosan induced a dose-dependent NBT reduction in pronephric leucocytes. Parasite extract caused a slight but significantly ($p < 0.05$) elevated NBT reduction, indicating the presence of reactive oxygen metabolites (ROM, Table 3).

Enzyme reactions

Positive enzyme reactions were found for 8 of 19 tested enzymes (Table 4). Strong reactions in the Apizym-test were seen for acid phosphatase, esterases, phosphohydrolase, glucuronidase, glucosaminidase and arylamidases. Cells were also esterase-positive when tested on slides.

Proteins and peptides

The dot-blot test showed significant immunoreactivity in the peritoneal macrophage supernatant for complement factor C3 and a very weak reaction for Il-1. No reaction for other substances were found (Table 5).

DISCUSSION

As the *in vitro* survival of non-exposed *Gyrodactylus derjavini* was of longest duration at temperatures between 11 and 3°C, the former temperature was selected for the macrophage experiments for 3 reasons: First, the natural survival time of parasites should be substantial to reveal any effect of experimental exposure. Second, immunoreactivity of trout is better between 10 and 20°C compared to the interval 1–5°C (O'Neil 1985, Andersen & Buchmann 1998); and third, rainbow trout are capable of mounting a significant response to *G. derjavini* at 11°C (Buchmann 1997, Buchmann & Bresciani 1998). Under these conditions it was shown that peritoneal macrophages were able to colonize *G. derjavini* within 3 h whether or

Table 2. Chemotaxis assays. Number of macrophages on the polycarbonate membrane following 90 min incubation at 17°C with chemoattractants in the lower compartment of a modified Boyden chamber. Mean and SD of 9 fields (400× magnification) counted. *Significantly different from controls ($p < 0.05$)

Chemoattractant	Mean no. of cells field^{-1}	SD
Zymosan activated serum (ZAS)	73.3*	16.2
Zymosan activated serum (ZAS)	100.8*	30.5
Fresh untreated serum	49.3*	3.8
Fresh untreated serum	47.6*	23.7
Gyrodactylus derjavini L-15	50.3*	12.3
Gyrodactylus derjavini water	7.6	10.5
Control (water)	9.0	1.7
Control (L-15 medium)	8.0	1.0
Control (L-15 medium)	1.0	0.7

not the parasite was opsonized with rainbow trout serum. Similar colonization of helminths of the genera *Diplostomum* and *Sanguinicola* by fish leucocytes were reported by Whyte et al. (1989) and Richards et al. (1996a), respectively. In addition, the presence of peritoneal macrophages in the incubation medium was lethal for the parasites and this adverse effect of activated macrophages was at least partly related to the contents of the cell culture supernatant. This is in accordance with the adverse effect of leucocyte culture medium towards the acanthocephalan *Paratenuisentis ambiguus* described by Hamers et al. (1992). When the supernatant was partially analyzed, the presence of complement factor C3 was detected. As the alternative complement factors have been shown lethal to *G. derjavini* (Buchmann 1998), this component could explain the lethal effect of the supernatant. This was stressed by the heat lability of the lethal factor in the cell culture supernatant. Further, during the opsonization process in the present study the devastating effect of complement containing serum was confirmed, as evidenced by the SEM studies showing damaged and perforated parasites. Production of complement factor C3 by monocytes and macrophages has previously been described in human and murine systems, and these cells are considered to be the main extra-hepatic source of complement (Lappin & Whaley 1993). It is noteworthy that untreated supernatant from non-activated pronephric leucocytes did not show any lethal effect to the parasites within 3 h. The possibility cannot, therefore, be ruled out that the squalene injection used for the isolation activates the peritoneal macrophages to a certain extent. Collection of peritoneal exudate cells after injection with Freund's incomplete adjuvant also resulted in cells with a certain state of activation (Chung & Secombes 1987). Thus, the collection procedure and the source of cells are likely to influence the result of *in vitro* exposure of parasites to host cells. The proportion of neutrophils was lower in the peritoneal harvest compared to the pronephros, providing a more uniform composition of cells. In addition, concentration of cells on a Percoll gradient (as conducted in this study) excludes lymphocytes normally found in peritoneal lavages (Afonso et al. 1997).

It is not known whether macrophages found in the cephalic ducts were attracted to the mannose-rich regions in this head organ as demonstrated by Buchmann (1998) or if some other factors present in those structures were responsible for macrophage attachment. However, the rich concentration of not previously described cilia and microvilli in the cephalic ducts demonstrated in this work is noteworthy and may be associated with mannose. Fish leucocytes are known to possess receptors for carbohydrates, including mannose-specific receptors (Saggers & Gould 1989, Dannevig et al. 1994). However, as we know that C3 attach readily to the mannose-rich regions of the parasite (Buchmann 1998) and fish leucocytes carry C3 receptors (Johnson & Smith 1984, Saggers & Gould 1989, Matsuyama et al. 1992), another explanation is possible. The macrophages could attach to C3 (released by the macrophages) which is binding to the mannose-rich regions in the ducts. Through the release of C3 from the cells the invading target would be opsonized and accessible for attachment. Although C3 produced by macrophages in the fish skin could play an important role in the host response to gyrodactylids, the rich enzyme activity in rainbow trout macrophages is notable. Esterases, arylamidases and and phosphatases could have adverse effects on para-

Table 3. Respiratory burst activity evidenced by nitro blue tetrazolium (NBT) reduction in pronephros leucocytes from rainbow trout following exposure to phorbol myristate acetate (PMA), zymosan or *Gyrodactylus derjavini* extract. Absorbance (abs) detected at 690 nm. *Significantly different from control ($p < 0.05$)

	PMA (1 µg ml^{-1})	Zymosan (0.5 mg ml^{-1})	Zymosan (2.0 mg ml^{-1})	Zymosan (5.0 mg ml^{-1})	*G. derjavini* extract	Control (L-15)
No. of wells	8	2	2	2	8	8
Mean abs	0.17*	0.16	0.25	0.61	0.11*	0.09
SD	0.01	0.01	0.01	0.09	0.02	0.02

Table 4. Enzyme activity in peritoneal macrophages. Enzyme reactions are ranked on a scale from 0 (no reaction) to 5 (strong reaction)

Enzyme	Reaction
Alcaline phosphatase	1
Esterase	3
Esterase lipase	3
Lipase	1
Leucine arylamidase	5
Valine arylamidase	5
Cystine arylamidase	1
Trypsin	0
Chymotrypsin	0
Acid phosphatase	5
Naphthol-AS-BI-phosphohydrolase	4
Galactosidae alpha	0
Galactosidae beta	1
Glucuronidase alpha	4
Glucosidase alpha	0
Glucosidase beta	0
N-acetyl-beta-glucosaminidase	3
Mannosidase alpha	0
Fucosidase alpha	0
Control	0

site performance. It is noteworthy that activities of these enzymes (among others) were detected in extracts of mucus and epithelium from rainbow trout fins (Buchmann & Bresciani 1998). A number of studies have previously shown activity due to esterases and/or acid phosphatases (Meseguer et al. 1994, Dalmo & Seljelid 1995, Wang et al. 1995, Afonso et al. 1997, Sørensen et al. 1997) in fish leucocytes.

The cytokine production by macrophages is also likely to be of importance. It is suggested that the neighbouring mucous cells proliferate (Balm et al. 1995) and secrete their products due to the release of Il-1 from macrophages (Cohan et al. 1991). The presence of IL-1-like molecules has been seen in blood from various fishes (Secombes et al. 1996), and infected rainbow trout skin (Buchmann & Bresciani 1998), and production of IL-1 by macrophages was previously detected in carp (Verburg-van Kemenade et al. 1995). A possible involvement of TNF (tumor necrosis factor; Jang et al. 1995) in this system cannot be rejected, but no reaction with rabbit anti-human TNF was found in the present study on the macrophage supernatant.

Table 5. Dot-blot immunoreactivity in supernatant from macrophage suspension after 8 h incubation. –: no reaction, +: positive reaction, (+): weak reaction

Rabbit antibody to:	Source	Dilution	Reaction
Somatostatin	DAKO N-1551	Prediluted	–
ACTH	DAKO N-1531	Prediluted	–
Prolactin	DAKO N-1549	Prediluted	–
Human CG	DAKO N-1534	Prediluted	–
Synaptophysin	DAKO N-1566	Prediluted	–
Human IL-1 alpha	Sigma I-5018	1:500	(+)
Human TNF-alpha	Sigma T-8300	1:500	–
Human CRP	DAKO A-073	1:100	–
Salmon Ig	Buchmann & Pedersen (1994)	1:1000	–
Rainbow trout C3	Jensen & Koch (1992)	1:1000	+

The chemotaxis assays confirm previous studies. Griffin (1984) showed that trout leucocytes were more attracted by fresh serum than by heat-inactivated serum. In addition, activated fish serum attracts leucocytes in all likelihood through the release of C3a and C5a (Obenauf & Smith 1992), corresponding to the enhanced cell migration towards ZAS in the present work. Bacterial pathogens have been demonstrated as potent chemoattractants for leucocytes (Lamas & Ellis 1994, Weeks-Perkins & Ellis 1995, Klesius & Sealey 1996), and metazoan products are known to attract fish leucocytes (Hamers et al. 1992). Likewise, Richards et al. (1996b) recorded a marked polarization of carp leucocytes in response to *Sanguinicola inermis*. The present study showed that substances from *Gyrodactylus derjavini* will induce membrane attachment of leucocytes and initiate migration.

Respiratory burst of macrophages and neutrophils induced by bacteria have been described widely (Sharp & Secombes 1993, Lamas & Ellis 1994). Extracts from *Gyrodactylus derjavini* were seen to elicit a weak production of ROM as evidenced by NBT reduction in leucocytes. Although this reaction was relatively low compared to the action of zymosan, the possibility that ROM could be a part of the host response against gyrodactylids cannot be ruled out. This would correspond to the respiratory burst induction in rainbow trout against other metazoan parasites, as shown for *Diplostomum spathaceum* (Whyte et al. 1989) and *Diphyllobothrium dendriticum* (Sharp et al. 1991).

The results, therefore, support the suggestion that skin immunity in rainbow trout towards *Gyrodactylus derjavini* infection is based on an initial activation of epithelial cells and leucocytes in the host epidermis. Subsequently, complement and IL-1 are released from these cells. The cytokine in turn induces hyperplasia and mucus secretion from goblet cells as the first line of defence. The complement factors produced bind to carbohydrate epitopes of the parasite, which is adversely affected by these molecules. Whether or not macrophages bind to the parasites *in vivo* is not known but the possibility cannot be excluded at present. At least the present study has shown that fish macrophages are able to interact with molecules or structures in *G. derjavini*,

which is biologically relevant. The ROM and enzymes produced by these cells could take part in the response. As a result the parasite is suggested to detach from the skin or colonize other skin areas (cornea and tail fin) where macrophages and mucous cells are less abundant (Buchmann & Bresciani 1998). The pivotal role of complement (Harris et al. 1997, Buchmann 1998) corresponds well with studies on the host response to *Cryptobia salmositica* by Woo (1996). As the type and amount of this host factor also will explain differences in innate resistance (within or between species) to parasitic flagellates (Woo 1996), it is now relevant to suggest that similar conditions exist in salmonid/gyrodactylid relations. Thus, the observed difference in susceptibility of Baltic and Atlantic strains of *Salmo salar* to *G. salaris* (Bakke et al. 1991) and of various salmonids to *G. derjavini* (Buchmann & Uldal 1987) could at least partly be explained by these molecules. Differences in complement types, complement quantities and complement binding affinities to a certain parasite species could contribute to the observed species difference in susceptibility. The leucocytes in the skin would then, due to their complement production, be one of the responsible factors in this complex problem.

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LITERATURE CITED

Afonso A, Ellis AE, Silva MT (1997) The leucocyte population of the unstimulated peritoneal cavity of rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immunol 7:335–348

Andersen PS, Buchmann K (1998) Temperature dependent population growth of *Gyrodactylus derjavini* on rainbow trout. J Helminthol 72:9–14

Bakke TA, Jansen PA, Hansen LP (1990) Difference in the host resistance of Atlantic salmon, *Salmo salar* L., to the monogenean *Gyrodactylus salaris* Malmberg, 1957. J Fish Biol 37:577–587

Balm PHM, Lieshoute EV, Lokate J, Wendelaar Bonga SE (1995) Bacterial lipopolysaccharide (LPS) and interleukin 1 (IL-1) exert multiple physiological effects in the Tilapia *Oreochromis mossambicus* (Teleostei). J Comp Physiol B 165:85–92

Buchmann K (1988) Interactions between the gill parasitic monogeneans *Pseudodactylogyrus anguillae* and *P. bini* and the fish host *Anguilla anguilla*. Bull Eur Assoc Fish Pathol 8:98–100

Buchmann K (1993) A note on the humoral immune response of infected *Anguilla anguilla* against the gill monogenean *Pseudodactylogyrus bini*. Fish Shellfish Immunol 3:397–399

Buchmann K (1997) Population increase of *Gyrodactylus derjavini* on rainbow trout induced by testosterone treatment of the host. Dis Aquat Org 30:145–150

Buchmann K (1998) Binding and lethal effect of complement from *Oncorhynchus mykiss* on *Gyrodactylus derjavini* (Platyhelminthes: Monogenea). Dis Aquat Org 32:195–200

Buchmann K, Bresciani J (1998) Microenvironment of *Gyrodactylus derjavini* on rainbow trout *Oncorhynchus mykiss*: association between mucous cell density in skin and site selection. Parasitol Res 84:17–24

Buchmann K, Pedersen K (1994) A study on teleost phylogeny using specific antisera. J Fish Biol 45:901–903

Buchmann K, Uldal A (1997) *Gyrodactylus derjavini* infections in four salmonids: comparative host susceptibility and site selection of parasites. Dis Aquat Org 28:201–209

Cohan VL, Scott AL, Dinarello CA, Prendergast RA (1991) Interleukin-1 is a mucus secretagogue. Cell Immunol 136: 425–434

Chung S, Secombes CJ (1987) Activation of rainbow trout macrophages. J Fish Biol 31(Suppl A):51–56

Cross ML, Matthews RA (1993) Localized leucocyte response to *Ichthyophthirius multifiliis* establishment in immune carp *Cyprinus carpio* L. Vet Immunol Immunopathol 38: 341–358

Dalmo RA, Seljelid R (1995) The immunomodulatory effect of LPS, laminaran and sulphated laminaran (beta(1,3)-D-glucan) on Atlantic salmon, *Salmo salar* L., macrophages in vitro. J Fish Dis 18:175–185

Dannevig BH, Lauve A, Press CM, Landsverk T (1994) Receptor-mediated endocytosis and phagocytosis by rainbow trout head kidney sinusoidal cells. Fish Shellfish Immunol 4:3–18

Griffin BR (1984) Random and directed migration of trout (*Salmo gairdneri*) leukocytes: activation by antibody, complement and normal serum components. Dev Comp Immunol 8:589–597

Hamers R, Lehmann J, Stürenberg FJ, Taraschewski H (1992) *In vitro* study of the migratory and adherent responses of fish leucocytes to the eel-pathogenic acanthocephalan *Paratenuisentis ambiguus* (van Cleave, 1921) Bullock et Samuel, 1975 (Eoacanthocephala:Tenuisentidae). Fish Shellfish Immunol 2:43–51

Harris PD, Soleng A, Bakke TA (1997) Susceptibility of *Gyrodactylus salaris* to host complement. Bull Scand Soc Parasitol 7:69

Hoole D (1994) Tapeworm infections in fish: past and future problems. In: Pike AW, Lewis JW (eds) Parasitic diseases of fish. Samara Publishing Ltd, Tresaith, p 119–140

Jang SI, Mulero V, Hardie LJ, Secombes CJ (1995) Inhibition of rainbow trout phagocyte responsiveness to human necrosis factor a(hTNF a) with monoclonal antibodies to the hTNFa 55 kDa receptor. Fish Shellfish Immunol 5: 61–69

Jensen LB, Koch C (1992) Use of monoclonal and polyclonal antibodies to analyse the degradation of rainbow trout C3 in inulin-activated serum. Fish Shellfish Immunol 2: 241–249

Johnson E, Smith P (1984) Attachment and phagocytosis by salmon macrophages of agarose beads coated with human C3b and C3bi. Dev Comp Immunol 8:623–630

Klesius PH, Sealey WM (1996) Chemotactic and chemokinetic responses of Channel catfish macrophages to exoantigen from *Edwardsiella ictaluri*. J Aquat Anim Health 8: 314–318

Lamas J, Ellis AE (1994) Atlantic salmon (*Salmo salar*) neutrophil responses to *Aeromonas salmonicida*. Fish Shellfish Immunol 4:201–219

Lappin D, Whaley K (1993) Complement and the mononuclear phagocyte system. In: Horton MA (ed) Blood cell biochemistry, Vol 5. Macrophages and related cells. Plenum Press, New York, p 115–148

Lindenstrøm T, Buchmann K (1998) Dexamethasone treatment increases susceptibility of rainbow trout, *Oncorhyn-*

chus mykiss (Walbaum), to infections with *Gyrodactylus derjavini* Mikailov. J Fish Dis 21:29–38
Matsuyama H, Yano T, Yamakawa T, Nakao M (1992) Opsonic effect on the third complement component (C3) of carp (*Cyprinus carpio*) on phagocytosis by neutrophils. Fish Shellfish Immunol 2:69–78
Meseguer J, Lopez-Ruiz A, Esteban MA (1994) Cytochemical characterization of leucocytes from the seawater teleost, gilthead seabream (*Sparus aurata* L.). Histochemistry 102:37–44
Morley NJ, Hoole D (1997) The *in vitro* effect of *Khawia sinensis* on leucocyte activity in carp (*Cyprinus carpio*). J Helminthol 71:47–52
Obenauf SD, Smith SH (1992) Migratory response of nurse shark leucocytes to activated mammalian sera and porcine C5a. Fish Shellfish Immunol 2:173–182
O'Neill JG (1985) An *in vitro* study of polymorphonuclear phagocytosis and the effect of temperature. In: Manning MJ, Tatner MF (eds) Fish immunology. Academic Press, London, p 47–55
Ramakrishna NR, Burt MDB, MacKinnon BM (1993) Cell-mediated immune response of rainbow trout (*Oncorhynchus mykiss*) to larval *Pseudoterranova decipiens* (Nematoda; Ascaroidea) following sensitization to live sealworm, sealworm extract, and non-homologous extracts. Can J Fish Aquat Sci 50:60–65
Richards DT, Hoole D, Lewis JW, Ewens E, Arme C (1996a) Adherence of carp leucocytes to adults and cercariae of the blood fluke *Sanguinicola inermis*. J Helminthol 70: 63–67
Richards DT, Hoole D, Lewis JW, Ewens E, Arme C (1996b) *In vitro* polarization of carp leucocytes in response to the blood fluke *Sanguinicola inermis* Plehn, 1905 (Trematoda: Sanguinicolidae). Parasitology 112:509–513
Saggers BA, Gould ML (1989) The attachment of microorganisms to macrophages isolated from the tilapia *Oreochromis spilurus* Gunther. J Fish Biol 35:287–294
Sakai DK (1992) Repertoire of complement in immunological defense mechanisms of fish. Annu Rev Fish Dis 2:223–247
Secombes CJ (1990) Isolation of salmonid macrophages and analysis of their killing activity. In: Stolen JS, Fletcher TC, Anderson DP, Roberson BS, van Muiswinkel WB (eds) Techniques in fish immunology. SOS Publications, Fairhaven, p 137–155
Secombes CJ, Chappell LH (1996) Fish immune responses to experimental and natural infection with helminth parasites. Annu Rev Fish Dis 6:167–177
Secombes CJ, Hardie LJ, Daniels G (1996) Cytokines in fish: an update. Fish Shellfish Immunol 6:291–304
Sharp GJE, Secombes CJ (1993) The role of reactive oxygen species in the killing of the bacterial fish pathogen *Aeromonas salmonicida* by rainbow trout macrophages. Fish Shellfish Immunol 3:119–129
Sharp GJE, Pike AW, Secombes CJ (1991) Rainbow trout (*Oncorhynchus mykiss* (Walbaum, 1792)) leucocyte interactions with metacestode stages of *Diphyllobothrium dendriticum* (Nitzsch, 1824), (Cestoda, Pseudophyllidea). Fish Shellfish Immunol 1:195–211
Steinhagen D, Hespe K (1997) Carp coccidiosis: activity of phagocytic cells from common carp infected with *Goussia carpelli*. Dis Aquat Org 31:155–159
Sørensen KK, Sveinbjørnsson B, Dalmo RA, Smedsrød B, Bertheussen K (1997) Isolation, cultivation and characterization of head kidney macrophages from Atlantic cod, *Gadus morhua* L. J Fish Dis 20:93–107
Taylor MJ, Hoole D (1995) The chemiluminescence of cyprinid leucocytes in response to zymosan and extracts of *Ligula intestinalis* (Cestoda). Fish Shellfish Immunol 5: 191–198
Verburg-van Kemenade BML, Weyts FAA, Debets R, Flik G (1995) Carp macrophages and neutrophilic granulocytes secrete an interleukin-1-like factor. Dev Comp Immunol 19:59–70
Vladimirov VL (1971) The immunity of fishes in the case of dactylogyrosis. Parasitologiya 5:51–58 (English translation: Riverdale 1971, Parasitology 1:58–68)
Wang G, Kim JH, Sameshima M, Ogawa K (1997) Detection of antibodies against the monogenean *Heterobothrium okamotoi* in Tiger puffer by ELISA. Fish Pathol 32:179–180
Wang R, Neumann NF, Shen Q, Belosevic M (1995) Establishment and characterization of a macrophage cell line from the goldfish. Fish Shellfish Immunol 5:329–346
Weeks-Perkins BA, Ellis AE (1995) Chemotactic responses of Atlantic salmon (*Salmo salar*) macrophages to virulent and attenuated strains of *Aeromonas salmonicida*. Fish Shellfish Immunol 5:313–323
Whyte SK, Chappell LH, Secombes CJ (1989) Cytotoxic reactions of rainbow trout, *Salmo gairdneri* Richardson, macrophages for larvae of the eye fluke *Diplostomum spathaceum* (Digenea). J Fish Biol 35:333–345
Woo PTK (1996) Protective immune responses of fish to parasitic flagellates. Annu Rev Fish Dis 6:121–131
Wood BP, Matthews RA (1987) The immune response of the thick-lipped grey mullet, *Chelon labrosus* (Risso, 1826), to metacercarial infections of *Cryptocotyle lingua* (Creplin, 1825). J Fish Biol 31(Suppl A):175–183

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