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Impact of PCB on resistance to *Flavobacterium psychrophilum* after experimental infection of rainbow trout *Oncorhynchus mykiss* eggs by nanoinjection

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ABSTRACT: The effects of sublethal exposure of a commercial blend of polychlorinated biphenyls (PCB), i.e. Clophen A50, on disease resistance to the aetiological agent of rainbow trout fry syndrome, *Flavobacterium psychrophilum*, were investigated. Newly fertilised rainbow trout *Oncorhynchus mykiss* eggs were nanoinjected with 2 doses of Clophen A50 (0.4 or 2 µg egg^{-1}) and/or 100 colony forming units of *F. psychrophilum*. The mean cumulative mortality in control groups, and groups exposed to the lower dose of Clophen A50 (0.4 µg egg^{-1}) was below 5.0%. The mean cumulative mortality in groups exposed to the higher dose of Clophen A50 (2.0 µg egg^{-1}) was 5.8%, which was not significantly different from the control groups. In all groups infected with *F. psychrophilum*, with or without exposure to Clophen A50, significantly higher cumulative mortalities compared with control groups were recorded. No differences in mortality were recorded between groups exposed to bacteria alone or bacteria in combination with the higher dose of Clophen A50 (21.6 and 20.4%, respectively). Decreased disease resistance was recorded in groups exposed to *F. psychrophilum* and the lower dose of Clophen A50, with a mean cumulative mortality of 56.0%. These results could be due to non dose-dependent effects on the immune system, or toxic effects of PCB or their metabolites on the bacteria in groups exposed to the higher dose of Clophen A50. The present study indicates that maternal transfer of PCB might affect disease resistance to vertically transmitted *F. psychrophilum.*

KEY WORDS: *Flavobacterium psychrophilum* · Rainbow trout fry syndrome · RTFS · Polychlorinated biphenyls · PCB · Disease resistance · Nanoinjection · Rainbow trout

INTRODUCTION

Polychlorinated biphenyls (PCB) are environmental pollutants of considerable importance all over the world, even though their use has been restricted in most countries since the 1970s. Due to their high persistence and strong lipophilic properties, PCB bioaccumulate in aquatic food webs. Even if decreasing levels of PCB have been observed during the last 30 yr, they still constitute a major contaminant in fish (Bignert et al. 1998). PCB are well-known immunomodulators in mammals (Vos 1977, Luster & Rosenthal 1993). Effects on the innate as well as the acquired immune system have also been shown in fish (Thuvander & Carlstein 1991, Thuvander et al. 1993, Arkoosh et al. 1994, Lacroix et al. 2001, Regala et al. 2001, Duffy et al. 2002). Furthermore, atrophy of lymphoid tissue in the spleen and toxic effects on thymocytes have been observed in fish exposed to PCB (Nestel & Budd 1975, Spitsbergen et al. 1988, Sweet et al. 1998). Studies on disease resistance in connection with PCB exposure have been performed with varying results. Decreased, increased and

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unaltered susceptibility to infections have been observed (Snarski 1982, Mayer et al. 1985, Spitsbergen et al. 1988, Arkoosh et al. 2001, Powell et al. 2003).

Infections with the bacterium *Flavobacterium psychrophilum*, the aetiological agent of rainbow trout fry syndrome (RTFS) and bacterial cold-water disease (BCWD), are a problem in salmonid aquaculture worldwide. RTFS is a septicaemic disease in rainbow trout (*Oncorhynchus mykiss*) fry that can cause mortalities up to 70% (Lorenzen et al. 1991, Bruno 1992). Disease outbreaks often occur during the first 2 mo of feeding (Lorenzen et al. 1991). Main pathological findings are an enlarged spleen, pale gills, liver and kidney, and a haemorrhagic protruding anus (Lorenzen et al. 1991). *F. psychrophilum* has been isolated from internal organs and sexual products of salmonid brood fish (Holt 1987, Rangdale et al. 1996, Brown et al. 1997, Ekman et al. 1999). The bacterium has also been isolated from the inside of fertilised eggs (Brown et al. 1997, Kumagai et al. 2000), strongly indicating that vertical transmission occurs.

The developing fry can be exposed to lipophilic pollutants, like PCB, by transfer from the female to the egg before ovulation (Niimi 1983, Miller 1993). Micro- and nanoinjection techniques into eggs or newly hatched fry have been used to mimic this maternal transfer of lipophilic contaminants (Walker et al. 1996, Norrgren et al. 1993, Engwall et al. 1994) as well as vertical transmission of bacteria, i.e. *Renibacterium salmoninarum* (Brown et al. 1990) and *Flavobacterium psychrophilum* (Ekman et al. 2003). The nanoinjection technique makes it possible to administer very small volumes into the newly fertilised egg without causing adverse effects.

Organic lipophilic compounds, such as PCB, need to be biotransformed into more water-soluble forms before they can be excreted. The cytochrome P450-mono-oxygenase system in the liver is one of the most important enzyme systems involved in this process. One commonly used method for measuring the catalytic activity of this system is the ethoxyresorufin *O*-deethylase (EROD) assay, which has been used as a biomarker for exposure to environmental pollutants (Norrgren et al. 1993, Buchelli & Fent 1995).

The aim of this study was to use nanoinjection techniques to investigate interactions between *Flavobacterium psychrophilum* and a commercial blend of PCB (Clophen A50) by simultaneous exposure in newly fertilised rainbow trout eggs.

MATERIALS AND METHODS

Preparation of PCB solution. The PCB solution was prepared by dissolving Clophen A50 (Bayer) in triolein (Sigma Chemical) to a concentration of 40 mg ml^{-1}. This solution was used for the injections in the high dose groups. The low dose groups were injected with an aliquot diluted with triolein to a concentration of 8.0 mg ml^{-1}.

Preparation of bacterial suspensions. An isolate of *Flavobacterium psychrophilum* (F9) originally isolated from a diseased juvenile rainbow trout, was stored at –80°C in tryptone and yeast extract salt (TYES) broth (Holt et al. 1993) with 17% glycerol added. Bacteria were taken from the frozen batch and inoculated in TYES broth at 15°C on a shaker. After 48 h, the bacteria were harvested by centrifugation at 1500 × *g* for 10 min at 5°C and washed twice in 0.9% NaCl. The suspension was adjusted with 0.9% NaCl to an absorbance of 0.4, using a spectrophotometer (Shimadzu UV-1601PC) at 525 nm corresponding to approximately 2×10^8 colony forming units (CFU) ml^{-1}. The bacterial suspension was diluted 1:100 with 0.9% NaCl before injection. Drop inoculation on TYES agar in triplicate was performed to estimate the number of viable bacteria in the suspension.

Nanoinjection procedure. The nanoinjection technique has been described in detail by Åkerman & Balk (1995) and Walker et al. (1996). A fine needle of aluminium silicate glass was held in a micromanipulator (WR-87, Narishige Scientific Instrument Laboratory) and the injection volume controlled with a pico-injector (PLI-100, Medical Systems). The injection was performed under a stereomicroscope (Leica MZ8). A total of 50 nl bacterial suspension and/or Clophen A50 solution was injected into the yolk of individual eggs.

Experimental design. Rainbow trout eggs and milt were obtained from a commercial fish farm connected to the Swedish fish-health control programme (U.-P. Wichardt, Fish-health control programme, Fiskhälsan FH AB; Älvkarleby, Sweden, pers. comm.). Eggs from 1 female were fertilised with milt from 1 male. After water hardening, at the start of the experiment, 40 control eggs were sampled for bacteriological examination to confirm that the eggs were not infected with the bacterium. The remaining eggs were placed in prepared holes in 1% agarose gel, cast in square Petri dishes (Åkerman & Balk 1995, Walker et al. 1996) and divided into 9 experimental groups in duplicates (total 18 groups) with 69 to 72 eggs in each group. The eggs were incubated at Stockholm University in flow-through water at a water temperature of 8 ± 0.5°C until the injections were performed after fertilisation on Days 1 to 3.

After the injections, the eggs were transported to the Swedish University of Agricultural Sciences, Uppsala, and each group was separately incubated in 5 l aquaria in flow-through water at 10 ± 1°C. On Day 12 after fertilisation, unfertilised eggs were removed from the Petri dishes, leaving 57 to 66 fertilised eggs in each group. The fertilisation rate in the groups varied

between 81.9 and 93.1% with a mean value of 88.4%. After yolk-sac absorption, the fry were fed commercial fish food (Aller Aqua AB) 3 to 5 times a day.

Two days after fertilisation, the Clophen A50 solution was injected into the yolk of the eggs. Two groups were injected with 0.4 µg Clophen A50 egg^{-1} (Ld) and 2 groups with 2 µg Clophen A50 egg^{-1} (Hd). On Day 3 after fertilisation, 100 CFU *Flavobacterium psychrophilum* was injected into eggs in 1 Ld and Hd group (FpLd and FpHd), respectively. One group was injected with bacteria only (Fp). Control groups were injected with triolein, 0.9% NaCl or triolein + 0.9% NaCl. One additional control group was left uninjected. All groups were made in duplicate.

The eggs were inspected daily and monitored for mortality. Dead eggs were sampled and cultured for the presence of *Flavobacterium psychrophilum*. After hatching, fry were inspected 3 times a day and mortality was recorded. Dead and moribund fry were examined for gross pathological findings, cultured for the presence of *F. psychrophilum* or fixed in 10% phosphate-buffered formalin (pH 7.2 to 7.4) for morphological studies. Fry that died at night were not used for morphological studies due to post-mortem changes. Livers were dissected 16 d post-hatching (p.h.) for EROD activity measurements. The experiment was terminated 110 d after fertilisation (65 d p.h.) and the remaining fry were sampled for bacteriology and morphological studies.

Bacteriology. After being proven to be surface-sterile as described by Ekman et al. (2003), eggs sampled for bacteriological examination at the start of the experiment and eggs that died 5 d or more before expected hatching were examined for the presence of *Flavobacterium psychrophilum*. Eggs that died 5 d or less before expected hatching were disinfected with 5% Buffodine (Evans Vanodine International) for 20 min and the embryo was aseptically removed from the egg. The whole embryo was placed in a test tube with 3 ml TYES, crushed with a sterile glass rod and incubated at 15°C for 7 d. Dead and moribund fry were aseptically sampled from the brain and yolk (yolk sac fry) or kidney and spleen (fry after yolk sac absorption) with a plastic loop and inoculated on TYES agar for 7 d at 15°C and 5% horse blood agar for 7 d at 20°C. At the end of the experiment, samples for bacteriology from kidney and spleen were inoculated on TYES agar and 5% horse blood agar.

Identification of re-isolated *Flavobacterium psychrophilum* was performed with morphological and phenotypic characteristics including colony morphology, Gram staining, production of cytochrome oxidase (Bactidrop™Oxidase, Remel), catalase (30% H_2O_2), flexirubin-like pigment (20% KOH), reactivity in the API-zym gallery (bioMérieux) and ability to grow at 6 and 30°C in TYES broth. The results were compared with the results of Bernardet & Kerouault (1989) and with the characteristics of the type strain NCIMB 1947^T.

Morphology and immunohistochemistry. Fixed fry were processed and embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E). Between 5 and 10 sections of each fry were examined. The heads from 10 fry in the triolein control group, Ld and Hd groups, sampled at the end of the experiment, were separately embedded in order to evaluate any changes in morphology in the thymus due to PCB exposure.

Immunohistochemical stainings for detection of *Flavobacterium psychrophilum* were performed on sections mounted on SuperFrost® Plus glass (Menzel). An avidin-biotin immunoperoxidase kit, VECTASTAIN® elite ABC kit (Vector Laboratories), was used according to the manufacturer's instructions. Endogenous peroxidase activity was quenched with 3% H_2O_2 for 5 min. A commercial polyclonal antibody against *F. psychrophilum* (RFP01, Microtek International), diluted 1:5000 in 0.05 M Tris-buffer (pH 7.6), was used as the primary antibody. The antibody was replaced with nonimmune rabbit serum or Tris-buffer in negative controls. As chromogen, 3-amino-9-ethylcarbazole (AEC) was used, and Mayer's haematoxylin was used as counterstain.

EROD analysis. The yolk sac fry were decapitated and the livers removed. Three livers were pooled in 300 µl 0.25 M sucrose and immediately homogenized (glass-Teflon homogenizer, # 18, Kontes) at 0°C and frozen in liquid nitrogen. In total, 15 livers from each sampled group (7 + 8 from each duplicate) were analysed. All groups, except the uninjected controls were analysed. The samples were stored at –140°C and rapidly thawed just before enzymatic analysis. EROD activity was measured according to Prough et al. (1978) and the protein content was determined according to Lowry et al. (1951).

Statistics. To evaluate any differences in mortality between different groups, Kaplan-Meier survival probability curves were compared with the Logrank (Mantel-Haenszel) test. A statistical comparison of EROD values was performed with the Mann-Whitney *U* test. p-values < 0.05 were considered statistically significant for all tests.

RESULTS

Mortality

In all control groups, the total mortality was below 5%. The mean cumulative mortality in the Ld group was 4.0% (3.1 and 4.9% in duplicates) and in the Hd

group 5.8% (4.9 and 6.6% in duplicates), which was not significantly different from controls (Fig. 1). The groups infected with *Flavobacterium psychrophilum* showed significantly higher cumulative mortality compared with controls (Fig. 1). The mean mortality was 21.6% (20.2 and 23.0% in duplicates) in the Fp groups and 20.4% (19.0 and 21.9% in duplicates) in the FpHd groups. The mean mortality in the FpLd groups, 56.0% (53.3 and 58.6% in duplicates), was significantly higher than the mortality in the Fp and FpHd groups. No significant differences in mortality were recorded between the duplicates.

Bacteriology

No *Flavobacterium psychrophilum* was isolated from dead eggs or dead/moribund fry in any of the controls, Ld or Hd groups. All bacteriologically examined dead eggs/embryos and dead or moribund fry from the infected groups yielded growth of *F. psychrophilum*. No other known fish-pathogenic bacteria were isolated.

Gross pathological findings

Gross pathological findings of dead and moribund yolk sac fry and feeding fry are summarised in Table 1. Precipitates in the yolk were present in dead and moribund fry from all groups. A common finding in dead and moribund fry in infected groups was a lesion on the yolk sac with visible leakage of yolk (Table 1). In these fry, the yolk was often totally coagulated. In addition, some fry in infected groups had a small protrusion of the yolk sac without visible leakage of yolk (Fig. 2). Oedema of the yolk sac was present in infected as well as in Clophen A50 exposed groups (Table 1). In dead and moribund feeding fry, pale gills, liver and kidney, an enlarged spleen and an empty gastro-intestinal tract were recorded at necropsy (Table 1). Only sporadic mortalities were recorded in control and Clophen A50 exposed feeding fry and no significant gross pathology findings were recorded (Table 1). Fry examined at the end of the experiment did not show any gross pathological signs.

Table 1. *Oncorhynchus mykiss*. Gross pathology findings in dead fry exposed to 0.4 (Ld) or 2 µg (Hd) Clophen A50 egg^{-1} and/or 100 colony forming units (CFU) *Flavobacterium psychrophilum* (Fp, FpLd, FpHd). Number of fry with pathological finding and total number of dead or moribund fry (shown in parentheses). Results of duplicates are summarised. The results from the different control groups (uninjected, triolein, NaCl and triolein+NaCl) are summarised into 1 control group

	Gross pathology finding	Controls	Ld	Hd	Fp	FpLd	FpHd
Yolk sac fry	Precipitates in yolk sac	6(11)	1(4)	2(4)	3(5)	27(31)	13(14)
	Haemorrhages in yolk sac	0(11)	0(4)	0(4)	2(5)	13(31)	5(14)
	Oedema in yolk sac	0(11)	2(4)	3(4)	1(5)	6(31)	3(14)
	Protrusion of yolk sac	0(11)	0(4)	0(4)	0(5)	2(31)	1(14)
	Leakage of yolk	0(11)	0(4)	0(4)	2(5)	8(31)	3(14)
Feeding fry	Dark pigmentation	0(1)	1(1)	0(2)	5(7)	9(12)	2(3)
	Pale gills	0(1)	0(1)	0(2)	7(7)	12(12)	3(3)
	Pale liver	0(1)	0(1)	0(2)	6(7)	9(12)	2(3)
	Pale kidney	0(1)	0(1)	0(2)	2(7)	4(12)	0(3)
	Enlarged spleen	0(1)	0(1)	0(2)	6(7)	10(12)	3(3)

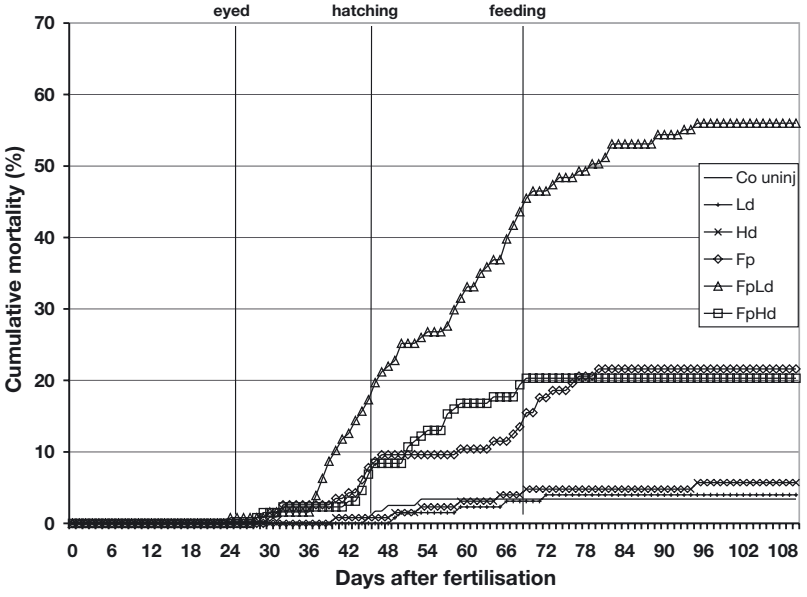

Fig. 1. *Oncorhynchus mykiss*. Cumulative mortality in eggs and fry nanoinjected with 0.4 µg (Ld) and 2 µg (Hd) Clophen A50 egg^{-1} and/or 100 colony forming units (CFU) *Flavobacterium psychrophilum* egg^{-1} (FpLd, FpHd, Fp)

Fig. 2. *Oncorhynchus mykiss* yolk sac fry experimentally infected with *Flavobacterium psychrophilum* (100 CFU) by nanoinjection in the newly fertilised egg. Protrusion of the yolk sac (arrow) and yolk sac oedema (*)

Histopathological findings and immunohistochemistry

No significant histopathological signs were recorded in dead fry from control groups. In the Ld and Hd groups, no pathological signs other than oedema in the yolk sac were seen. No positive immunohistochemical staining was obtained in fry from either the control, Ld or Hd groups.

Dead and moribund fry in Fp, FpLd and FpHd groups showed similar histopathological changes. Large amounts of immunohistochemically positive-stained bacteria were present in the yolk. Oedema in the yolk sac was occasionally observed. Focal areas on the yolk sac with a marked hyperplasia of the epidermis often accompanied by spongiosis were often present. Vesicle formations, where the epidermis had lost connection with the underlying tissue, were seen in some individuals (Fig. 3). Inside the vesicle, immunohistochemically positive-stained phagocytes were present (Fig. 3). In yolk sac fry with macroscopically visible leakage of yolk, parts of the epidermis covering the yolk were sloughed off, and the yolk sac was ulcerated with subsequent leakage of yolk (Fig. 4). Fibrosis in the dermis and hypodermis with necrotic cells present were seen adjacent to the ulceration (Fig. 4). Positively stained phagocytes were dispersed in the epidermis and dermis. In the spleen, congestion and haemorrhage were present, often together with small areas of necrosis. Numerous immunohistochemically positive-stained bacteria were seen as well as positively stained phagocytes. In the kidney, necrosis of tubular epithelium was often present. Occasionally, small areas of necrosis of the haematopoietic tissue in the kidney were seen. Positively stained phagocytes were present in sinusoids and peritubular capillaries as well as in the haematopoietic tissue. Eosinophilic droplets were often present in the kidney tubular epithelium. In the liver, positively stained phagocytes were commonly present in capillaries and sinusoids. No pathological changes due to PCB exposure were noted in infected groups exposed to Clophen A50 or groups exposed to Clophen A50 alone. Furthermore, no pathological changes in the thymus, due to Clophen A50 exposure, were observed. Surviving fry did not show any significant pathological changes at termination of the experiment, and no positive immunohistochemical staining was observed.

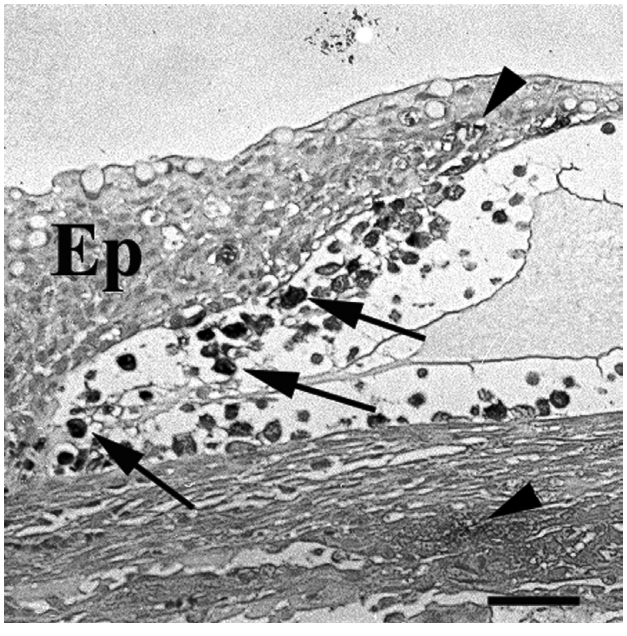

Fig. 3. *Oncorhynchus mykiss* yolk sac fry exposed to Clophen A50 and *Flavobacterium psychrophilum* (100 CFU) by nanoinjection into the newly fertilised egg. Immunohistochemical staining with a polyclonal antibody towards *F. psychrophilum* (RFP01). Avidin-biotin complex method, 3-amino-9-ethylcarbazole (AEC) as choromogen and Mayer's haematoxylin as counterstain. Positive-stained phagocytes are present in a vesicle in the epidermis covering the yolk (arrows). Positive-stained free bacteria are present in the epidermis (Ep) and dermis (arrowheads). Scale bar = 50 µm

EROD induction

EROD activity of control groups and the Fp group was in the range of 26 to 44 pmol resorufin min^{-1} mg

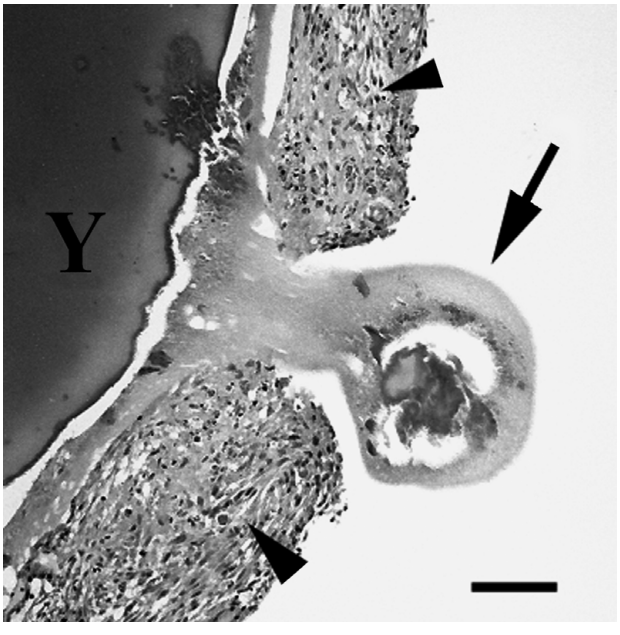

Fig. 4. *Oncorhynchus mykiss* yolk sac fry exposed to Clophen A50 (0.4 µg) and *Flavobacterium psychrophilum* (100 CFU) using nanoinjection in the newly fertilised egg. Lesion of the yolk sac (Y) with loss of epidermis, leakage of yolk material (arrow) and fibrosis of dermis and hypodermis (arrowheads) (H&E). Scale bar = 100 µm

protein^{-1} (Fig. 5). In all PCB exposed groups, the EROD activity was significantly higher than in their respective control group. In groups exposed to the low dose of Clophen A50, a 10- to 20-fold increase in EROD activity was observed and in groups exposed to the high dose of Clophen A50 a 40- to 60-fold increase compared with the control was recorded (Fig. 5). The EEOD activity in the high dose group was significantly different from the low dose group.

DISCUSSION

Previous studies on effects of PCB on disease resistance in fish have yielded contradictory results. Decreased disease resistance in Chinook salmon *Oncorhynchus tschawytscha* challenged with *Listonella anguillarum* (formerly *Vibrio anguillarum*) after intraperitoneal injection of PCB has been reported (Arkoosh et al. 2001). Unaltered disease resistance to infectious haematopoietic necrosis virus (IHNV) and *L. anguillarum* has been shown in rainbow trout and Coho salmon, respectively, after exposure to PCB via contaminated food (Spitsbergen et al. 1988, Powell et al. 2003). Finally, increased disease resistance has been reported in rainbow trout challenged with *Aeromonas hydrophila* and *Yersinia ruckeri* after exposure to PCB through water or food (Snarski 1982, Mayer et al. 1985). In the present study, the highest mortality was recorded in groups infected with *Flavobacterium psychrophilum* in combination with exposure to the lower dose (0.4 µg egg^{-1}) of Clophen A50. No effect on mortality was recorded in the group exposed to bacteria in combination with the higher dose of Clophen A50 (2 µg egg^{-1}) compared with groups exposed to bacteria alone. The nonspecific defence mechanisms are very important during the embryonic stage and in newly hatched fry (Tatner 1996). The presence of functional phagocytes has been described as early as 4 d p.h. in rainbow trout fry (Tatner & Manning 1985). The decreased disease resistance at the lower dose of Clophen A50 exposure in this study might be explained as being due to an impaired phagocytic function. PCB exposure has been shown to have a negative impact on phagocytosis in fish with a decrease in number of actively phagocytosing macrophages in the head kidney and peritoneal cavity (Jones et al. 1979, Lacroix et al. 2001) and a suppression of the oxidative burst activity of macrophages in the head kidney (Rice & Schlenk 1995, Regala et al. 2001). Negative effects on the humoral immune system might also have contributed to the decreased disease resistance. Mature B-cells with immunoglobulins on their surface were demonstrated 8 d before hatching (at 14°C) (Castillo et al. 1993), although full immunocompetence of rainbow trout is not developed until later (Tatner 1996). Suppression of the humoral immune system after exposure to Clophen A50, with a reduced antibody response to *L. anguillarum*, has been shown in rainbow trout (Thuvander et al. 1993). Furthermore, suppressed primary and secondary plaque-forming cell responses of leucocytes to the T-independent antigen TNP-keyhole limpet hemocyanin after exposure to the commercial PCB mixture Aroclor 1254, has been reported (Arkoosh et al. 1994, 2001). Exposure to the higher dose (2 µg egg^{-1}) of Clophen A50 in this study resulted in unaltered disease resistance to *F. psychrophilum*. The negative effects on the immune system might not always be dose dependent, as shown in a study on the phagocytic activity of rainbow trout blood leucocytes after chronic exposure to the herbicide Linuron (Falk et al. 1990). Exposure to the lowest dose of Linuron affected the phagocytic capacity negatively, while at the higher doses, no differences were shown compared with controls (Falk et al. 1990). Furthermore, it cannot be excluded that either Clophen A50 or its metabolites have toxic effects on the bacteria that could have contributed to the results. However, Mayer et al. (1985) did not observe any bacteriostatic or bactericidal effects of PCB on *Y. ruckeri* at concentrations considerably higher

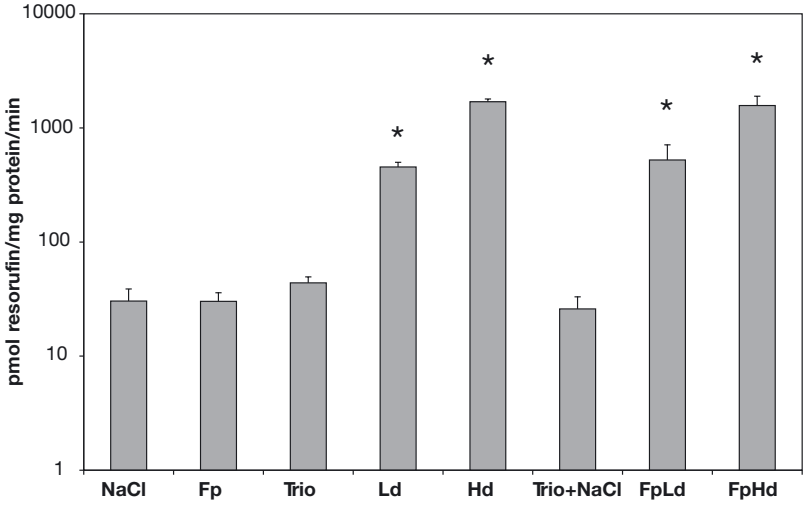

Fig. 5. *Oncorhynchus mykiss*. Ethoxyresorufin *O*-deethylase (EROD) induction (mean + SD) in yolk sac fry exposed to 0.4 µg (Ld) or 2 µg (Hd) Clophen A50 egg^{-1} and/or 100 colony forming units (CFU) *Flavobacterium psychrophilum* egg^{-1} (Fp, FpLd, FpHd). Controls were injected with NaCl, triolein (Trio) or Trio + NaCl. Livers were sampled 16 d p.h. Three livers were pooled and in total 15 livers per group were examined. *$p < 0.05$ compared with control

than those used in this experiment. To our knowledge this is the first study where a method of mimicking the maternal transfer of PCB and vertical transmission of bacteria has been used to evaluate any impact on disease resistance. It might be possible that exposure to contaminants before the immune system has developed, has caused as yet unknown effects. The developing immune system in mammals has been shown to be highly sensitive to environmental contaminants (Ross et al. 1997) and the same could be expected in fish.

In an experimental infection with *Flavobacterium psychrophilum* using the nanoinjection method (Ekman et al. 2003) the clinical signs of disease and pathological findings of dead and moribund fry are in accordance with those described in the present study. However, lesions on the yolk sac with vesicles, ulcerations and leakage of yolk were not noted in the previous study. *F. psychrophilum* is known to be highly proteolytic with the ability to degrade albumin, casein, collagen, fibrinogen, gelatin and haemoglobin (Pacha 1968, Bertolini et al. 1995, Dalsgaard & Madsen 2000). Many isolates such as the isolate used in this study, can also degrade elastin. It is likely that proteolytic enzymes are involved in the pathogenesis of the yolk sac lesions.

In this study, yolk sac oedema was present in groups exposed to Clophen A50, *Flavobacterium psychrophilum* or a combination of both. Yolk sac oedema has previously been reported in connection with exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Walker et al. 1991) and PCB (Wilson & Tillitt 1996). Yolk sac oedema has also been seen in association with experimental *F. psychrophilum* infection (Ekman et al. 2003). Furthermore, *F. psychrophilum* has been suggested as being part of the aetiology of blue-sac disease, characterised by yolk sac oedema, in lake trout *Salvelinus namaycush* (Symula et al. 1990).

The mortality in the groups injected with 100 CFU bacteria was lower in this experiment compared to earlier results in a study by Ekman et al. (2003). A passive transfer of immunity from the female to the offspring has been indicated (Mor & Avtalion 1990, Castillo et al. 1993). The eggs in this and the previous study by Ekman et al. (2003) originated from 1 female each. It cannot be ruled out that the eggs in this study originated from a female that had been exposed to *Flavobacterium psychrophilum* previously during her life. Genetic variation in disease resistance has been reported (Chevassus & Dorson 1990, Gjedrem 2000), and might also be a possible explanation for the observed differences in susceptibility.

No enhanced mortality was recorded in the groups exposed to Clophen A50 even at the highest dose (2 µg egg^{-1}). This is in accordance with earlier studies where injection of 4 µg Clophen A50 per embryo did not result in mortalities significantly higher than controls (Norrgren et al. 1993). The exposure to PCB resulted in an induction of the cytochrome P450-mediated enzyme activity measured by EROD activity. The EROD activities are at the same level as reported by Norrgren et al. (1993) after microinjection of Clophen A50 in rainbow trout embryos. Exposure to bacteria did not affect the EROD induction.

Depletion of lymphoid tissue in the thymus and spleen has been recorded in rainbow trout exposed to PCB (Nestel & Budd 1975, Thuvander et al. 1993). In this study, no microscopic changes in the thymus or spleen were recorded following Clophen A50 exposure. The alteration in disease resistance might be due to functional disturbances rather than depletion of lymphoid tissue or degenerative changes.

PCB levels up to 2.7 mg g^{-1} fresh weight have been reported in rainbow trout eggs (Hogan & Brauhn 1975), whereas concentrations up to 8.3 mg g^{-1} fresh weight have been reported in Chinook salmon eggs from the highly contaminated Lake Michigan (Miller 1993). The injected doses in the present study of 0.4 and 2 mg egg^{-1} theoretically correspond to concentrations of approximately 5 and 25 mg g^{-1} fresh weight, respectively. Consequently at least the lower Clophen A50 dose used in this study may be environmentally relevant.

This study indicates that exposure to Clophen A50 might affect disease resistance to vertically transmitted *Flavobacterium psychrophilum*.

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