Francisella noatunensis in Atlantic cod (*Gadus morhua* L.); waterborne transmission and immune responses

Terje Ellingsen[a,1], Makoto Inami[a,b,1], Mona Cecilie Gjessing[c], Koen Van Nieuwenhove[a], Rannveig Larsen[a], Marit Seppola[d], Vera Lund[d] and Merete Bjørgan Schrøder[a,*]

[a] Faculty of Bioscience, Fishery and Economics, University of Tromsø, 9037 Tromsø, Norway

[b] Faculty of Biosciences and Aquaculture, University of Nordland, 8049 Bodø, Norway

[c] Section of Fish Health, National Veterinary Institute, Pb 750 Sentrum, 0106 Oslo, Norway

[d] Nofima Marin, Muninbakken 9-13, N-9291 Tromsø, Norway

[*] Corresponding author Tel.: +47-776-44000 fax: +47-776-46020

Email address: merete.schroder@uit.no (M. B. Schrøder).

[1] Both authors contributed equally to the work.

Abstract

This is the first report that confirms horizontal waterborne transmission of francisellosis in Atlantic cod. To investigate the transmission of disease, particle reduced water was transferred from a tank with intraperitoneally infected cod to a tank with healthy cod. Waterborne transmission was confirmed in the effluent group using immunohistochemistry and real-time quantitative PCR (RT-qPCR) detecting *Francisella noatunensis*. The bacteria were located inside of accumulated macrophage-like cells. In addition, specific and high antibody responses against live and inactivated bacteria were observed in which the live bacteria gave the highest antibody titres. Oil adjuvant had no effect on the antibody responses against inactivated *F. noatunensis*. The antigen epitope was a 20-25 kDa component suggested to be a lipopolysaccharide detected with Western blot, Coomassie blue, Sypro Ruby and Silver staining. Systemic immune reactions were investigated by measuring the expression of *IFN-γ*, *IL-1β* and *IL-10* genes with RT-qPCR. After i.p. injection of live bacteria, a significant up-regulation of *IFN-γ* and *IL-1β* expression was observed from 15 to 60 days post infection in spleen and head kidney. In intestine, *IFN-γ* was significantly up-regulated after 60 days whereas rectum showed no significant differences in expression. Elevated expression of *IL-10* was observed in all the organs tested but was only significantly up-regulated at 60 days post infection in intestine from i.p. infected fish. Up-regulation of *IL-1β* and *IFN-γ* was observed in spleen whereas intestine and rectum showed a down-regulation in cohabitant fish after 60 days. *IL-10* was up-regulated in intestine of cohabitant fish from day 30 to day 60. These results indicate that *F. noatunensis* infection provokes both specific antibody responses and long term inflammatory responses in cod. The present study provides new knowledge about infection routes and shows that both humoral and cellular defence mechanisms are triggered by *F. noatunensis* in cod.

Keywords: Atlantic cod, *Francisella noatunensis*, Waterborne transmission, Antibody responses, Immune gene expression, Cytokines

1. Introduction

Farming of Atlantic cod has been commercialized since the early 1980's but has confronted several difficulties. These include an unsteady market, low prices due to the availability of cheaper wild-caught fish, early sexual maturation and bacterial diseases like vibriosis, atypical furunculosis and francisellosis. Francisellosis which was first reported in 2004 in Norway [1, 2] has now become a major problem in the cod farming industry.

The disease is caused by *Francisella noatunensis* and develops gradually, sometimes without clinical signs of disease. Pathological changes are characterised by white nodules in different parts of the fish, including spleen, head kidney and heart. The bacterium seem to establish mainly intracellularly in macrophage-like cells (MLC) in cod [1, 2] and no efficient vaccine has been developed so far. Challenge experiments have shown presence of *F. noatunensis* in the skin and mucus of infected cod and that the bacterium is horizontally transmitted to cohabitants [3]. Further, there are also strong indications of horizontal transmission in fish farms. Knowledge about the bacterium, the infection routes and the immune responses in cod is of vital importance for better control of the disease and for development of vaccines.

The immune system of Atlantic cod seems to differ from several other bony fish species as the antibody responses are shown to be absent or low after immunization with *Vibrio salmonicida* [4], *Vibrio anguillarum* [5] and hapten carriers [6, 7]. In contrast to this, newer findings demonstrate an elevation of antibody levels against inactivated *Aeromonas salmonicida*, *V. anguillarum* and *F. noatunensis* [8]. However, the individual antibody responses against *A. salmonicida* and *V. anguillarum* varied from no to high responses, whereas those against *F. noatunensis* were high and specific for all individuals. It is still unknown what kind of impact the humoral responses in cod have on *F. noatunensis*. Several immune genes have been characterized and analysed from cod in the recent years, including cytokines and chemokines [9, 10, 11, 12], interferon stimulated genes [13] and antimicrobial peptides [14].

Immune gene regulation has also been studied in cod intestine and this work shows that cod lack a second gut segment and that the rectal tissue of Atlantic cod differs from other parts of the gut and has a different distribution of immune cells compared to the distal intestine [15].

The aim of this study was to investigate the possibility of waterborne transmission of *F. noatunensis* in cod. In addition we wanted to enlighten some aspects of the immune responses against live and inactivated bacteria including antibody responses and expression of interferon-γ (*IFN-γ*), interleukin-1β (*IL-1β*) and interleukin-10 (*IL-10*). Presence and location of the bacteria were demonstrated by immunohistochemistry and real-time quantitative PCR (RT-qPCR). Enzyme-linked Immunosorbent Assay (ELISA) and Western blot were used to study the antibody responses whereas RT-qPCR was employed to investigate the gene expression in selected tissues.

2. Materials and methods

2.1. Fish

Unvaccinated Atlantic cod (*Gadus morhua* L.) without any clinical signs of disease were obtained from the National Cod Breeding Centre (Tromsø, Norway). The fish were transferred to the Aquaculture research station (Tromsø, Norway) where they were kept in 300 L tanks with filtered seawater at 12 °C, 24 h light regime and daily feeding rate (Dana feed A/S, Horsens, Denmark). Before the experiment started, the fish were transferred to the fish health facilities and acclimatized for 10 days at 12 °C. The fish had an average weight of 160 g at the start of the experiment and 218 g at the end. Prior to marking, immunization, sampling and challenge, the fish were anesthetized with 60 mg L^{-1} of Metacainum (Norsk Medisinaldepot, Oslo, Norway). The immunized fish were marked with Visible Implant Fluorescent Elastomer (Northwest Marine Technology Inc., Washington, USA).

2.2. Bacteria

Francisella noatunensis isolate NCIMB 14265 was obtained from the National Veterinary Institute (Oslo, Norway). The bacteria were grown on Cystein Heart agar plates supplemented with 5% blood (CHAB) [16] for seven days at 22 °C. A single colony was transferred to 10 ml of liquid medium adapted for *F. noatunensis* (provided by PHARMAQ AS, Oslo, Norway) and further incubated for approximately 20 h at 16 °C on gentle shaking until optical density at 600 nm (OD_{600nm}) reached a value of 1.0. The culture was centrifuged and diluted with 0.9% NaCl (saline) to OD_{600nm} 0.2 corresponding to approximately 10^9 bacteria ml^{-1}. Infection doses were adjusted by diluting the bacteria suspension to 10^6 bacteria ml^{-1}. Colony forming units (cfu) of the infection doses were determined using CHAB plates which were incubated at 22 °C for seven days.

F. noatunensis used for immunization was cultured as described above, thereafter inactivated by adding 37% formaldehyde to a final concentration of 0.5% and incubated at 12 °C overnight on gentle shaking. Inactivation was confirmed by spreading 100 µl of inactivated bacterial suspension on CHAB plates followed by five days of incubation at room temperature.

2.3. Immunization and infection

2.3.1. Immunization

Two formulations of *F. noatunensis* were prepared for immunization; one containing oil adjuvant (provided by PHARMAQ AS, Oslo, Norway) and one with saline. Each formulation was prepared with two concentrations of bacterins (Table 1A). Four groups of 15 fish were anesthetized, marked with fluorescent implant intraperitoneally (i.p.), immunised and transferred to a 300 L tank together with a control group of 15 fish injected with saline and oil. Saline injected controls were sampled from the control tank as described below.

2.3.2. Infection and transmission of disease

For infection studies, 75 fish were i.p. injected with 0.1 ml of live *F. noatunensis* (5×10^6 bacteria ml^{-1}) per fish and thereafter transferred to the 500 L main tank (Table 1B). The colony forming unit count showed that 3.3×10^4 viable bacteria were injected per

fish. A group of 15 non-infected fish were transferred to the same tank and referred to as the cohabitant group. The water from the main tank was transferred through an aerator to a 300 L receiving tank (effluent tank) after removing feces and feed particles by sedimentation. After running the system for 24 h, 70 untreated fish were transferred to the receiving tank and denoted as the effluent group. As control for the i.p. infected, cohabitant and effluent groups, 51 fish were anesthetized, i.p. injected with saline and transferred to a 300 L tank.

2.4. Sampling

For all fish, blood was collected using vacutainer tubes without anticoagulant, and allowed to coagulate overnight at 4 °C before centrifugation. The sera were individually frozen at -20 °C and equal amounts of individual sera were collected to prepare pooled samples for further use. In addition, samples from spleen, head kidney, intestine and rectum were collected, transferred to RNA*later*® (Ambion, Austin, USA) and incubated at 4°C for 24 h before stored at -80°C. For histological examination, three mm slices of spleen were kept on formaldehyde. External and internal clinical signs of francisellosis were observed and recorded individually. Prior to the experiment 10 healthy non-injected fish were sampled as a 0-control.

Ten fish were sampled from the infected group, the saline injected controls at 1, 2, 4, 7, 15 and 30 days post infection (d.p.i.) and the rest at 60 d.p.i. Six and 9 fish were sampled from the cohabitant group at 30 d.p.i. and at 60 d.p.i., respectively. Six fish were sampled from the effluent group at day 30, 15 fish at day 60 and 15 fish at day 90. Blood sampling was performed at 30 and 60 d.p.i. from the immunized fish.

2.5. RNA isolation

Total RNA was isolated from spleen, head kidney, intestine and rectum using RNeasy mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's description including DNase treatment. The quality of total RNA was verified using 1% agarose gel electrophoresis and the concentration was measured by NanoDrop® ND-1000 spectrophotometer (NanoDrop® Technologies Inc., Wilmington, NC, USA). Reverse transcription was performed in a total volume of 20 µl with 500 ng RNA using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA, USA) in

accordance with the manufacturers protocol. The cycling parameters were as following; 25 °C for 5 min, 42 °C for 60 min and 85 °C for 5 min. The cDNA were diluted three times in nuclease free water (Ambion, Austin, TX, USA) for further use in RT-qPCR. The absence of genomic DNA in the RNA (non-template control) was verified by a selection of samples being subjected to RT-qPCR without prior cDNA synthesis.

2.6. RT-qPCR analysis and statistics

All reagents and equipment for RT-qPCR was obtained from Applied Biosystems unless otherwise stated. RT-qPCR was performed in a total reaction volume of 20 µl using 8 µl of diluted cDNA, 10 µl Fast SYBR green PCR Master Mix and 1 µl of 10 µM forward and reverse primers. Elongation factor 1α, 18S, and cod beta actin were tested as the housekeeping genes, and 18S was chosen as the most stable housekeeping gene for the samples in the present study. Detection of *F. noatunensis* RNA was performed with minor changes as described by Ottem et al. [17]. Primer sequences and references are listed in Table 2. The amplification efficiency of each primer pair was approximately equal and near 100%, allowing the use of the $2^{-\Delta\Delta CT}$ method (comparative CT method) to calculate the relative gene expression. All gene expression data were analyzed with the SDS 2.3 software and exported to Microsoft Excel (Microsoft, Redmond, WA, USA) for further analysis. The 0-control served as calibrator. Significance levels ($p \leq 0.05$) were determined by exact 2-sample permutation test computed in R (http://www.r-project.org).

2.7. Histological staining and immunohistochemical detection of F. noatunensis

Sections of spleen from one control fish, two cohabitant fish sampled at 60 d.p.i. and two effluent fish sampled at 60 d.p.i. and 90 d.p.i. were fixed in 4% paraformaldehyde at 4 °C for 10-16 h, transferred to 70% EtOH and thereafter embedded in paraffin. Three µm-thick sections were cut and stained with haematoxylin and eosin (H&E) for histology. Immunohistochemistry for *F. noatunensis* was performed on dewaxed and rehydrated paraffin sections subsequent to antigen retrieval by microwave heating in citrate buffer as described by Shi et al [18]. The primary antibody (provided by Dr. Zerihun, National Veterinary Institute, Oslo, Norway) was raised in rabbit against *F. noatunensis* whole bacteria. Staining was performed using a biotinylated anti-rabbit immunoglobulin

secondary antibody (Dako, Glostrup, Denmark), a streptavidin-alkaline-phosphatase (GE Healthcare, Chalfont St. Giles, UK) and fast red visualisation system.

2.8. Enzyme-linked immunosorbent assay (ELISA)

A whole cell ELISA was performed as described by Schrøder et al. [8] and was used to determine the content of specific antibodies against *F. noatunensis* in cod sera. ELISA was carried out with pooled and individual sera from i.p. infected groups, immunised groups and control fish. Individual sera data were exported to Microsoft Excel where mean and standard errors were calculated.

2.9. Western-blot analysis

The specificity of the antibody responses was determined by immunostaining of cell lysate from *F. noatunensis* using pooled sera from groups with high antibody titres (ELISA). Individual sera were also tested from i.p. infected fish at 60 d.p.i. Western blot was performed as described by Lund et al. [19] with the exception that bacterial cells were suspended in 10 mM Tris-base pH 8. Gels were stained for proteins using Coomassie blue (Pierce, Rockford, IL, USA), Sypro Ruby and Silver staining (BioRad, Hercules, CA, USA).

3. Results

3.1. Pathological changes

A few, small white nodules were observed in spleen and head kidney from three out of 10 fish from the i.p. infected group at 15 d.p.i., whereas many nodules were observed in all the 10 fish sampled from the i.p. infected group at 30 and 60 d.p.i. No visible nodules were observed in the cohabitant or effluent groups at 30 and 60 d.p.i. At 90 d.p.i. several nodules were observed in spleen and head kidney from the effluent group. Two fish died during the experiment due to swim bladder problems according to examination of a veterinarian while no fish died of the infection.

3.2. Histopathological changes and detection of F. noatunensis in infected fish

Bacteria were re-isolated from the i.p. infected fish as early as one d.p.i. and the number of positive individuals increased for each sampling. Bacterial streaks from both head kidney and blood formed colonies on CHAB-plates. No bacteria were re-isolated from any of the control fish.

Presence of *F. noatunensis* was also demonstrated by RT-qPCR (Fig. 1). In the i.p. infected group *F. noatunensis* could be detected at one d.p.i. and the copy number of Fc50 amplicon was greatly increased for each time point. Bacterial RNA was first detected in the cohabitant group at 30 d.p.i. and in the effluent group at 90 d.p.i. (Fig. 1). No bacterial RNA was detected in the untreated or saline injected control fish.

Histological examination showed a few, small aggregations of cells in the red pulp (connective tissue) containing MLCs from the cohabitant group sampled at 60 d.p.i. Intracellular vacuole-like structures with bacteria staining positive for *F. noatunensis* were detected in the MLCs. The white nodules in the spleen of fish from the effluent group sampled at 90 d.p.i. were composed of aggregates of many cells including MLCs. Several of the MLCs contained intracellular vacuole-like structures with bacteria staining for *F. noatunensis* (Fig. 2a). No staining for bacteria was observed in the spleen of control fish (Fig. 2b).

3.3. Antibody responses

Specific antibody responses to *F. noatunensis* in serum pools from the various groups were tested by ELISA. The pooled samples corresponded very well with the mean of the individual sera and are displayed in Fig.3. Fish i.p. infected with live *F. noatunensis* showed an elevated antibody titre at 30 d.p.i. and the highest titre was observed at 60 d.p.i. (Fig. 3). Serum pools sampled at 60 d.p.i. from fish injected with the high concentrations of inactivated bacteria showed an antibody titre similar to the serum pools from i.p. infected fish at 30 d.p.i. Oil adjuvant seemed to have no effect on the level of antibodies compared to the saline formulation. None of the cohabitants showed any significant antibody responses at the studied time points (results not shown). In the groups immunized with inactivated or live bacteria there were both high and low responders at 30 d.p.i., whereas at 60 d.p.i. all individuals seemed to have equally high

responses (results not shown). No individuals of the control groups revealed specific antibody responses.

Antibody specificities were investigated by immunostaining of Western blot with *F. noatunensis* lysate using pooled sera from the groups with high antibody responses. A single broad band with a molecular weight of 20-25 kDa was detected with sera from fish injected with inactivated bacteria or live bacteria at 60 d.p.i. (Fig. 4). No visible bands were detected with sera from the infected fish at 30 d.p.i. All individual sera from the infected group gave positive staining for a similar component (results not shown), whereas all controls were negative. The 20-25 kDa molecule was not detected on gels stained with Coomassie blue but it was visible with Sypro Ruby and Silver staining (results not shown).

3.4. Immune gene expression

Expression of *IFN-γ*, *IL-1β* and *IL-10* was measured in organs from fish in the i.p. infected, cohabitant and effluent groups using RT-qPCR. In Fig.5 significance was denoted with asterisks when compared to untreated 0-contol fish and with plus sign when compared to individual saline control fish.

Fish i.p. infected with *F. noatunensis* showed a significant up-regulation of *IFN-γ* expression in spleen and head kidney from 7 d.p.i. (spleen) and 15 d.p.i. (head kidney) to 60 d.p.i. Both in spleen and head kidney the highest expression values were approximately 15 fold (Fig. 5). In intestine a significant up-regulation of *IFN-γ* was detected from 30 d.p.i. (18-fold) to 60 d.p.i. (7-fold) whereas in rectum there were no significant differences in the gene expression. In the cohabitant group there was a significant up-regulation of *IFN-γ* at 60 d.p.i. in head kidney (7-fold) whereas in intestine there was a significant down-regulation at 30 and 60 d.p.i. (Fig. 6C). In the effluent group, *IFN-γ* was significantly down-regulated at 30 d.p.i. in intestine and at 60 d.p.i. in rectum (Fig. 6D).

In i.p. infected fish, an increased expression of *IL-1β* was observed from 15 to 60 d.p.i. in spleen (25 to 70-fold) and from 7 to 60 d.p.i. in head kidney (10 to 45-fold). Intestine and rectum showed no significant differences in expression (Fig. 5). In the cohabitant group there was a significant up-regulation of *IL-1β* at 60 d.p.i. in both spleen and head

kidney, 17- fold and 8-fold respectively (Fig. 6A, B). *IL-1β* was down-regulated at 30 and 60 d.p.i.in intestine and at 60 d.p.i. in rectum (Fig. 6C, D). In the effluent group, significant down-regulations were observed at 30 d.p.i. in intestine and at 60 d.p.i. in rectum (Fig. 6D).

Expression of *IL-10* showed a significant down-regulation at 2 and 4 d.p.i. in rectum of i.p. infected fish (Fig. 5). In the cohabitants the expression of *IL-10* was down-regulated at 30 and 60 d.p.i. in rectum while in the effluent group it was down-regulated at 60 d.p.i. in spleen and head kidney and at 30 and 60 d.p.i. in the rectum (Fig. 6A, B). In intestine *IL-10* was up-regulated from 30 d.p.i to 60 d.p.i. in the cohabitant fish.

4. Discussion

4.1. Waterborne transmission

In the present study, waterborne transmission of francisellosis in Atlantic cod through particle reduced water was confirmed. Apparently, cod do not have to be in close proximity to diseased fish or feces to be infected with francisellosis. Presence of *F. noatunensis* was confirmed in the i.p. infected, cohabitant and effluent groups by RT-qPCR using *F. noatunensis* specific primers and by immunohistochemistry. *F. noatunensis* was detectable by RT-qPCR prior to the macroscopically visible changes and the bacteria were observed inside macrophage-like cells (MLCs). A former study showed that *F. noatunensis* could be detected at 38 d.p.i. in cohabitants by CHAB cultivation and PCR whereas the first white nodules were observed at 51 d.p.i. [20]. These results are in line with the present study as *F. noatunensis* was detected by RT-qPCR at 30 d.p.i. whereas the histopathological changes were observed at 60 d.p.i. in the group infected through cohabitation. The waterborne transmission of francisellosis implies that the infection may occur via the mucosal route such as the intestine, skin, mouth and gills.

As we have confirmed that francisellosis is horizontally transferred by water, the direction of ocean currents must be considered when establishing new cod farms or when taking action during outbreaks of disease as is also shown for pancreas disease in salmon [21]. Moreover, vaccination may contribute to prevent the horizontal spread of diseases

but currently there are no vaccines available against francisellosis in cod. Therefore, further effort in developing vaccines against *F. noatunensis* is of great importance and possible vertical transmission of francisellosis should be investigated.

4.2 Immune response

The present study showed that the antibody responses against injected live and inactivated bacteria were approximately equal at 30 d.p.i. whereas live bacteria gave higher antibody titres than inactivated bacteria at 60 d.p.i. The stronger response to live bacteria could be due to a dose difference between the continuously growing live bacteria and the inactivated bacteria. Previous findings have shown that cod respond poorly to various inactivated bacteria [4, 5]. Conversely, high specific antibody responses were found in cod immunized with inactivated *F. noatunensis* [8] and this is in line with the present study. The responses to inactivated bacteria showed no difference in the groups injected with bacteria in oil compared to bacteria in saline. Previous research has shown that oil adjuvants are important for the antibody response in cod against *A. salmonicida* [19] and *V. anguillarum* [19, 22]. However, oil adjuvant was not necessary to gain at least short time protection against vibriosis and furunculosis [23].

Serum pools from immunized and infected cod reacted with a 20-25 kDa *F. noatunensis* component on Western blot. This component was suggested to be of lipopolysaccharide (LPS) nature since it was not detected by protein staining whereas it was visible with Sypro Ruby and Silver Staining as also seen earlier in cod [8]. In a former study a component of similar molecular weight from *Francisella victoria* in tilapia (*Oreochromis niloticus niloticus*) was characterized as lipooligosaccharide [24].

To date, the main antibody responses to pathogenic bacteria in cod seem to be directed against LPS as described for *A. salmonicida* and *V. anguillarum* [5, 19]. It has been suggested that MHC class II and CD4 is absent in cod [25]. Based on these observations it could be hypothesised that cod lack T-cell dependent antibody responses because MHC class II and CD4 is essential for these responses. On the other hand, some reports have shown weak responses against the A-layer protein of *A. salmonicida* [19, 26]. Interestingly, the A-layer is composed of regularly arranged surface subunit proteins, thus

making it possible to activate the antibody response in a T-cell independent manner. Another possibility is that other molecules have acquired similar functions.

The mammalian pathogen *F. tularensis,* which is related to *F. noatunensis*, escapes acidification and degradation by disrupting the phagosomes of the phagocytes and then migrates to the cytoplasm [27, 28]. Phagocytes and epithelial cells have also been suggested to be the host cells for *F. noatunensis* in cod [1, 2] as supported by this experiment (Fig. 2) and a recent study that confirms the migration of *F. noatunensis* from the phagosome to the cytoplasm in cod leukocytes [29].

Up-regulation of *IFN-γ* was observed from 7 to 60 d.p.i. in spleen, head kidney and intestine of i.p. infected fish and in head kidney sampled from cohabitants at 60 d.p.i. IFN-γ was down-regulated in intestine and rectum sampled from the cohabitants and the effluent groups. In mammals, IFN-γ is one of the most important macrophage activating cytokines and is responsible for the development of naïve T lymphocytes to $CD4^+$ T_H1-type [30]. Similar roles of IFN-γ are discussed in fish [31] such as rainbow trout (*Oncorhynchus mykiss*) where it enhanced respiratory burst activity [32]. In cod, up-regulation of *IFN-γ* in gill epithelial cells was reported 3 h post infection with *V. anguillarum* and *V. salmonicida* while no significant differences were seen 24 h post infection [33]. In addition, up-regulation of *IFN-γ* in cod head kidney was reported 24 h after injection of formalin inactivated *V. anguillarum* [10]. Compared to these earlier reports, our findings show a late up-regulation of *IFN-γ*.

Up-regulation of *IL-1β* was observed from 7 to 60 d.p.i. in spleen and head kidney of i.p. infected fish and at 60 d.p.i.of the cohabitants in the same organs. *IL-1β* was down-regulated in intestine and rectum of both cohabitants and effluent groups. IL-1β plays an important role in inflammatory regulation in fish [34] and is one of the key factors of the innate immune system. Quick up-regulation of *IL-1β* has been reported by Seppola et al. where an 800-fold increase was observed in spleen of cod 24 h after inactivated *V. anguillarum* injection [9]. Feng et al. also reported that the highest up-regulation of *IL-1β* was observed in spleen 6 h post injection of formalin-killed atypical *A. salmonicida* in cod [11]. In mammals, gene expression of *IL-1β* is strongly induced by bacterial LPS [35], which is also described in trout leukocytes [36]. The late up-regulation of *IL-1β* and *IFN-γ* observed in the present study was in contrast to earlier work which suggest a fast up-

regulation of these genes by different pathogens in epithelial cells from gills [33]. The late up-regulation might be because of dose differences or that the bacterium itself might have mechanisms to down-regulate or at least delay the gene expression.

In the present study, IL-*10* showed no significant increase of expression but a clear trend towards up-regulation could be observed in spleen and intestine of i.p. infected fish. In the cohabitant and effluent groups *IL-10* seem to be down-regulated. IL-10 is known to be an anti-inflammatory cytokine mainly produced by phagocytes. It enhances B cell survival, proliferation, inhibits T_H1 response and antibody production in mammals. In addition IL-10 is involved in the regulation of the JAK-STAT signalling pathway [37]. Although IL-10 is known to be an immunosuppressive cytokine, it has been suggested that it acts like an inflammatory cytokine in fish [38]. However, a recent study on goldfish (*Carassius auratus* L.) showed that a recombinant form of IL-10 functioned as an anti-inflammatory cytokine [39]. In our study expression of *IL-10* seemed to increase from 15 d.p.i. but the results were not significant. It is possible that *IL-10* could become up-regulated stronger at a later stage and suppress the expression of inflammatory cytokines. Interestingly, up-regulation of *IL-10* and a possible suppression of *IFN-γ* and *IL-1β* were observed in the intestine of cohabitant fish from 30 d.p.i. IL-10 has been reported to be active in mucosal surfaces in humans to counterbalance inflammatory cytokines and protect the tissue from inflammatory reactions [40, 41, 42].

The generally lower expression of cytokines in intestine and rectum compared to the spleen and head kidney could be due to the lower number of immune cells in gastrointestinal organs as reported in Atlantic cod [15]. Even though the expression level was not as high as in spleen or head kidney, a certain regulation of cytokines were observed in intestine and rectum and this suggests an involvement of the intestinal mucosal immune system during *F. noatunensis* infections.

The present study is the first report which confirms horizontal waterborne transmission of *F. noatunensis* in cod and thus provides new knowledge about *F. noatunensis* infection routes. The disease was transmitted through water and the fish need not come into contact with diseased fish or feces to be infected. In addition, high and specific antibody responses and regulation of immune genes were observed in infected

cod suggesting that both humoral and cellular immune responses are triggered by this pathogen. The biological effect of these responses needs further investigation.

Acknowledgments

This study was supported by Norwegian Research Council project number 183204 and partly by 19672/S40. We would like to thank the staff at the Fish Health Laboratory for taking care of fish and Dr. D. Colquhoun, National Veterinary Institute, for confirming the reisolation of *Francisella* from infected fish. We would also like to thank Prof. M. Greenacre, Department of Economics and Business, Universitat Pompeu Fabra, for helping us with the statistics.

References

1. Olsen AB, Mikalsen J, Rode M, Alfjorden A, Hoel E, Straum-Lie K, Haldorsen R, Colquhoun DJ. A novel systemic granulomatous inflammatory disease in farmed Atlantic cod, *Gadus morhua* L., associated with a bacterium belonging to the genus *Francisella*. J Fish Dis. 2006;29:307-11.
2. Nylund A, Ottem KF, Watanabe K, Karlsbakk E, Krossøy B. *Francisella* sp. (Family Francisellaceae) causing mortality in Norwegian cod (*Gadus morhua*) farming. Arch Microbiol. 2006;185:383-92.
3. Nylund A. and Ottem K. F. Francisellosis a new cod (*Gadus morhua*) disease caused by *Francisella* n.sp. DIPNET 2006; 45.
4. Schrøder MB, Espelid S, Jørgensen TØ. Two serotypes of *Vibrio salmonicida* isolated from diseased cod (*Gadus morhua* L.); virulence, immunological studies and vaccination experiments. Fish Shellfish Immunol. 1992;2:211-21.
5. Espelid S, Rødseth OM, Jørgensen TØ. Vaccination experiments and studies of the humoral immune responses in cod, *Gadus morhua* L., to four strains of monoclonal-defined *Vibrio anguillarum*. J Fish Dis. 1991;14:185-97.
6. Magnadóttir B, Jónsdóttir H, Helgason S, Björnsson B, Solem ST, Pilström L. Immune parameters of immunised cod (*Gadus morhua* L.). Fish Shellfish Immunol. 2001;11:75-89.
7. Pilström L, Petersson A. Isolation and partial characterization of immunoglobulin from cod (*Gadus morhua* L.). Dev Comp Immunol. 1991;15:143-52.
8. Schrøder MB, Ellingsen T, Mikkelsen H, Norderhus EA, Lund V. Comparison of antibody responses in Atlantic cod (*Gadus morhua* L.) to *Vibrio anguillarum*, *Aeromonas salmonicida* and *Francisella* sp. Fish Shellfish Immunol. 2009;27:112-9.
9. Seppola M, Larsen AN, Steiro K, Robertsen B, Jensen I. Characterisation and expression analysis of the interleukin genes, IL-1beta, IL-8 and IL-10, in Atlantic cod (*Gadus morhua* L.). Mol Immunol. 2008;45:887-97.
10. Furnes C, Seppola M, Robertsen B. Molecular characterisation and expression analysis of interferon gamma in Atlantic cod (*Gadus morhua*). Fish Shellfish Immunol. 2009;26:285-92.
11. Feng CY, Johnson SC, Hori TS, Rise M, Hall JR, Gamperl AK, et al. Identification and analysis of differentially expressed genes in immune tissues of Atlantic cod stimulated with formalin-killed, atypical *Aeromonas salmonicida*. Physiol Genomics. 2009 37:149-63.
12. Borza T., Stone C., Rise M. L., Bowman S. and Johnson S. C. Atlantic cod (*Gadus morhua*) CC chemokines: Diversity and expression analysis. Dev Comp Immunol. 2010;34:904-13.
13. Seppola M, Stenvik J, Steiro K, Solstad T, Robertsen B, Jensen I. Sequence and expression analysis of an interferon stimulated gene (ISG15) from Atlantic cod (*Gadus morhua* L.). Dev Comp Immunol. 2007;31:156-71.
14. Solstad T, Larsen AN, Seppola M, Jørgensen TØ. Identification, cloning and expression analysis of a hepcidin cDNA of the Atlantic cod (*Gadus morhua* L.). Fish Shellfish Immunol. 2008;25:298-310.
15. Inami M, Taverne-Thiele AJ, Schrøder MB, Kiron V, Rombout JH. Immunological differences in intestine and rectum of Atlantic cod (*Gadus morhua* L.). Fish Shellfish Immunol. 2009;26:751-9.

16. Mikalsen J, Olsen AB, Tengs T, Colquhoun DJ. *Francisella philomiragia* subsp. *noatunensis* subsp. nov., isolated from farmed Atlantic cod (*Gadus morhua* L.). Int J Syst Evol Microbiol. 2007;57:1960-5.
17. Ottem KF, Nylund A, Isaksen TE, Karlsbakk E, Bergh Ø. Occurrence of *Francisella piscicida* in farmed and wild Atlantic cod, *Gadus morhua* L., in Norway. J Fish Dis. 2008;31:525-34.
18. Shi SR, Chaiwun B, Young L, Cote RJ, Taylor CR. Antigen retrieval technique utilizing citrate buffer or urea solution for immunohistochemical demonstration of androgen receptor in formalin-fixed paraffin sections. J Histochem Cytochem. 1993; 41:1599-604.
19. Lund V, Børdal S, Kjellsen O, Mikkelsen H, Schrøder MB. Comparison of antibody responses in Atlantic cod (*Gadus morhua* L.) to *Aeromonas salmonicida* and *Vibrio anguillarum*. Dev Comp Immunol. 2006;30:1145-55.
20. Mikalsen J, Olsen AB, Rudra H, Moldal T, Lund H, Djønne B, Bergh Ø, Colquhoun1 DJ. Virulence and pathogenicity of *Francisella philomiragia* subsp. noatunensis for Atlantic cod, *Gadus morhua* L., and laboratory mice. J Fish Di. 2009;32:377-81.
21. Viljugrein H, Staalstrøm A, Molvaelr J, Urke HA, Jansen PA. Integration of hydrodynamics into a statistical model on the spread of pancreas disease (PD) in salmon farming. Dis Aquat Organ. 2009;88:35-44.
22. Gudmundsdóttir S, Magnadóttir B, Björnsdóttir B, Arnadóttir H, Gudmundsdóttir BK. Specific and natural antibody response of cod juveniles vaccinated against *Vibrio anguillarum*. Fish Shellfish Immunol. 2009;26:619-24.
23. Mikkelsen H, Schrøder MB, Lund V. Vibriosis and atypical furunculosis vaccines; efficacy, specificity and side effects in Atlantic cod, *Gadus morhua* L. Aquaculture. 2004;242:81-91.
24. Kay W, Petersen BO, Duus JO, Perry MB, Vinogradov E. Characterization of the lipopolysaccharide and beta-glucan of the fish pathogen *Francisella victoria*. FEBS J. 2006;273:3002-13.
25. Pilström L, Warr GW, Strömberg S. Why is the antibody response of Atlantic cod so poor? The search for a genetic explanation. Fisheries Sci. 2005 71:961-71.
26. Lund V, Arnesen JA, Mikkelsen H, Gravningen K, Brown L, Schrøder MB. Atypical furunculosis vaccines for Atlantic cod (*Gadus morhua*); vaccine efficacy and antibody responses. Vaccine. 2008;26:6791-9.
27. Golovliov I, Baranov V, Krocova Z, Kovarova H, Sjöstedt A. An attenuated strain of the facultative intracellular bacterium *Francisella tularensis* can escape the phagosome of monocytic cells. Infect Immun. 2003;71:5940-50.
28. Clemens DL, Lee BY, Horwitz MA. Virulent and avirulent strains of *Francisella tularensis* prevent acidification and maturation of their phagosomes and escape into the cytoplasm in human macrophages. Infect Immun. 2004;72:3204-17.
29. Furevik A, Pettersen EF, Colquhoun D, Wergeland HI. The intracellular lifestyle of Francisella noatunensis in Atlantic cod (*Gadus morhua* L.) leucocytes. Fish Shellfish Immunol. 2010.
30. Schoenborn JR, Wilson CB, Frederick WA. Regulation of Interferon-γ During Innate and Adaptive Immune Responses. Adv Immunol. 2007;96:41-101.

31. Robertsen B. The interferon system of teleost fish. Fish Shellfish Immunol. 2006;20:172-91.
32. Zou J, Carrington A, Collet B, Dijkstra JM, Yoshiura Y, Bols N, Secombes C. Identification and bioactivities of IFN-gamma in rainbow trout *Oncorhynchus mykiss*: The first Th1-type cytokine characterized functionally in fish. J Immunol. 2005;175:2484-94.
33. Caipang CM, Lazado CC, Brinchmann MF, Kiron V. Infection-induced changes in expression of antibacterial and cytokine genes in the gill epithelial cells of Atlantic cod, *Gadus morhua* during incubation with bacterial pathogens. Comp Biochem Physiol B Biochem Mol Biol. 2010;156:319-25.
34. Huising MO, Stet RJM, Savelkoul HFJ, Verburg-van Kemenade BML. The molecular evolution of the interleukin-1 family of cytokines; IL-18 in teleost fish. Dev Comp Immunol. 2004;28:395-413.
35. Davidson J, Kerr A, Guy K, Rotondo D. Prostaglandin and fatty acid modulation of Escherichia coli O157 phagocytosis by human monocytic cells. Immunology. 1998;94:228-34.
36. Secombes CJ, Zou J, Laing K, Daniels GD, Cunningham C. Cytokine genes in fish. Aquaculture. 1999;172:93-102.
37. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol. 2001;19:683-765.
38. Inoue Y, Kamota S, Ito K, Yoshiura Y, Ototake M, Moritomo T, Nakanishi T. Molecular cloning and expression analysis of rainbow trout (*Oncorhynchus mykiss*) interleukin-10 cDNAs. Fish Shellfish Immunol. 2005;18:335-44.
39. Grayfer L, Hodgkinson JW, Hitchen SJ, Belosevic M. Characterization and functional analysis of goldfish (*Carassius auratus* L.) interleukin-10. Mol Immunol. 2011;48:563-71.
40. Jarry A, Bossard C, Bou-Hanna C, Masson D, Espaze E,. Denis MG, Laboisse CL. Mucosal IL-10 and TGF-β play crucial roles in preventing LPS-driven, IFN-γ-mediated epithelial damage in human colon explants. J Clin Invest. 2008;118:1132-1142.
41. Melgar S, Yeung MM, Bas A, Forsberg G, Suhr O, Oberg A, Hammarstrom S, Danielsson A, Hammarstrom ML. Over-expression of IL-10 in mucosal T cells of patients with active ulcerative colitis. Clin Exp Immunol. 2003;134:127-37.
42. Bodger K, Wyatt JI, Heatley RV. Gastric mucosal secretion of IL-10: relations to histopathology, Helicobacter pylori status, and tumor necrosis factor-alpha secretion. Gut. 1997;40:739-44.

Figure legends

Figure 1 Detection of *F. noatunensis* RNA by real-time PCR in head kidney of i.p. infected with live *F. noatunensis*, cohabitants, effluent groups and healthy cod (controls) at various time points.

Figure 2 Immunohistochemical detection of *Francisella* antigen in spleen of effluent group sampled after 90 days (a). Note aggregations of macrophage-like cells (MLCs; arrows) and staining of bacteria (red) often within vacuole-like structures in the MLCs. Control group shows no staining for bacteria (b).

Figure 3 Antibody response in Atlantic cod to inactivated or live injected *F. noatunensis*. Antibody levels in serum pools (N=10-15) were measured by ELISA against inactivated *F. noatunensis* cells. ◇ Saline 60 d.p.i. ● Inactivated *F. noatunensis* (5×10^9 ml^{-1}) in saline 60 d.p.i. ▲ Inactivated *F. noatunensis* (5×10^9 ml^{-1}) in oil 60 d.p.i × Live *F. noatunensis* 30 d.p.i. ○ Live *F. noatunensis* 60 d.p.i.

Figure 4 Immunostaining of Western blot of *F. noatunensis* lysate incubated with pooled sera from group A: Saline control 60 d.p.i. B: Inactivated *F. noatunensis* (5×10^9 ml^{-1}) in saline 60 d.p.i. C: Inactivated *F. noatunensis* (5×10^9 ml^{-1}) in oil 60 d.p.i. D: Live *F. noatunensis* (1×10^6 ml^{-1}) 30 d.p.i. E: Live *F. noatunensis* (1×10^6 ml^{-1}) 60 d.p.i. F: All blue marker shown in kDa.

Figure 5 Relative gene expression of *IFN-γ*, *IL-1β*, and *IL-10* in different organs after live *F. noatunensis* injection. ■ 0-control □ saline injected control ▩ i.p. infected fish. The gene expression was normalized with non-injected fish. Relative expression of each gene was compared to the expression of 18s. Bars show averages ± SD of n = 6 or 10 fish. Significant differences ($p \leq 0.05$) between 0-control and infected groups are denoted with an asterisk. Significant differences ($p \leq 0.05$) between individual control (saline injected

control) and infected groups are denoted with a plus sign. Note the different scales on the y-axes.

Figure 6 Relative gene expression of *IFN-γ* (□), *IL-1β* (▒), and *IL-10* (■) in different organs in water-borne groups. (A) Spleen, (B) Head kidney, (C) Intestine, (D) Rectum. The gene expression was normalized with non-injected fish. Relative expression of each gene was compared to the expression of 18s. Bars show averages ± SD of n = 6 or 10 fish. Significant differences ($p \leq 0.05$) between individual control (saline injected control) and infected groups are denoted with a plus sign. Note the different scales on the y-axes.

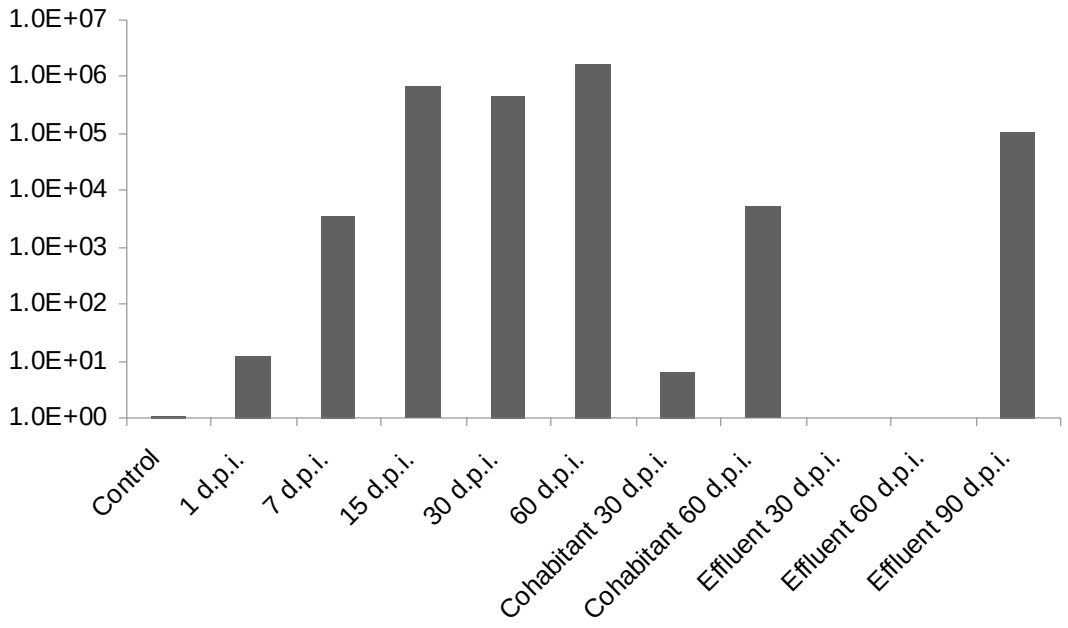

Figure 1

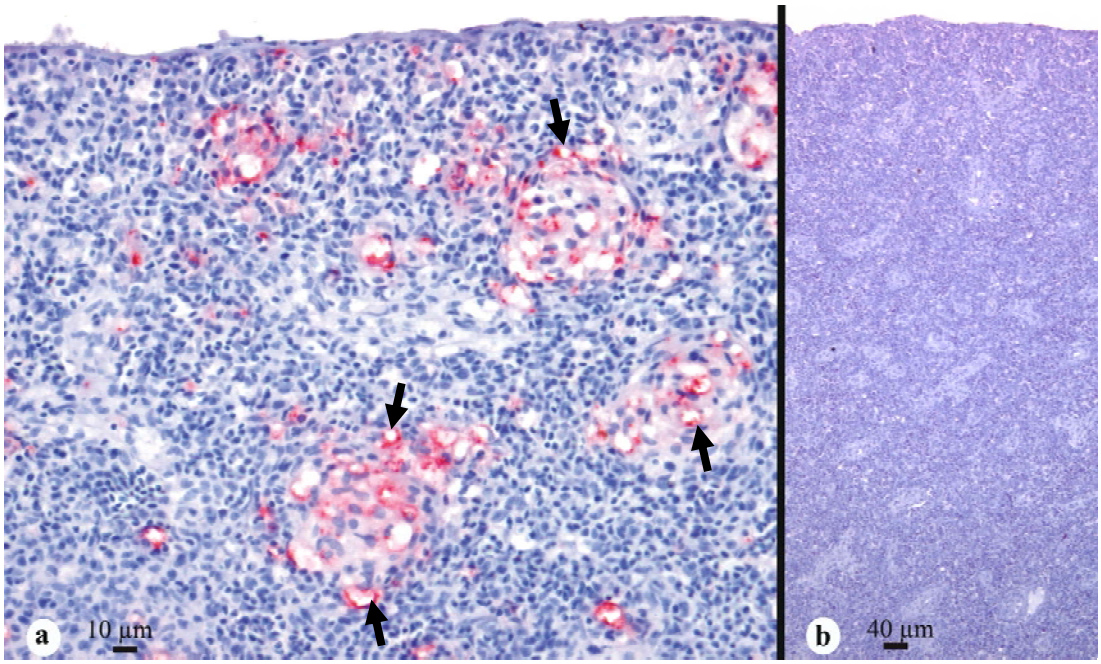

Figure 2

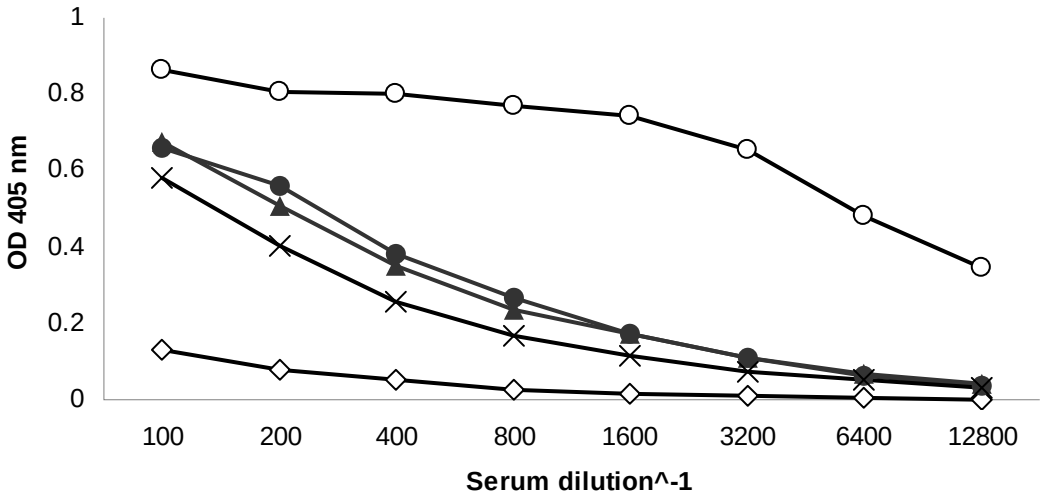

Figure 3

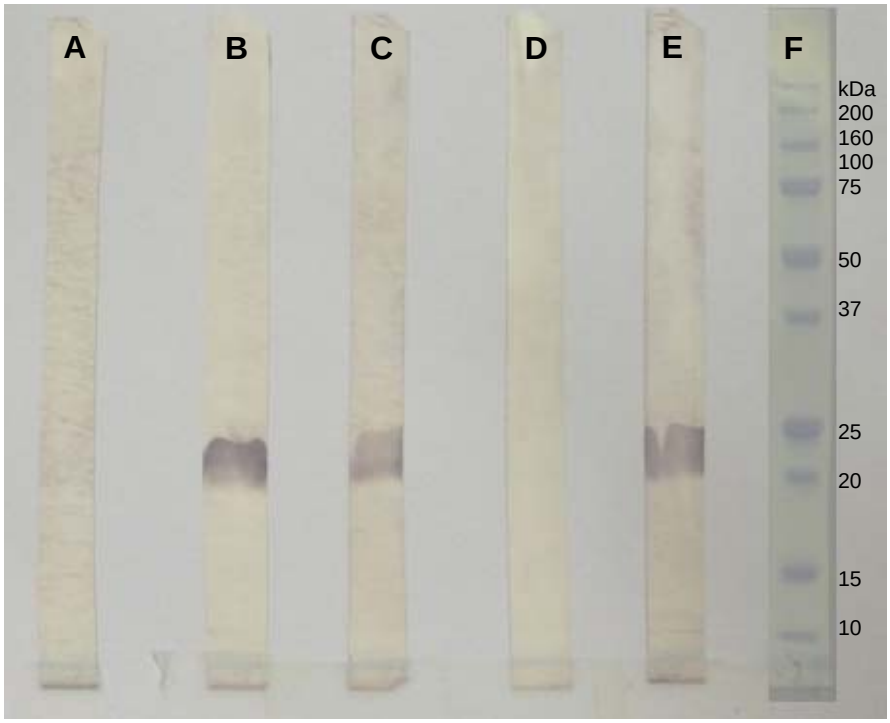

Figure 4

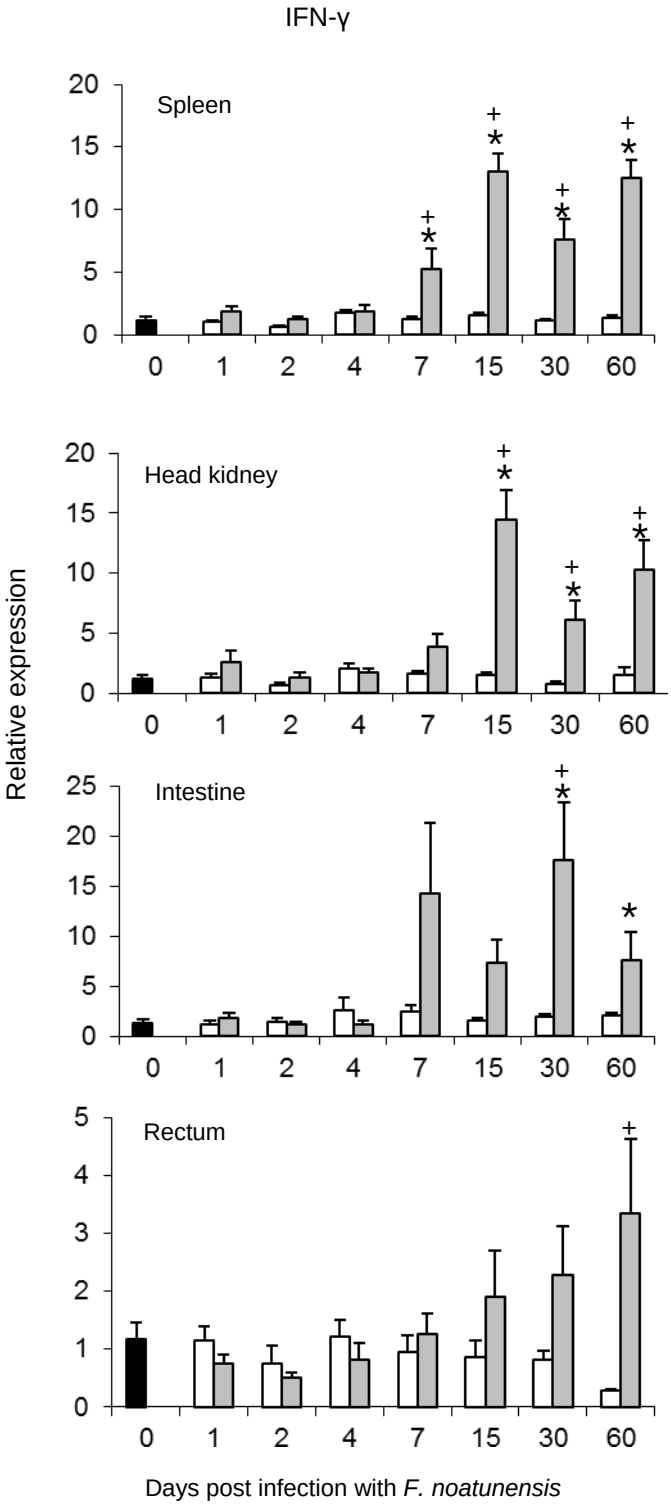
IFN-γ
Spleen
Head kidney
Intestine
Rectum
Relative expression
Days post infection with F. noatunensis

Figure 5

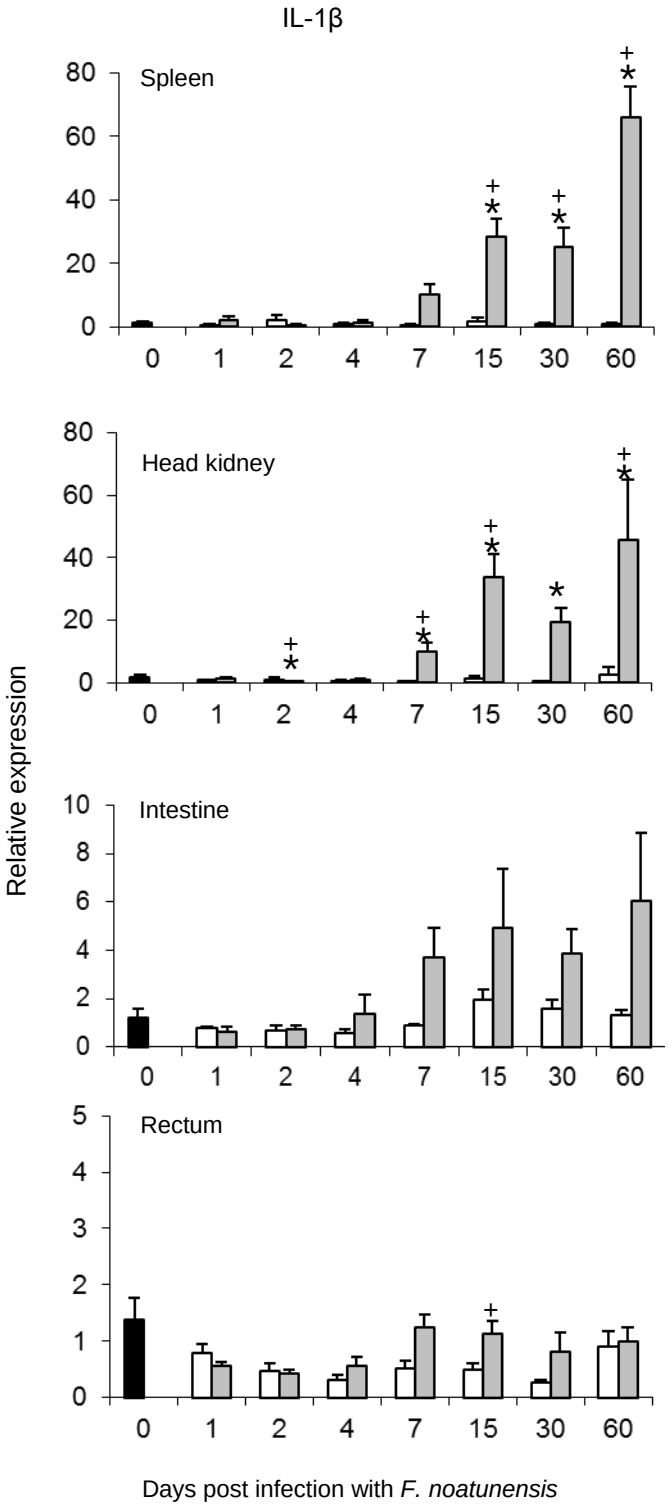

Figure 5 continue

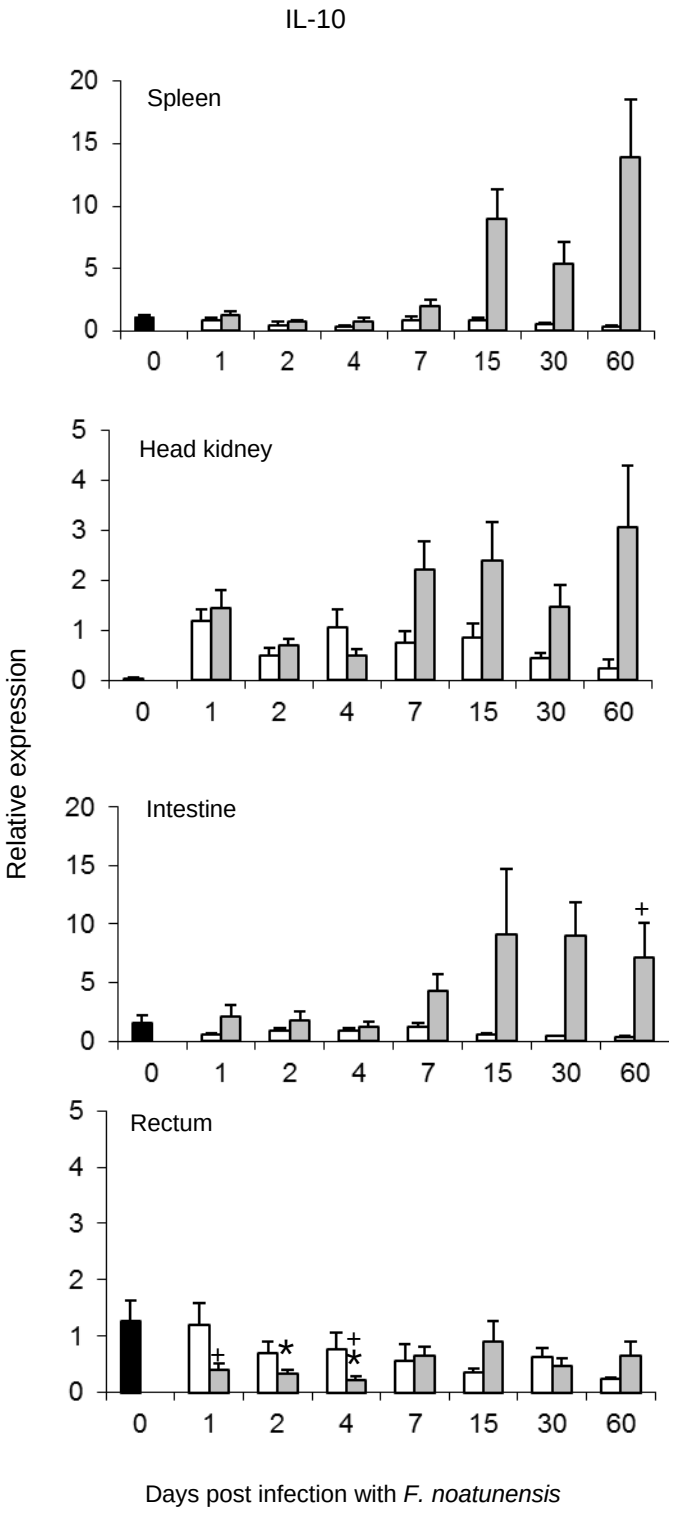

Figure 5 continue

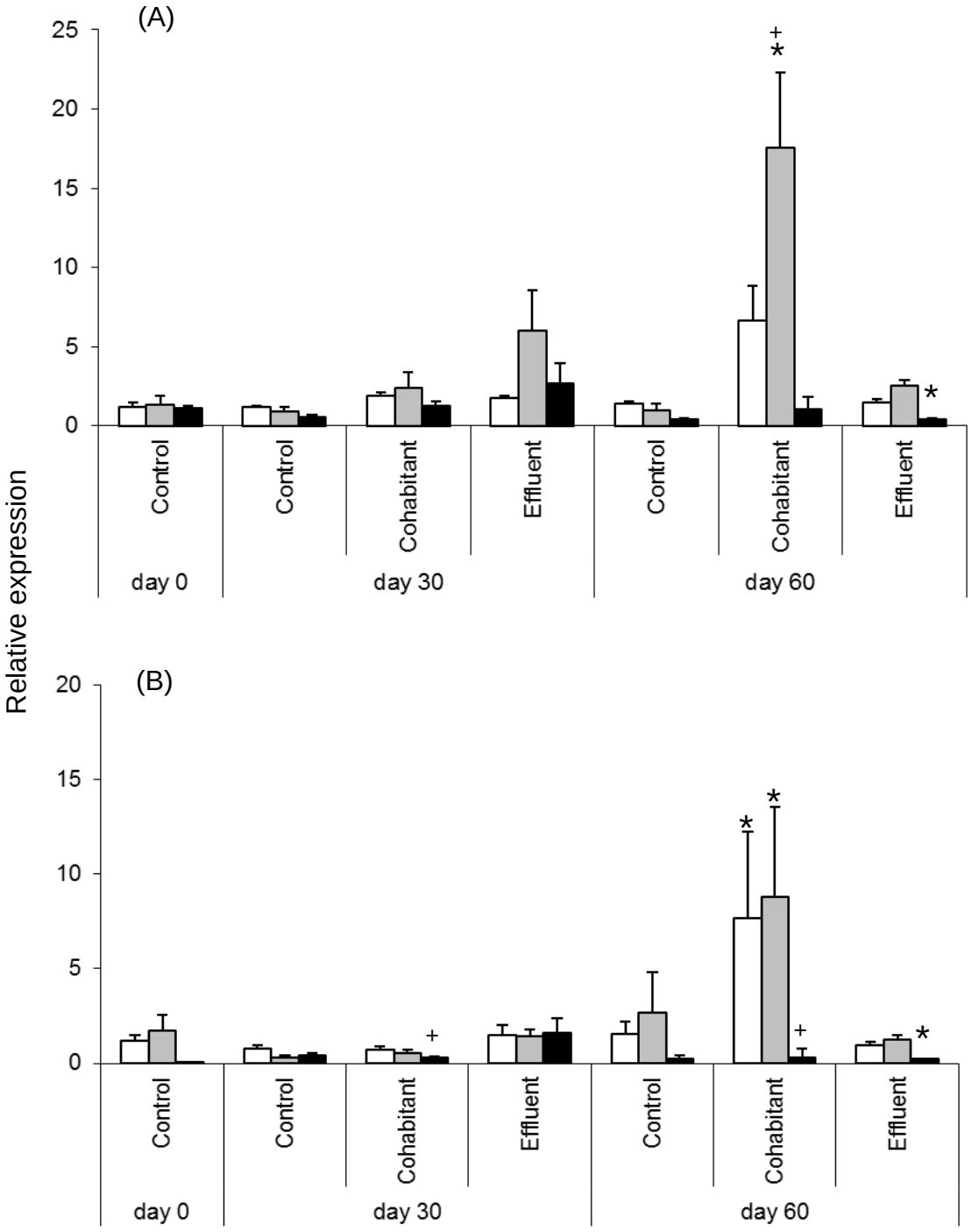
(A)
25
20
15
10
5
0
Relative expression
Control
Control
Cohabitant
Effluent
Control
Cohabitant
Effluent
day 0
day 30
day 60
(B)
20
15
10
5
0
Control
Control
Cohabitant
Effluent
Control
Cohabitant
Effluent
day 0
day 30
day 60

Figure 6

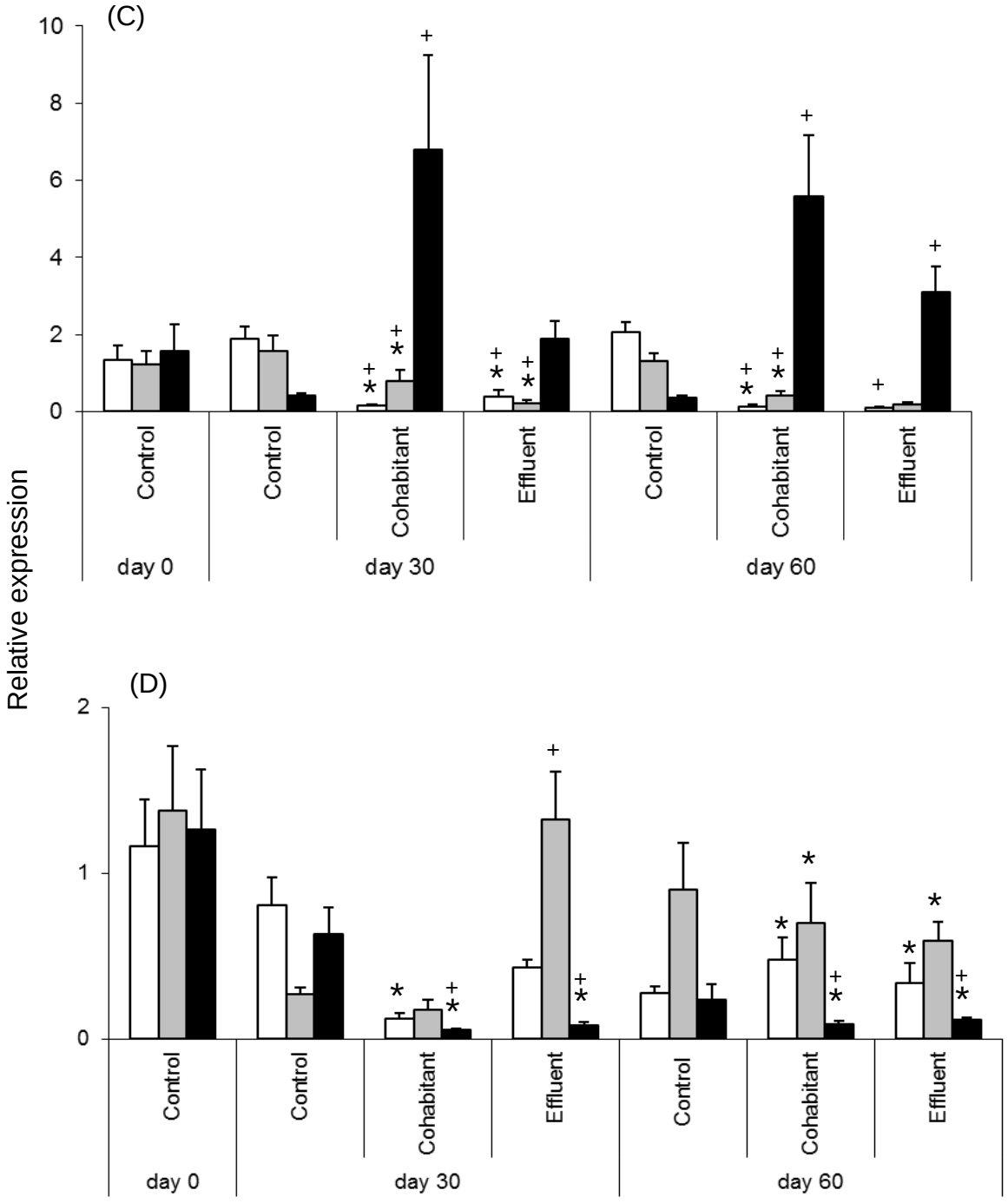

Figure 6 continue