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IMMUNOHISTOCHEMICAL DETECTION AND GENE EXPRESSION OF TNFα IN TURBOT (*Scophthalmus maximus*) ENTEROMYXOSIS

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Abstract

Enteromyxum scophthalmi (Myxozoa) constitutes one of the most devastating pathogens for turbot (*Scophthalmus maximus*, L.) aquaculture. This parasite causes a severe intestinal parasitosis that leads to a cachectic syndrome with high morbidity and mortality rates for which no therapeutic options are available. Presence of inflammatory infiltrates, increased apoptotic rates and epithelial detaching have been described at intestinal level, as well as leukocyte depletion in lymphohaematopoietic organs. Previous investigations on enteromyxosis in turbot showed the high susceptibility of this species to the parasite and reported the existence of a dysregulated immune response against the parasite. The pleiotropic cytokine tumour necrosis factor alpha (TNFα) plays a major role in immune response and is involved in a wide range of biological activities. In teleost, the gene expression of this cytokine has been found regulated under several pathological conditions. Teleost TNFα shows some analogous functions with its mammalian counterparts, but the extent of its activities is still poorly understood. Cytokines are generally considered as a double-edge sword and TNFα has been implicated in the pathogenesis of different inflammatory diseases as well as in wasting syndromes described in mammals. The aim of this work was to analyse the expression of TNFα during enteromyxosis with molecular (Q-PCR) and morphological (immunohistochemistry) tools. Kidney, spleen and pyloric caeca from turbot with moderate and severe infections were analysed and compared to healthy naïve fish.

TNFα expression was increased in both spleen and kidney in the earlier stages of the disease, whereas in severely infected fish, the expression decreased, especially in kidney. At the intestinal level, an increase in the number of TNFα-positive cells was noticed, which was proportional to the infiltration of inflammatory cells. The results demonstrate the involvement of TNFα in the immune response to *E. scophthalmi* in turbot, which could be related to the development of the clinic signs and lesions.

Keywords: *Tumour necrosis factor alpha; cytokine; turbot; enteromyxosis; inflammatory response; Q-PCR; immunohistochemistry*

1. Introduction

Enteromyxosis caused by the myxozoan parasite *Enteromyxum scophthalmi* poses a serious threat for turbot (*Scophthalmus maximus*, L.) aquaculture. Parasitic forms invade the digestive tract, being the infection first detected in pyloric caeca or anterior intestine and subsequently spreading along the entire gut [1, 2]. In contrast to other myxozoan species, *Enteromyxum* spp. can be directly transmitted from fish to fish, and up to now, no effective therapeutic options are available to control this parasitosis [3, 4]. The disease leads to a cachectic syndrome characterized by weight loss, anorexia and amyotrophy [3]. Turbot presents elevated rate of morbidity and mortality and the disease can affect up to 100 % of fish in a farming unit [5, 6]. Compared to gilthead sea bream (*Sparus aurata*, L.) infected by *E. leei*, turbot shows a higher susceptibility to enteromyxosis associated to more severe lesions [3]. Microscopically, the main lesion is catarrhal enteritis of increasing severity throughout the disease, characterized by severe inflammatory infiltrates and detachment of the lining epithelium, along with high parasite burden in late stages of the disease. In these stages, leukocyte depletion in the lymphohaematopoietic organs is also a common finding [1, 7, 8]. Previous studies on the immune response and host-parasite interaction have provided evidences of a dysfunctional immune response against the parasite. Turbot appears unable to mount an effective systemic adaptive response [8-11], while locally the immune response seems to be exacerbated, contributing to the development of lesions [7, 9, 12]. Tumour necrosis factor alpha (TNFα) is a cytokine that acts in a broad range of signalling events within cells, being involved in cell activation, proliferation death and survival [13]. It plays a pivotal role in the organization and functions of the immune system, mainly as a major pro-inflammatory cytokine, acting at early stages of the inflammatory reaction and orchestrating the subsequent cascade of events [13, 14]. This cytokine has been described as a double-edge sword, since its functions are essential for a proper immune response, but it is also clearly associated with the development of clinical signs and lesions in different human diseases [14-18]. TNFα is clearly implicated in the pathogenesis of inflammatory bowel diseases (IBDs) [19], which share with enteromyxosis the dysregulated immune response and the intestinal lesions, as well as in wasting diseases [16], characterized as enteromyxosis by anorexia, weight loss and amyotrophy. For these conditions, the immunomodulatory therapies, often consisting in specific blockade on TNFα action, have raised in many cases as the most effective [20-

23]. Also in a model of IBD described in zebrafish (*Danio rerio*, Hamilton), TNFα expression was found increased and immunomodulatory therapies showed positive results [24]. In fact, in the different fish species where TNFα has been identified, this cytokine showed similar immune-related functions to its mammalian counterpart [25-30]. Nevertheless, the complex and widespread biological activities accomplished in mammals are still poor described in teleosts. The regulation of its expression has been reported in several piscine parasitic diseases [31-33], including enteromyxosis by *E. leei* in gilthead sea bream [34]. The aim of this study was to investigate the involvement of TNFα in turbot enteromyxosis combining Q-PCR and immunohistochemistry to analyse its expression in target organs (pyloric caeca, kidney and spleen).

2. Materials and methods

Experimental design and histopathology

The experimental setup and sampling procedures were previously described [9]. Briefly, recipient (R) turbot were experimentally-infected by oral route [4] and tissue samples were collected at different time points in Bouin's fluid and RNAlater for histopathological and molecular techniques, respectively. The status of control (C, not exposed to infection) and R fish was assessed by light microscopy on H&E and toluidine blue stained sections. R fish were classified into three groups (slight, moderately and severely infected) according to the histopathological grading described by Bermúdez *et al.* [1]. For this study, spleen, kidney and pyloric caeca from 8 C and 8 R turbot at 24 and 42 days post-inoculation (DPI) were used. In order to increase the uniformity of the samples, R turbot at 24 DPI were chosen among those graded as moderately infected and R turbot at 42 DPI among those graded as severely infected. The experiment was carried out in accordance with national (Royal Decree RD1201/2005, for the protection of animals used in scientific experiments) and institutional regulations (CSIC, IATS Review Board) and the current European Union legislation on handling experimental animals.

Immunohistochemical detection of TNFα

Paraffin sections (3 μm thick) from Bouin's fixed tissue samples were dewaxed in xylene and rehydrated through a graded ethanol series. IHC was carried out with a previously developed protocol [35], using an automated stainer (Dako Autostainer, Dako, Glostrup, Denmark) after the antigen retrieval step, in order to standardize the immunostaining. Briefly, primary antibody (1:600 working dilution, rabbit polyclonal antibody to human TNFα, ab6671, Abcam, Cambridge, UK) was incubated during 2 h at room temperature. After 30 min incubation with a HRP-labelled secondary antibody, the peroxidase reaction was developed with a diaminobenzidine-positive chromogen (EnVision+ System-HRP kit, K 4011; Dako), achieving the desired signal after 1 min of incubation. The sections were washed three times for 5 min in 0.1 M phosphate buffered saline containing 0.05% Tween-20 between all subsequent steps. After

counterstaining with haematoxylin, sections were unloaded by the Autostainer, dehydrated and coverslipped with DePeX mounting medium (Gurr®, BDH Prolabo, VWR International, Ltd. UK). In order to test the specificity of the immunoreaction, positive (swine tissue) and negative (replacement of the primary antibody by PBS) controls were included.

Gene expression

Tissue samples preserved in RNAlater were kept at 4ºC during 24 h and stored at -20ºC until RNA extraction. Total RNA was extracted from tissues of C and R fish using TRIZOL Reagent (Life Technologies, Carlsbad, CA, USA) according to manufacturer's recommendations. RNA was quantified using NanoDrop® ND-1000 spectrophotometer (NanoDrop® Technologies Inc.) and its quality was checked in an Agilent BioAnalizer (Agilent Technologies, USA). Good quality RNA (RIN > 7.5) was reverse transcribed (1 µg) into cDNA by random primers using AffinityScript Multiple Temperature cDNA Synthesis kit following the supplier's protocol (Agilent Technologies). The Q-PCR analysis was carried out in a MX3005P thermocycler (Stratagene) using 2 µl of cDNA per reaction and 300 nM of each primer in a final volume of 20 µl according to the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies) manufacturer's instructions. The constitutively expressed ribosomal protein S4 (RPS4), proved to be stably expressed in turbot [36, 37], was chosen as the house-keeping gene for sample normalisation. TNFα primers (sense: 5'-GGGTGGATGTGGAAGGTGAT-3'; antisense: 5'-GGCCTCTGTTTGGCTTGACT-3') were designed based on the mRNA sequence of turbot TNFα (GenBank accession number FJ654645) [38]. Each sample was performed in triplicate for accuracy and error estimation including one reverse-transcription-negative control for each gene. Fluorescence readings at the end of each cycle were used to estimate threshold cycle values (Ct). Values were normalized to RPS4 and fold change in transcript level determined with the relative quantitative method (ΔΔCt) [39] using data from C fish as reference values. Prior to quantitative analysis, a standard curve was constructed using six serial dilutions of cDNA (from 1,000 to 0.01 ng) and the efficiency of each primer set was determined. Efficiencies of 90–110% were obtained by primer optimization. Each sample was analysed for primer–dimer, contamination, or mispriming by inspection of their dissociation curves.

Statistical analysis

The statistical analysis of gene expression was performed with SPSS Statistics 20.0 software (SPSS Inc., Chicago, Illinois, USA). Data were expressed as mean ± SEM, and significance of differences was determined by Student's *t*-test, after checking that data from C and R fish follow a normal distribution using Shapiro-Wilk test. Results were considered significant at $P < 0.05$.

3. Results

Histopathology

The 8 R turbot at 24 DPI selected for this study presented scarce parasitic forms in the lining epithelium of different regions of the gastrointestinal tract, more numerous in pyloric caeca and anterior intestine. In these regions, mild inflammatory infiltrates constituted by mononuclear cells were commonly observed in the lamina propria-submucosa and at the basis of the epithelium (Fig 1a, c). These cells were mainly consistent with lymphocytes within the lining epithelium, while the infiltrates in the lamina propria-submucosa were constituted by a heterogeneous population of macrophages and lymphocytes (Fig. 1c). In those areas where the presence of *E. scophthalmi* and inflammatory cells was higher, some intestinal folds showed changes in epithelial architecture (Fig. 1a) and some enterocytes presented apoptotic features. No significant histopathological changes were present in other organs.

On the other hand, the 8 R turbot at 42 DPI sampling point showed a heavy parasitic load along the entire gut lining epithelium, which presented the typical scallop shape and numerous areas of epithelial detachment (Fig. 1b). Mononuclear cells, mainly consistent with lymphocytes, were seen infiltrating the epithelium and the lamina-propria submucosa appeared thickened and edematous, with severe mixed inflammatory infiltrates (Fig. 1b). Leukocyte accumulation in blood vessels and dilatation of blood and lymphatic vessels were also often observed. Most enterocytes and detached cells in the intestinal lumen, showed altered morphology, consistent with apoptosis, like rounding up or shrinkage and nuclear alterations, namely hypertrophied nuclei, chromatin condensation, degradation of the nuclear envelope or nuclear fragmentation (Fig. 1d). Also, numerous inflammatory cells in the lamina propria-submucosa undergoing apoptotic death were reported. The histological diagnosis was moderate to severe parasitic catarrhal enteritis. In other organs, the most striking lesion was the leukocyte depletion found in the spleen and in the kidney, being generally more evident in the latter organ (Fig. 1f).

C turbot from both sampling points did not show any significant histological alteration in the sampled tissues.

Immunohistochemical detection of TNFα

Immunoreactivity against TNFα antibody was found in the three studied organs from both C and R turbot. A comparison between the different conditions is shown in Fig. 2 and the relative density of TNFα-positive ($TNF\alpha^+$) cells summarized in Table 1.

In the spleen, immunoreactive macrophage-like cells were observed in all analysed sections, and generally tended to cluster around blood vessels and melanomacrophage centres (Fig. 3a). Nevertheless, in moderately infected fish, a marked increase in $TNF\alpha^+$ cells was observed, which were diffusely distributed in the splenic parenchyma (Fig. 2). In severely infected specimens, on the contrary, the density of $TNF\alpha^+$ cells was comparable to C fish, with some areas of the spleen showing high concentration of labelled cells, but diminished in the areas suffering from cellular depletion (Fig. 2).

In kidney, numerous cells of the lymphohaematopoietic interstitial tissue were $TNF\alpha^+$ in C fish (Fig. 2). Turbot with moderate infection showed a similar or slightly augmented

number of immunoreactive cells, which often showed a more intense immunoreaction compared to C turbot (Fig. 2). In turbot with severe infection, which showed an evident cellular depletion affecting the interstitial tissue, the number of TNFα$^+$ cells was generally scarce (Fig. 2 and Fig. 3b). In all the studied fish, rodlet cells within the epithelium of renal tubules were occasionally immunostained (Fig. 3b), without a clear association with the healthy status of the specimens.

In pyloric caeca, the main TNFα$^+$ cell types were rounded macrophage-like cells in the lamina propria-submucosa and bullet-shape rodlet cells within the lining epithelium (Fig. 2 and Fig. 3c, d, e, f). In C fish, scarce numbers of immunoreactive cells were observed (Fig. 1). Moderately infected turbot showed increased numbers of TNFα$^+$ macrophage-like cells in the lamina-propria submucosa (Fig. 2 and Fig. 3c), that further augmented in those severely infected, in association with the inflammatory infiltrates in the lamina propria-submucosa (Fig. 2 and Fig. 3e). By contrast, TNFα$^+$ rodlet cell numbers were higher in turbot with moderate infection (Fig. 3d) than in those with severe infection, where this cell type was rarely observed. In the severely infected group, the lining epithelium often suffered critical damage, and TNFα+ cells were also found between those sloughed off into the intestinal lumen (Fig. 3e). Also, monocyte-like immunoreactive cells were commonly observed in blood vessels (Fig. 3f).

Gene expression

TNFα expression was detected in the three organs of all the analysed fish. Fig. 4 shows the TNFα expression pattern in the three organs of R and C in the two sampling points. The expression of TNFα in the kidney and spleen of moderately infected turbot was significantly higher than in C fish ($P < 0.05$), whereas no significant differences were found in severely infected turbot. In this latter group, however, it is interesting to denote that TNFα expression was decreased in the kidney, though not statistically enough ($P = 0.052$). In pyloric caeca, there was a general increasing trend in both infected groups, especially in moderately infected turbot, but the high individual variability resulted in no statistically significant differences.

4. Discussion

Turbot enteromyxosis is characterized by catarrhal enteritis of increasing severity alongside the disease course. The histopathological findings observed in the infected fish represent the characteristic evolution of the disease, including the development of moderate to severe cellular depletion in the lymphohaematopoietic organs [1]. At intestinal level, progressive increase of the parasite load and inflammatory infiltration were observed, associated to alteration of the lining epithelium architecture, enhanced presence of apoptotic figures and epithelial detachment. In moderately infected turbot, the parasitization and lesions development were more emphasized in pyloric caeca and anterior intestine, in accordance with previous observations reporting the beginning of the infection in these regions [1, 2]. As well, the results confirmed the oral route as the most effective way for infecting turbot, with more homogeneous prevalence rates and

lesions [2, 4], which allowed the selection of a proper number of specimens with analogous lesions for this study. Different reports point towards the involvement of a dysregulated response in the pathogenesis of turbot enteromyxosis [9, 10, 12]. The multifunctional cytokine TNFα is considered a key mediator of host response to infection [13], and this role should be confirmed also in teleosts, where changes in TNFα expression have been reported in bacterial, viral and parasitic diseases [31, 34, 40-42]. In turbot, TNFα has been cloned by Ordás *et al.* [38], who have also performed gene expression assays and studied some functions of the obtained recombinant protein (rTNFα). rTNFα was able to recruit and activate inflammatory cells, as well to enhance nitric oxide production by macrophages. In the current study, the main lymphohaematopoietic organs (spleen and kidney) showed an increased gene expression of TNFα in turbot with moderate infection, which was coincident with the results of the immunohistochemistry. This demonstrates the involvement of this cytokine in the development of the immune response against *E. scophthalmi* in this stage of the infection. Turbot kidney physiologically presents numerous TNFα$^+$ cells in the lymphohaematopoietic interstitial tissue [35], so their increase in infected specimens did not appear dramatic, but a more intense labelling was often noticed. Spleen, on the other hand, presented the highest increase in TNFα gene expression and a remarkable increase of immunoreactive cells. This fact may reflect the major role of this organ in antigen trapping and presentation in teleosts [43], which is probably enhanced in this phase of the disease. Therefore, TNFα may be suggested to drive the induction of a systemic response against enteromyxosis in turbot, as seen in mammalian species [13, 14], by activating and recruiting inflammatory cells to the site of infection. Immunohistochemistry showed the progressive increase in TNFα$^+$ cells in pyloric caeca of infected fish, including the mobilization of labelled monocyte-like cells in blood vessels. On the other hand, TNFα gene expression in this location did not result significantly different from control in any sampling point and just an increasing trend was noticed. Although the higher individual variability might have influenced this result, the lack of a clear increase in TNFα gene expression also suggests that part of the numerous TNFα-containing cells in the intestine of infected turbot may have been recruited from other localizations. The gene expression would occur before these cells reach the digestive tract, where they arrive containing a preformed pool of TNFα. The existence of a preformed intracellular pool of TNFα, ready to be released and not necessarily associated with gene expression, have been described in rainbow trout (*Oncorhynchus mykiss*) macrophages [44].

Intestinal rodlet cells were previously shown to be TNFα$^+$ [35] and to increase in numbers in early stages of turbot enteromyxosis [1]. In this study, an increase in the number of TNFα$^+$ rodlet cells was observed in pyloric caeca of moderately infected turbot, supporting the hypothesis of their role in the defence response against the parasite. As well, the paucity of immunoreactive rodlet cells noticed in fish with severe infection is in accordance with the decrease of this cell type previously observed in highly parasitized turbot, and attributed to the damage in the epithelium that becomes unable to support these cells [1].

In mammals, TNFα is involved in the establishment of the inflammatory reaction and in the physiologic and pathologic adaptation of the cells to inflammation at intestinal level [19]. Particularly, this cytokine plays a critical role in the pathophysiology of IBDs, being involved in intestinal epithelial shedding and barrier dysfunction [45, 46]. These conditions are characterized by a dysregulated immune response, and monoclonal antibodies against TNFα have been proved as effective tools for treatment [20, 21]. Also, TNFα expression was found increased in a zebrafish larvae model of IBD, and these fish showed a positive response to immunomodulatory treatment [24]. In turbot enteromyxosis, scalloped shape of the intestinal epithelium and detachment of the lining epithelium are characteristic lesions [1], as well as there are evidences of alterations in the expressions of the cell junctions proteins [47]. TNFα has been demonstrated to cause loss of intestinal epithelial barrier by acting in the modulation of tight-junctions [19], a lesion that might explain the pathophysiology of enteromyxosis in different species [3, 48]. In gilthead sea bream, experimentally-infected by *E. leei*, TNFα expression was found increased in the intestine both at 17 and 64 DPI [34], nonetheless this species does not show severe detachment of intestinal cells [49] and the disease usually has a subclinical development and not very high mortality rates [3]. Nonetheless, the analysis of immune-relevant genes expression suggested that an anti-inflammatory phase occurs in gilthead sea bream enteromyxosis that may mitigate the deleterious effects of a prolonged intestinal inflammation [34, 50]. This would not occur in turbot enteromyxosis where several genes involved in promoting inflammation were found still up-regulated at intestinal level in late stages of the disease [9]. As well, increased expression of inducible nitric oxide synthase (iNOS) has been reported in severe infection [12]. Modulation of iNOS expression by TNFα has been documented in mammal species [51], as well as rTNFα has been shown to enhance nitric oxide production by macrophage in turbot [38]. Nitric oxide may be an additional factor contributing to epithelial injury by altering cell junctions and inducing apoptosis of enterocytes [12, 52, 53].

Apoptosis has been recognized by means of histological [1], immunohistochemical [7] and molecular [9] techniques as one of the mechanism that plays a main role in the pathogenesis of turbot enteromyxosis at intestinal level. Apoptotic cells, immunoreactive to active caspase-3, are increased in the lining epithelium and between the inflammatory infiltrates of the intestine of infected fish [7]. In addition, the gene codifying for caspase-3 and other pro-apoptotic genes, included members of TNF family, were found up-regulated in pyloric caeca of severely parasitized fish [9]. The turbot employed in the present work also showed an enhanced presence of apoptotic enterocytes associated to the presence of the parasite and the inflammatory reaction, as well as, apoptotic inflammatory cells where often observed in lamina propria-submucosa of severely infected fish. The importance of TNFα signalling in modulating programmed cell death of intestinal cells has been reported in several mammalian parasitic diseases [54-56] and in IBDs [57]. Whether apoptosis may significantly affect lymphohaematopoietic organs of parasitized turbot, on the contrary, is still unclear [1, 7, 9]. In this study, we did not appreciate an clear increase in the apoptotic rate in kidney

and spleen from infected fish, altough slightly enhanced figures were reported for some specimen. In any case, the biology of TNFα signalling is complex, being involved in both cell survival and apoptosis [17, 18]. Regarding apoptosis of leukocytes, for example, blockade of TNFα is postulated to be beneficial in IBDs by promoting T lymphocyte apoptosis [58], whereas in other diseases, like swine fever, this cytokine was found responsible for leukocytic apoptosis and the consequent lymphoid depletion [59]. In turbot enteromyxosis, leukocytic death by apoptosis in the intestine may trigger the migration of these cells from lymphohaematopoietic organs, and this mechanism together with the local action of apoptosis were suggested to contribute to the cell depletion observed in kidney and spleen [1, 7, 8, 10]. In the current study, after the initial significant increase in moderately infected fish, a subsequent decreasing trend in TNFα expression was detected in the kidney of severely infected fish by both the techniques used. According to the histological and immunohistochemical results, this decrease seems to be mostly due to the severe cell depletion suffered by this organ. In fact, the number of $TNF\alpha^{+}$ cells appeared proportionally not so scarce, given the severe loss of interstitial tissue. Similar findings were observed in the spleen of these animals, though less pronounced. Cellular depletion in lymphohaematopoietic organs is a main lesion of advanced enteromyxosis in turbot, and is involved in dysfunctions of the immune response, such as the decrease of IgM-positive cells [10] or the depression of several immune-related genes [9]. Interestingly, the same lesion occurs in *E. leei*-infected sharpsnout sea bream (*Diplodus puntazzo*, Cetti) [60], a species that, as turbot, presents a high susceptibility to this myxozoan parasitosis, but not in diseased gilthead sea bream. In this sense, it is noteworthy to highlight that in *E. leei*-infected gilthead sea bream no significant differences in TNFα expression were found in blood or lymphohaematopietic organs (head kidney and spleen) at any time point (17 and 64 DPI in anal infection and 113 DPI in effluent infection) [34, 50]. In fact, all the changes found in the expression of cytokines were at the local intestinal level. The action of TNFα as positive or negative regulator of haematopoiesis is still poorly understood, even in mammals, where it appears to depend on a delicate balance of length of exposure to TNFα, progenitor cell type, stage of cell cycle and presence of other regulators [13, 61]. Nevertheless, evidence exists that chronic inflammatory cytokine signalling may leads to haematopoietic stem cells dysfunction [62].

Enteromyxoses in different species, including turbot, present a chronic course leading to a cachectic syndrome with weight loss, anorexia and muscle atrophy [3]. Inflammatory cytokines are considered the main mediators of cachexia [16, 22]. TNFα in particular, which was formerly named as cachectin [61], promotes different catabolic responses, inducing muscle loss, anorexia and down-regulation of the expression of anabolic hormones [16, 23, 63, 64]. Therefore, immunomodulatory therapies aimed to block the synthesis or action of TNFα and other inflammatory cytokines have been tested in several wasting diseases [22, 23], including parasitosis [65], with promising results. In enteromyxosis-induced cachectic syndrome, the implication of the immune response interacting with the neuroendocrine system is under debate [9, 66-69]. The results of

this study suggest that a possible involvement of TNFα in the pathophysiology of cachexia deserves further attention.

5. Conclusions

In the current work the regulation of the expression of the multifunctional cytokine TNFα during the infection of turbot by *E. scophthalmi* has been demonstrated. The combined use of Q-PCR and immunohistochemistry provided more feasible results and a more comprehensive picture of TNFα dynamics during the disease. The increased expression detected in earlier stages of enteromyxosis in spleen and kidney indicates the involvement of TNFα in the development of the immune response, probably driving the recruitment of inflammatory cells in the intestine, the target organ of the parasite. In this location, the accumulation of the inflammatory infiltrates containing $TNF\alpha^+$ cells suggests a prolonged exposure to TNFα that may be involved in the development of the lesions, namely apoptosis, epithelial shedding and intestinal barrier dysfunction. In advanced stages of enteromyxosis, the decreasing trend in $TNF\alpha^+$ cell numbers in both lymphohaematopoietic organs and of gene expression in kidney reflects the observed cell depletion. Turbot enteromyxosis appears to be characterized by a dysfunctional, exacerbated immune response. In similar conditions observed in other species TNFα plays a main role in the pathogenesis. This cytokine acts in the regulation of a wide spectrum of biological activities, which include immune response and haematopoiesis, but also feeding behaviour and metabolism. The extent of its implication in the development of the different clinical signs and lesions associated to enteromyxosis should be further addressed. This can set the basis for the implement of immunomodulatory therapies aimed to control this important parasitosis.

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590 Czech Republic; 2009.

591 TABLES

592 Table 1. Relative density of TNFα^{+} cells in control, moderately and severely *Enteromyxum*
593 *scopththalmi*-infected turbot.
594

FISH STATUS	SPLEEN	KIDNEY	PYLORIC CAECA	
			MØ-like	RC
Control	++	+++	+	+
Moderate	++++	+++/++++	++	++
Severe	+/++	++	+++	+

595

596 Relative density: +, low; ++, medium; +++, high; ++++, very high. MØ = macrophage, RC=
597 rodlet cells.

598 FIGURE LEGENDS

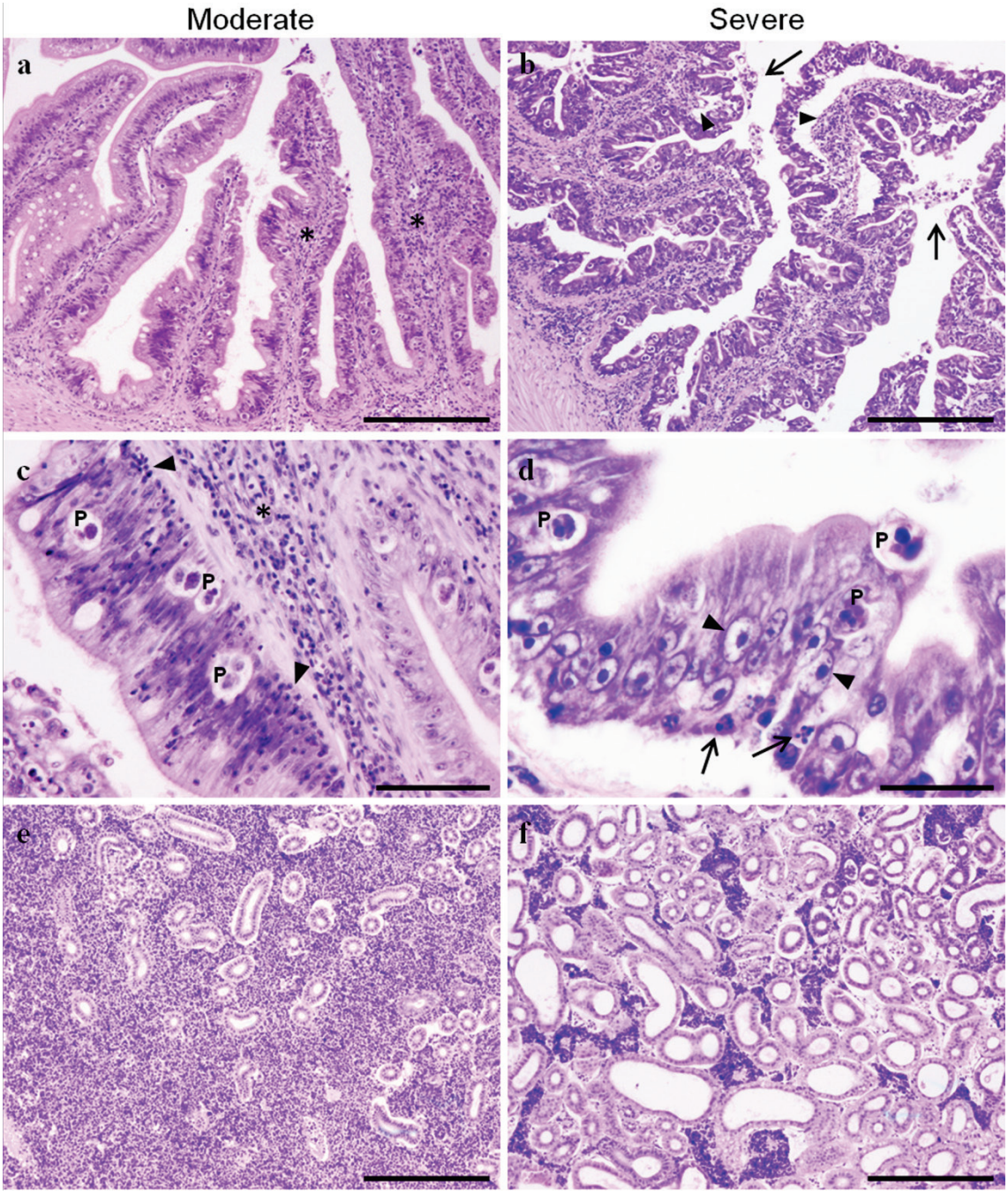
599 **Figure 1.** Histopathological findings in moderately (a, c, e) and severely (b, d, f)
600 *Enteromyxum scophthalmi*-infected turbot. Stained with H&E. a) Section of pyloric
601 caeca presenting features of moderate enteromyxosis, with some intestinal folds
602 (asterisks) presenting more severe inflammatory infiltrates with associated changes in
603 epithelial architecture (Scale bar = 200 μm). b) Pyloric caeca of a severely infected
604 turbot, showing high parasite load and inflammatory infiltration of the lamina propria-
605 submucosa. Note the marked alteration of the lining epithelium, which is also detached

606 from the basal lamina in some areas (arrowheads), as well as sloughed enterocytes can
607 be appreciated into the intestinal lumen (arrows) (Scale bar = 200 μm). c) Higher
608 magnification of pyloric caeca showing the infiltration of the epithelium (arrowheads)
609 and the lamina propria-submucosa (asterisk) by mononuclear inflammatory cells,
610 associated to the presence of the parasites (P) (Scale bar = 50 μm). d) Higher
611 magnification illustrating the presence of cells with apoptotic features in the epithelium
612 of pyloric caeca, which harbours several parasitic forms (P). Some of the cells at the
613 basis of the epithelium are shrunken with pyknotic, fragmented nuclei (arrows) while
614 other present hypertrophied nuclei (arrowheads) (Scale bar = 20 μm). e) Section of the
615 kidney of a moderately infected turbot, which do not present significant histological
616 alterations (Scale bar = 200 μm). f) Evident depletion of the lymphohaematopoietic
617 interstitial tissue in the kidney of a turbot with severe enteromyxosis (Scale bar = 200
618 μm).

619 **Figure 2.** Comparative photomicrographs of spleen, kidney and pyloric caeca from
620 control and parasitized turbot immunostained for TNFα (Scale bars = 200 μm).

621 **Figure 3.** Immunohistochemical detection of TNFα. a) $TNF\alpha^+$ macrophage-like cells
622 clustering around a melanomacrophage centre (arrow) and an arteriole (arrowhead) in
623 the spleen of a control fish (Scale bar = 50 μm). Higher magnification of labelled
624 macrophage-like cells (Scale bar = 10 μm). b) Photomicrographs of kidney from a
625 severely *Enteromyxum scophthalmi*-infected turbot showing a serious cell depletion in
626 the interstitial tissue associated to dilatation of renal tubules. Note the rounded
627 immunoreactive cells in the lymphohaematopoietic interstitial tissue and a $TNF\alpha^+$ rodlet
628 cell (arrowhead) in the epithelium of a tubule (Scale bar = 100 μm). c, d) Pyloric caeca
629 of moderately infected turbot. $TNF\alpha^+$ macrophage-like cells in the lamina propria-
630 submucosa (c, Scale bar = 20 μm) and high concentration of $TNF\alpha^+$ rodlet cells in the
631 lining epithelium (d, Scale bar = 50 μm). e, f) Pyloric caeca of severely *E. scophthalmi*-
632 infected fish (Scale bars = 100 μm). Intestinal folds showing a high parasitic burden in
633 the damaged lining epithelium, which presents areas of epithelial detachment (e,
634 arrowheads). $TNF\alpha^+$ cells can be seen between the inflammatory infiltrates in the
635 lamina propria-submucosa, and also in the intestinal lumen (e, arrow) together with
636 sloughed enterocytes and cellular debris. Notice the presence of numerous
637 immunoreactive monocyte-like cells in a blood vessel (f, asterisk) located in the lamina
638 propria-submucosa.

639 **Figure 4.** Bar graphs showing TNFα transcript levels in spleen (a), kidney (b) and
640 pyloric caeca (c) from control (white bars) and *Enteromyxum scophthalmi*-infected
641 (black bars) turbot at 24 (moderate infection) and 42 (severe infection) DPI. The
642 transcript levels of TNFα in control fish was used as references values (values >1 or <1
643 indicate increase or decrease with respect to the reference). Asterisks (*) indicate
644 statistically significant differences ($P < 0.05$) between control and infected groups from
645 the same sampling point.



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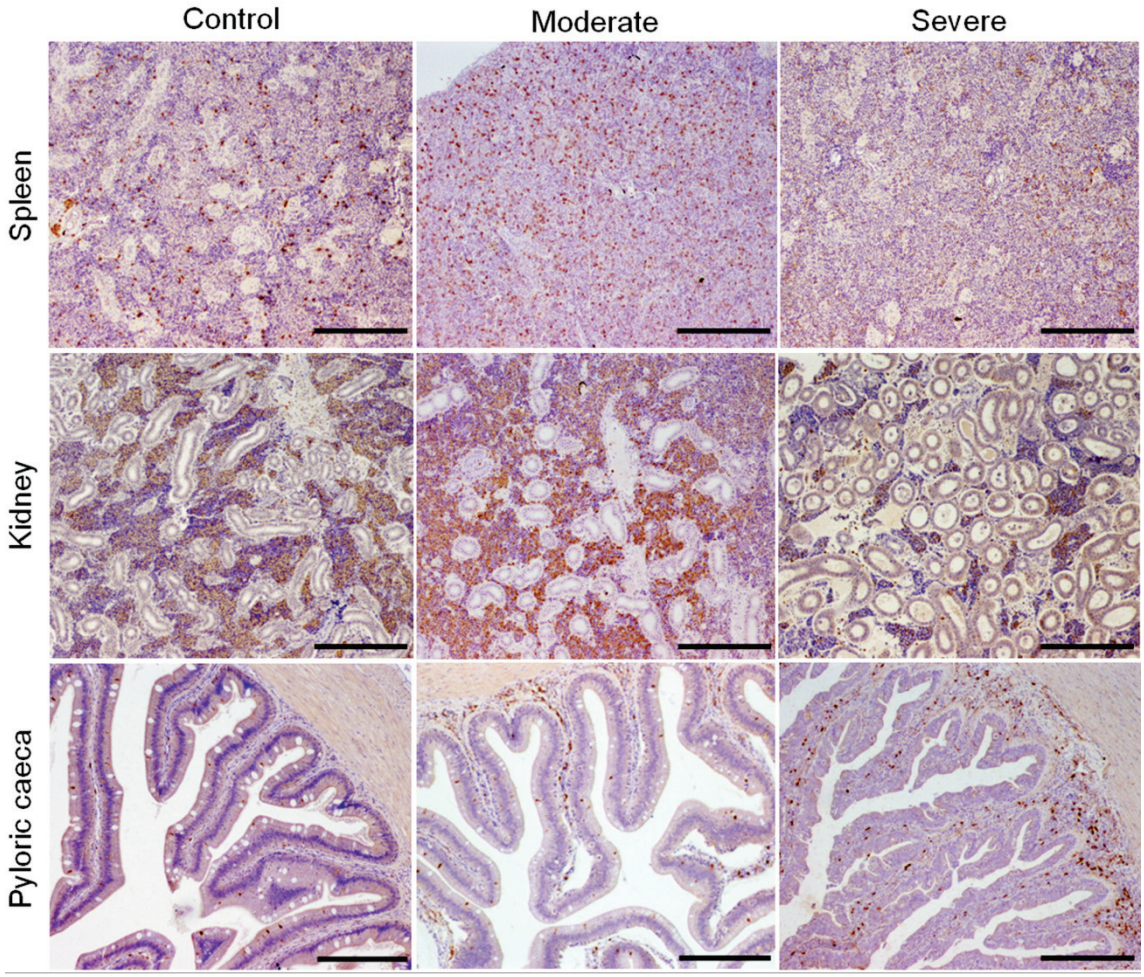

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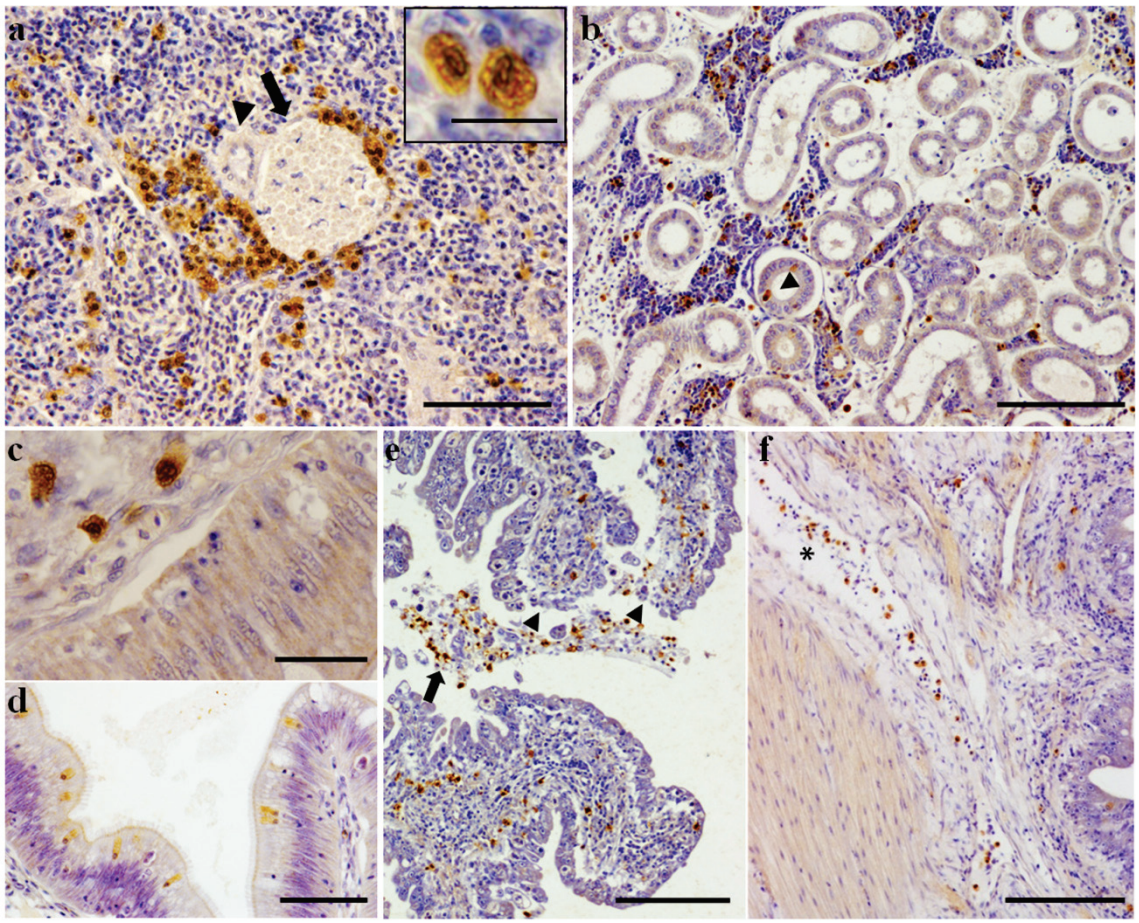

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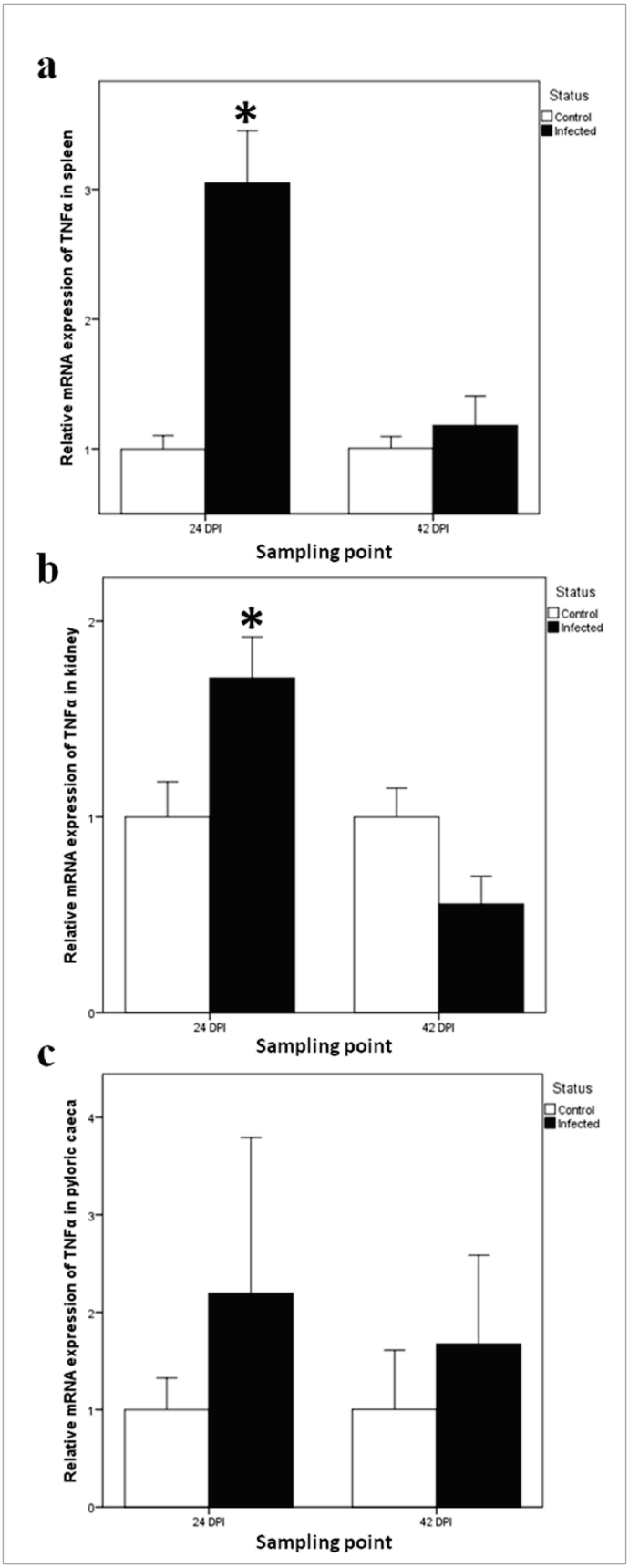

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