

1 **IMMUNOHISTOCHEMICAL DETECTION AND GENE**
2 **EXPRESSION OF TNF α IN TURBOT (*Scophthalmus maximus*)**
3 **ENTEROMYXOSIS**

4 Authors names (given name, family name) and order:

5 Paolo, Ronza¹; Roberto, Bermúdez²; Ana Paula, Losada¹; Ariadna, Sitjà-Bobadilla³;
6 Belén, G. Pardo⁴; María Isabel, Quiroga^{1*}

7 ¹Departamento de Ciencias Clínicas Veterinarias, Facultad de Veterinaria, Universidad
8 de Santiago de Compostela, 27002, Lugo, Spain.

9 ²Departamento de Anatomía y Producción Animal, Facultad de Veterinaria,
10 Universidad de Santiago de Compostela, 27002, Lugo, Spain.

11 ³Instituto de Acuicultura Torre de la Sal (IATS-CSIC), Ribera de Cabanes, Castellón
12 12595, Spain.

13 ⁴Departamento de Genética, Facultad de Veterinaria, Universidad de Santiago de
14 Compostela, 27002, Lugo, Spain.

15 *Corresponding author: misabel.quiroga@usc.es

16
17 **Abstract**

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

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

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

45 **1. Introduction**

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

78 23]. Also in a model of IBD described in zebrafish (*Danio rerio*, Hamilton), TNF α
79 expression was found increased and immunomodulatory therapies showed positive
80 results [24]. In fact, in the different fish species where TNF α has been identified, this
81 cytokine showed similar immune-related functions to its mammalian counterpart [25-
82 30]. Nevertheless, the complex and widespread biological activities accomplished in
83 mammals are still poor described in teleosts. The regulation of its expression has been
84 reported in several piscine parasitic diseases [31-33], including enteromyxosis by *E. leei*
85 in gilthead sea bream [34]. The aim of this study was to investigate the involvement of
86 TNF α in turbot enteromyxosis combining Q-PCR and immunohistochemistry to analyse
87 its expression in target organs (pyloric caeca, kidney and spleen).

88 **2. Materials and methods**

89 *Experimental design and histopathology*

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

105

106 *Immunohistochemical detection of TNF α*

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

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

123

124 *Gene expression*

125 Tissue samples preserved in RNAlater were kept at 4°C during 24 h and stored at -20°C
126 until RNA extraction. Total RNA was extracted from tissues of C and R fish using
127 TRIZOL Reagent (Life Technologies, Carlsbad, CA, USA) according to manufacturer's
128 recommendations. RNA was quantified using NanoDrop[®] ND-1000 spectrophotometer
129 (NanoDrop[®] Technologies Inc.) and its quality was checked in an Agilent BioAnalyzer
130 (Agilent Technologies, USA). Good quality RNA (RIN > 7.5) was reverse transcribed
131 (1 µg) into cDNA by random primers using AffinityScript Multiple Temperature cDNA
132 Synthesis kit following the supplier's protocol (Agilent Technologies). The Q-PCR
133 analysis was carried out in a MX3005P thermocycler (Stratagene) using 2 µl of cDNA
134 per reaction and 300 nM of each primer in a final volume of 20 µl according to the
135 Brilliant III Ultra-Fast SYBR[®] Green QPCR Master Mix (Agilent Technologies)
136 manufacturer's instructions. The constitutively expressed ribosomal protein S4 (RPS4),
137 proved to be stably expressed in turbot [36, 37], was chosen as the house-keeping gene
138 for sample normalisation. TNFα primers (sense: 5'-GGGTGGATGTGGAAGGTGAT-
139 3'; antisense: 5'-GGCCTCTGTTTGGCTTGA CT-3') were designed based on the
140 mRNA sequence of turbot TNFα (GenBank accession number FJ654645) [38]. Each
141 sample was performed in triplicate for accuracy and error estimation including one
142 reverse-transcription-negative control for each gene. Fluorescence readings at the end of
143 each cycle were used to estimate threshold cycle values (Ct). Values were normalized to
144 RPS4 and fold change in transcript level determined with the relative quantitative
145 method ($\Delta\Delta Ct$) [39] using data from C fish as reference values. Prior to quantitative
146 analysis, a standard curve was constructed using six serial dilutions of cDNA (from
147 1,000 to 0.01 ng) and the efficiency of each primer set was determined. Efficiencies of
148 90–110% were obtained by primer optimization. Each sample was analysed for primer-
149 dimer, contamination, or mispriming by inspection of their dissociation curves.

150 *Statistical analysis*

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

156 **3. Results**

157 *Histopathology*

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

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

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

186

187 *Immunohistochemical detection of TNF α*

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

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

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

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

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

218 *Gene expression*

219 TNF α expression was detected in the three organs of all the analysed fish. Fig. 4 shows
220 the TNF α expression pattern in the three organs of R and C in the two sampling points.
221 The expression of TNF α in the kidney and spleen of moderately infected turbot was
222 significantly higher than in *C* fish ($P < 0.05$), whereas no significant differences were
223 found in severely infected turbot. In this latter group, however, it is interesting to denote
224 that TNF α expression was decreased in the kidney, though not statistically enough ($P =$
225 0.052). In pyloric caeca, there was a general increasing trend in both infected groups,
226 especially in moderately infected turbot, but the high individual variability resulted in
227 no statistically significant differences.

228 **4. Discussion**

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

240 lesions [2, 4], which allowed the selection of a proper number of specimens with
241 analogous lesions for this study. Different reports point towards the involvement of a
242 dysregulated response in the pathogenesis of turbot enteromyxosis [9, 10, 12]. The
243 multifunctional cytokine TNF α is considered a key mediator of host response to
244 infection [13], and this role should be confirmed also in teleosts, where changes in
245 TNF α expression have been reported in bacterial, viral and parasitic diseases [31, 34,
246 40-42]. In turbot, TNF α has been cloned by Ords *et al.* [38], who have also performed
247 gene expression assays and studied some functions of the obtained recombinant protein
248 (rTNF α). rTNF α was able to recruit and activate inflammatory cells, as well to enhance
249 nitric oxide production by macrophages. In the current study, the main
250 lymphohaematopoietic organs (spleen and kidney) showed an increased gene expression
251 of TNF α in turbot with moderate infection, which was coincident with the results of the
252 immunohistochemistry. This demonstrates the involvement of this cytokine in the
253 development of the immune response against *E. scophthalmi* in this stage of the
254 infection. Turbot kidney physiologically presents numerous TNF α ⁺ cells in the
255 lymphohaematopoietic interstitial tissue [35], so their increase in infected specimens did
256 not appear dramatic, but a more intense labelling was often noticed. Spleen, on the other
257 hand, presented the highest increase in TNF α gene expression and a remarkable increase
258 of immunoreactive cells. This fact may reflect the major role of this organ in antigen
259 trapping and presentation in teleosts [43], which is probably enhanced in this phase of
260 the disease. Therefore, TNF α may be suggested to drive the induction of a systemic
261 response against enteromyxosis in turbot, as seen in mammalian species [13, 14], by
262 activating and recruiting inflammatory cells to the site of infection.
263 Immunohistochemistry showed the progressive increase in TNF α ⁺ cells in pyloric caeca
264 of infected fish, including the mobilization of labelled monocyte-like cells in blood
265 vessels. On the other hand, TNF α gene expression in this location did not result
266 significantly different from control in any sampling point and just an increasing trend
267 was noticed. Although the higher individual variability might have influenced this
268 result, the lack of a clear increase in TNF α gene expression also suggests that part of the
269 numerous TNF α -containing cells in the intestine of infected turbot may have been
270 recruited from other localizations. The gene expression would occur before these cells
271 reach the digestive tract, where they arrive containing a preformed pool of TNF α . The
272 existence of a preformed intracellular pool of TNF α , ready to be released and not
273 necessarily associated with gene expression, have been described in rainbow trout
274 (*Oncorhynchus mykiss*) macrophages [44].

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

283 In mammals, TNF α is involved in the establishment of the inflammatory reaction and in
284 the physiologic and pathologic adaptation of the cells to inflammation at intestinal level
285 [19]. Particularly, this cytokine plays a critical role in the pathophysiology of IBDs,
286 being involved in intestinal epithelial shedding and barrier dysfunction [45, 46]. These
287 conditions are characterized by a dysregulated immune response, and monoclonal
288 antibodies against TNF α have been proved as effective tools for treatment [20, 21].
289 Also, TNF α expression was found increased in a zebrafish larvae model of IBD, and
290 these fish showed a positive response to immunomodulatory treatment [24]. In turbot
291 enteromyxosis, scalloped shape of the intestinal epithelium and detachment of the lining
292 epithelium are characteristic lesions [1], as well as there are evidences of alterations in
293 the expressions of the cell junctions proteins [47]. TNF α has been demonstrated to
294 cause loss of intestinal epithelial barrier by acting in the modulation of tight-junctions
295 [19], a lesion that might explain the pathophysiology of enteromyxosis in different
296 species [3, 48]. In gilthead sea bream, experimentally-infected by *E. leei*, TNF α
297 expression was found increased in the intestine both at 17 and 64 DPI [34], nonetheless
298 this species does not show severe detachment of intestinal cells [49] and the disease
299 usually has a subclinical development and not very high mortality rates [3].
300 Nonetheless, the analysis of immune-relevant genes expression suggested that an anti-
301 inflammatory phase occurs in gilthead sea bream enteromyxosis that may mitigate the
302 deleterious effects of a prolonged intestinal inflammation [34, 50]. This would not occur
303 in turbot enteromyxosis where several genes involved in promoting inflammation were
304 found still up-regulated at intestinal level in late stages of the disease [9]. As well,
305 increased expression of inducible nitric oxide synthase (iNOS) has been reported in
306 severe infection [12]. Modulation of iNOS expression by TNF α has been documented in
307 mammal species [51], as well as rTNF α has been shown to enhance nitric oxide
308 production by macrophage in turbot [38]. Nitric oxide may be an additional factor
309 contributing to epithelial injury by altering cell junctions and inducing apoptosis of
310 enterocytes [12, 52, 53].

311 Apoptosis has been recognized by means of histological [1], immunohistochemical [7]
312 and molecular [9] techniques as one of the mechanism that plays a main role in the
313 pathogenesis of turbot enteromyxosis at intestinal level. Apoptotic cells,
314 immunoreactive to active caspase-3, are increased in the lining epithelium and between
315 the inflammatory infiltrates of the intestine of infected fish [7]. In addition, the gene
316 codifying for caspase-3 and other pro-apoptotic genes, included members of TNF
317 family, were found up-regulated in pyloric caeca of severely parasitized fish [9]. The
318 turbot employed in the present work also showed an enhanced presence of apoptotic
319 enterocytes associated to the presence of the parasite and the inflammatory reaction, as
320 well as, apoptotic inflammatory cells where often observed in lamina propria-
321 submucosa of severely infected fish. The importance of TNF α signalling in modulating
322 programmed cell death of intestinal cells has been reported in several mammalian
323 parasitic diseases [54-56] and in IBDs [57]. Whether apoptosis may significantly affect
324 lymphohaematopoietic organs of parasitized turbot, on the contrary, is still unclear [1, 7,
325 9]. In this study, we did not appreciate an clear increase in the apoptotic rate in kidney

326 and spleen from infected fish, although slightly enhanced figures were reported for some
327 specimen. In any case, the biology of TNF α signalling is complex, being involved in
328 both cell survival and apoptosis [17, 18]. Regarding apoptosis of leukocytes, for
329 example, blockade of TNF α is postulated to be beneficial in IBDs by promoting T
330 lymphocyte apoptosis [58], whereas in other diseases, like swine fever, this cytokine
331 was found responsible for leukocytic apoptosis and the consequent lymphoid depletion
332 [59]. In turbot enteromyxosis, leukocytic death by apoptosis in the intestine may trigger
333 the migration of these cells from lymphohaematopoietic organs, and this mechanism
334 together with the local action of apoptosis were suggested to contribute to the cell
335 depletion observed in kidney and spleen [1, 7, 8, 10]. In the current study, after the
336 initial significant increase in moderately infected fish, a subsequent decreasing trend in
337 TNF α expression was detected in the kidney of severely infected fish by both the
338 techniques used. According to the histological and immunohistochemical results, this
339 decrease seems to be mostly due to the severe cell depletion suffered by this organ. In
340 fact, the number of TNF α ⁺ cells appeared proportionally not so scarce, given the severe
341 loss of interstitial tissue. Similar findings were observed in the spleen of these animals,
342 though less pronounced. Cellular depletion in lymphohaematopoietic organs is a main
343 lesion of advanced enteromyxosis in turbot, and is involved in dysfunctions of the
344 immune response, such as the decrease of IgM-positive cells [10] or the depression of
345 several immune-related genes [9]. Interestingly, the same lesion occurs in *E. leei*-
346 infected sharpsnout sea bream (*Diplodus puntazzo*, Cetti) [60], a species that, as turbot,
347 presents a high susceptibility to this myxozoan parasitosis, but not in diseased gilthead
348 sea bream. In this sense, it is noteworthy to highlight that in *E. leei*-infected gilthead
349 sea bream no significant differences in TNF α expression were found in blood or
350 lymphohaematopoietic organs (head kidney and spleen) at any time point (17 and 64 DPI
351 in anal infection and 113 DPI in effluent infection) [34, 50]. In fact, all the changes
352 found in the expression of cytokines were at the local intestinal level. The action of
353 TNF α as positive or negative regulator of haematopoiesis is still poorly understood,
354 even in mammals, where it appears to depend on a delicate balance of length of
355 exposure to TNF α , progenitor cell type, stage of cell cycle and presence of other
356 regulators [13, 61]. Nevertheless, evidence exists that chronic inflammatory cytokine
357 signalling may leads to haematopoietic stem cells dysfunction [62].
358 Enteromyxoses in different species, including turbot, present a chronic course leading to
359 a cachectic syndrome with weight loss, anorexia and muscle atrophy [3]. Inflammatory
360 cytokines are considered the main mediators of cachexia [16, 22]. TNF α in particular,
361 which was formerly named as cachectin [61], promotes different catabolic responses,
362 inducing muscle loss, anorexia and down-regulation of the expression of anabolic
363 hormones [16, 23, 63, 64]. Therefore, immunomodulatory therapies aimed to block the
364 synthesis or action of TNF α and other inflammatory cytokines have been tested in
365 several wasting diseases [22, 23], including parasitosis [65], with promising results. In
366 enteromyxosis-induced cachectic syndrome, the implication of the immune response
367 interacting with the neuroendocrine system is under debate [9, 66-69]. The results of

368 this study suggest that a possible involvement of TNF α in the pathophysiology of
369 cachexia deserves further attention.

370 **5. Conclusions**

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

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591 TABLES

592 Table 1. Relative density of TNF α ⁺ cells in control, moderately and severely *Enteromyxum*
593 *scophthalmi*-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

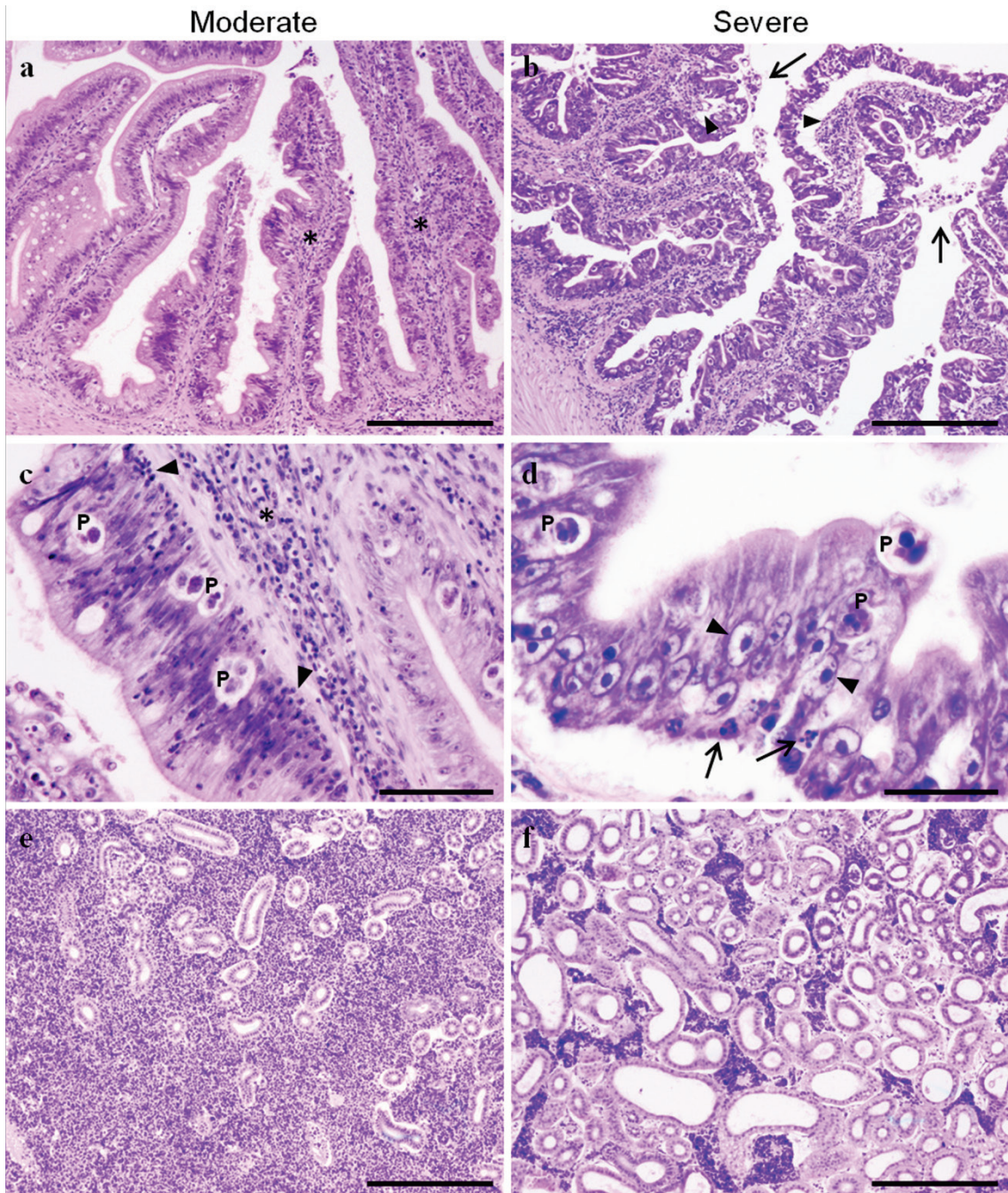
599 **Figure 1.** Histopathological findings in moderately (a, c, e) and severely (b, d, f)
600 *Enteromyxum scophthalmi*-infected turbot. Stained with H&E. a) Section of pyloric
601 caeca presenting features of moderate enteromyxosis, with some intestinal folds
602 (asterisks) presenting more severe inflammatory infiltrates with associated changes in
603 epithelial architecture (Scale bar = 200 μ m). 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 TNF α (Scale bars = 200 μm).

621 **Figure 3.** Immunohistochemical detection of TNF α . a) TNF α ⁺ 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 α ⁺ rodlet
 628 cell (arrowhead) in the epithelium of a tubule (Scale bar = 100 μm). c, d) Pyloric caeca
 629 of moderately infected turbot. TNF α ⁺ macrophage-like cells in the lamina propria-
 630 submucosa (c, Scale bar = 20 μm) and high concentration of TNF α ⁺ 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 α ⁺ 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.

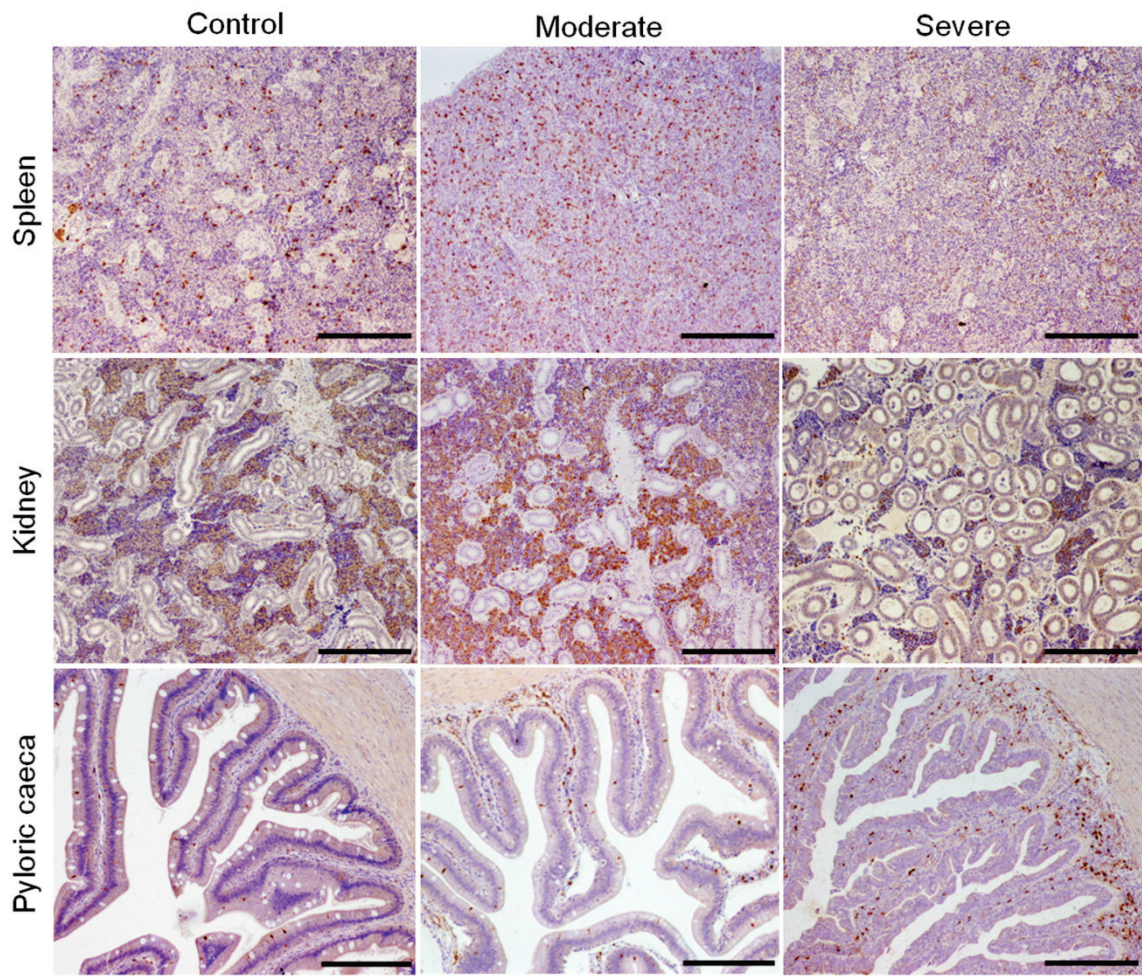
639 **Figure 4.** Bar graphs showing TNF α transcript levels in spleen (a), kidney (b) and
 640 pyloric caeca (c) from control (white bars) and *Enteromyxum scophthalmi*-infected
 641 (black bars) turbot at 24 (moderate infection) and 42 (severe infection) DPI. The
 642 transcript levels of TNF α in 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.



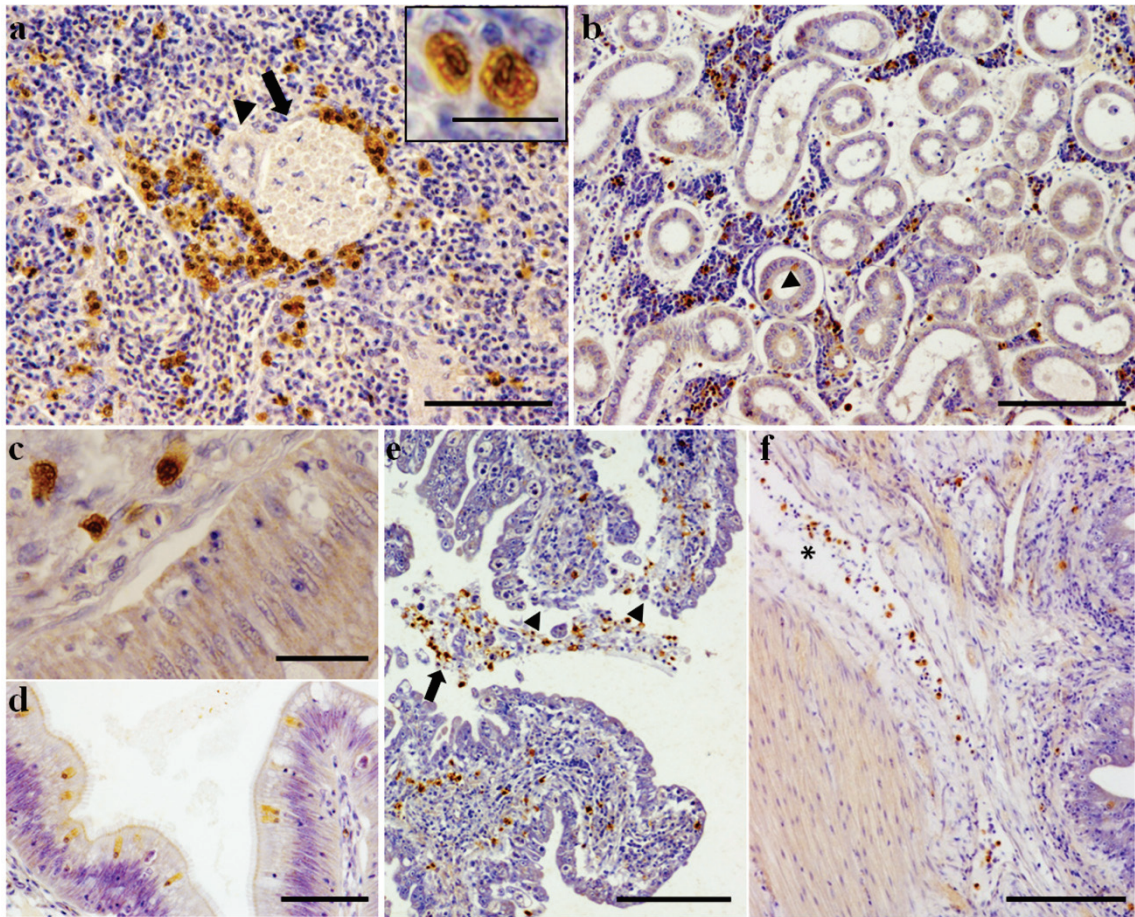
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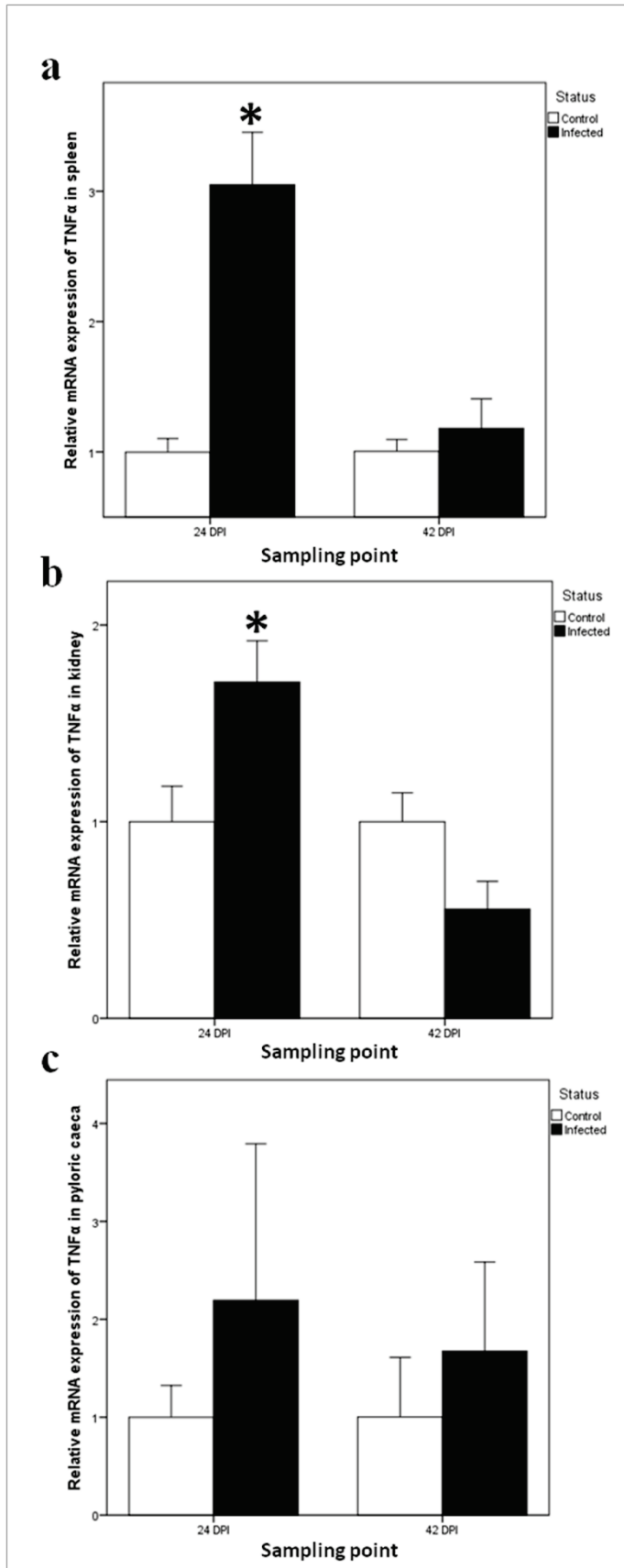
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