

1 **Immunohistochemical expression of E–cadherin in different**
2 **tissues of the teleost fish *Scophthalmus maximus***

3 Paolo Ronza^{a*}; Antonio Villamarín^b; Lucía Méndez^c; Belén G. Pardo^d; Roberto
4 Bermúdez^a; María Isabel Quiroga^a

5 ^aDepartamento de Anatomía, Producción Animal y Ciencias Clínicas Veterinaria,
6 Facultad de Veterinaria, Universidade de Santiago de Compostela, 27002 Lugo, Spain.

7 ^bDepartamento de Bioquímica y Biología Molecular, Facultad de Veterinaria,
8 Universidade de Santiago de Compostela, 27002 Lugo, Spain.

9 ^cInstituto de Investigaciones Marinas, Consejo Superior de Investigaciones Científicas
10 (IIM-CSIC), 36208 Vigo, Spain.

11 ^dDepartamento de Zoología, Genética y Antropología Física, Facultad de Veterinaria,
12 Universidade de Santiago de Compostela, 27002 Lugo, Spain.

13 *Corresponding author

14 E-mail: paolo.ronza@usc.es; Phone number: +34 982822306

15 **Abstract**

16 E–cadherin is an evolutionary conserved protein, whose main role as the principal
17 component of adherens junctions is supporting epithelial cell–cell adhesion. It is an
18 essential molecule for the maintenance of the epithelial barrier function and the analysis
19 of its immunohistochemical expression is a valuable resource in morphopathological,
20 ontogenetic and pathogenesis studies in mammals. As well, there is an increasing
21 understanding of the importance of E-cadherin in the physiology of the immune system

22 and the development of the immune response. Mucosal health is a primary issue in
23 aquaculture research; nevertheless, there is a lack of immunohistochemical studies of cell
24 junction proteins in fish species. In this work, an immunohistochemical technique was
25 optimized in Bouin- and formalin-fixed paraffin-embedded tissues of turbot
26 *Scophthalmus maximus*, employing a commercial antibody raised against human E-
27 cadherin. The specificity of the antibody in recognizing the molecule in this teleost
28 species was tested by western blot and mass spectrometry-based proteomic analyses. The
29 assays showed a good specificity and indicated that the antibody recognizes the well
30 conserved cytoplasmic domain of the protein. Immunohistochemistry showed the
31 localisation of E-cadherin at cell-cell contact in the epithelia of the different organs,
32 between the hepatocytes and the pancreatic acinar cells, as well as in the reticulo-
33 epithelial stroma of the thymus. Also, the immunoreaction was observed in the cells
34 constituting the melano-macrophage centres in the spleen and kidney. No
35 immunostaining was detected, as expected, only in the heart and brain. No significant
36 difference was noticed between the two fixative used for collecting the tissues samples.
37 This is the first description of E-cadherin immunohistochemical expression in several
38 tissues of a teleost. The immunohistochemical technique represents a useful tool to be
39 used in the different areas of fish health research.

40 Keyword: *Immunohistochemistry; cadherin-1; cell junctions; epithelial barriers; mucosal*
41 *health*

42

43 1. Introduction

44 Cell–cell adhesion is a fundamental structural feature of multicellular organisms,
45 mediated by a set of specialized membrane structures termed intercellular junctions.

46 Particularly, the ability of epithelial cells to organize into monolayered sheets is a
47 prerequisite for multicellularity, thereby providing tissue integrity, barrier function, and
48 tissue polarity in metazoan organisms (Bruser and Bogdan, 2017). Over the course of
49 morphological evolution, metazoan animals have diversified the architecture of their cell–
50 cell junctions, which includes tight junctions, adherens junctions (AJ), and desmosomes.

51 Among them, AJs are the only detected throughout the metazoan phyla, whereas other
52 junctional types show restricted phylogenetic distributions (Oda and Takeichi, 2011).

53 Thus, AJs could be considered the universal adhesion machinery for the generation and
54 maintenance of multicellular animal bodies. E-cadherin, a calcium–dependent
55 transmembrane protein, is the structural and functional core of AJs, mediating *trans*–
56 homophilic interactions between neighbouring cells. Its adhesive functions are mediated
57 by the extracellular cadherin repeat domains, while the highly conserved intracellular
58 domains form a complex with catenins linking E–cadherin to the actin cytoskeleton, being
59 so involved in junctional maintenance, dynamics, and plasticity of epithelial tissues
60 (Bruser and Bogdan, 2017; Oda and Takeichi, 2011; van Roy and Berx, 2008).

61 In teleost, the mucosal surfaces (skin, gills and gastrointestinal tract) constitute the first
62 line of defence against pathogens invasion and carry out multiple physiological processes,
63 such as osmoregulation, waste excretion and nutrient adsorption. For fish, even more than
64 for terrestrial species, mucosal barriers and their health are of primary importance to face
65 the continue interactions with the aquatic microbiota (Peatman and Beck, 2015). Almost
66 all of the most urgent areas of aquaculture research require a deeper understanding of

67 mucosal barriers, from pathology issues to vaccine delivery, nutrition, nutraceuticals and
68 microflora modulation (Peatman et al., 2015). The importance of E-cadherin as
69 diagnostic marker and the main role it plays in human diseases, from several cancer types
70 to different skin and gastrointestinal conditions, have been widely reported (Baniak et al.,
71 2016; Christou et al., 2017; Marezky et al., 2008; Singhai et al., 2011; Sugihara, 2016;
72 von Zeidler et al., 2014; Wu et al., 2007; Zbar et al., 2004). In fish pathology, E-cadherin
73 has recently been identified as a key molecule for resistance to infectious pancreatic
74 necrosis (IPN) in Atlantic salmon (Moen et al., 2015), and another work on the same
75 species reported modulated intestinal E-cadherin gene expression in response to an
76 experimental dietary treatment affecting intestinal fluid permeability (Hu et al., 2016).

77 The investigation of E-cadherin immunohistochemical expression is a primary focus in
78 morphological studies, including ontogenetic and morphopathological characterization of
79 the different organs, as well as an irreplaceable complement for studies based on gene
80 expression analysis (Bondow et al., 2012; Fuertes et al., 2013; Gassler et al., 2001;
81 Kuwahara et al., 2001; Sakamoto et al., 2008; Schneider et al., 2010). However, no
82 comprehensive study of E-cadherin immunolocalisation in teleost tissues has been
83 addressed and there is still a scarce knowledge of the physiological distribution of this
84 protein and its change under pathological conditions. In this study, an
85 immunohistochemical technique based on a commercial antibody was optimized in
86 Bouin- and formalin-fixed paraffin-embedded tissues from turbot (*Scophthalmus*
87 *maximus*), an economically-important marine species, and the distribution of E-cadherin
88 in different tissues of healthy specimens analysed.

89 **2. Materials and methods**

90 *2.1. Fish and sampling procedures*

91 For this study, 10 adult turbot ($2,127 \pm 182.2$ g mean weight) were employed. Fish were
92 euthanized by overexposure to tricaine methane sulfonate (MS222, Sigma–Aldrich,
93 Denmark) and necropsied. For histological examination and immunohistochemistry,
94 tissues samples from kidney, spleen, thymus, digestive tract, liver, pancreas, heart, gills,
95 brain and skin were collected. Samples were fixed in Bouin’s fluid at 4 °C or in formalin
96 during 24 hours and then paraffin–embedded. Histological analysis was performed on
97 H&E and toluidine blue–stained sections. For western blot analysis, samples (1 g) of
98 anterior and posterior intestine were collected and extensively washed with ice cold PBS
99 containing 1 mM of phenylmethylsulfonyl fluoride (PMSF) before storage at –80°C.

100 All experimental protocols were approved by the Institutional Animal Care and Use
101 Committee of the University of Santiago de Compostela (Spain).

102 *2.2. Western blotting and E-cadherin sequences analysis*

103 Western blot analysis was performed to assess the specificity of the primary antibody on
104 protein extracts from anterior and posterior intestine of turbot. Protein extracts from
105 human skin and intestine (ileum) were used as positive control. Tissues were
106 homogenized in ice–cold homogenization buffer (50 mM Tris–HCl, 150 mM NaCl, 5mM
107 EDTA, 1 mM dithiothreitol, 1 mM PMSF, 1 mM benzamidine hydrochloride hydrate, 5
108 $\mu\text{g}/\text{mL}$ pepstatin A, 5 $\mu\text{g}/\text{mL}$ aprotinin, 5 $\mu\text{g}/\text{mL}$ leupeptin, 1% triton X–100, 0,5 %
109 sodium deoxycholate, 0,1 % sodium dodecyl sulfate pH 7.4). Homogenates were
110 centrifuged at 35,000 g for 15 minutes at 4 °C, and supernatants filtered through gauze
111 and frozen in aliquots at –80° C until required. Protein concentrations were determined
112 by Bradford method according to manufacturer’s instruction (Bio–Rad, Hercules, CA,
113 USA).

114 Samples of protein extracts were mixed with 1/4 vol of 5X SDS sample buffer (250 mM
115 Tris-HCl, 8 % SDS, 40% glycerol, 20% β -mercaptoethanol, pH 6.8) and denatured by
116 heating at 37 °C for 20 min. Approximately 30 μ g of total protein per tissue were resolved
117 by SDS-PAGE using 7,5% polyacrylamide slabs-gel and proteins were transferred to
118 polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore, Bedford, MA,
119 USA) by applying a 275 mA current for 3 h at 4 °C. Sodium dodecyl sulphate (SDS) was
120 added (0,025%) to the transference buffer in order to facilitate the transfer of high
121 molecular weight proteins. After blocking for 2 h and 30 min at room temperature with
122 5% non-fat dry milk in TTBS (20 mM Tris-HCl buffer, pH 7.5, 0.15 M NaCl, 0.1%
123 Tween 20), membranes were incubated overnight at 4° C with the anti-human E-cadherin
124 primary antibody (mouse monoclonal antibody, clone NCH-38, M3612, Dako, Denmark)
125 diluted 1:500 with TTBS. The blots were then incubated for 1 h at room temperature with
126 anti-mouse secondary antibody conjugated to horseradish peroxidase (Sigma-Aldrich, St
127 Louis, MO, USA) diluted 1:50,000 with TTBS and, finally, developed with the
128 chemiluminiscent HRP substrate (Millipore) and exposed to X-ray film (Curix RP2 Plus;
129 Agfa-Gevaert, Mortsel, Belgium) for a few seconds. Membranes were washed 5 times
130 for 10 min with TTBS between subsequent steps. Negative control was performed by
131 substituting primary antibody with TTBS. In order to estimate the apparent molecular
132 weight of detectable bands, a sample of molecular weight standards (Unstained Broad
133 Range SDS-PAGE Standards, Bio-Rad) was also applied on the gel. The membranes were
134 finally stained with a Ponceau-S solution to mark the positions of the molecular weight
135 standards.

136 Additionally, for all tested samples SDS-PAGE and gel staining with Coomassie Brilliant
137 Blue R (Sigma-Aldrich) were carried out.

138 The amino acid sequence of turbot E-cadherin was inferred using ExPASy Translate tool
139 (<http://web.expasy.org/translate/>) from the nucleotide sequence (GenBank accession
140 number: MG137250) identified in a previous study (Robledo et al., 2014), and its
141 theoretical molecular weight estimated by ExPASy Compute pI/Mw tool
142 (http://web.expasy.org/compute_pi/). For comparison purposes, the amino acid sequence
143 alignments were performed by using the BLASTP 2.6.1 online program (Altschul et al.,
144 2005; Altschul et al., 1997).

145 *2.3. In-gel digestion and protein identification by nano-HPLC-ESI-IT-MS/MS*

146 Based on western blot results, the labelled bands of 110 kDa y 38 kDa obtained in turbot
147 were manually excised from SDS-PAGE Coomassie-stained gels. Proteins were in-gel
148 reduced, alkylated and digested with trypsin as previously described (Jensen et al., 1999).
149 Briefly, protein bands were three times washed with water and dehydrated with
150 acetonitrile. Gel plugs were then dehydrated in a vacuum centrifuge and finally
151 rehydrated with a 0.5 μ M solution of sequencing grade bovine trypsin (Promega,
152 Madison, WI) in 25 mM ammonium bicarbonate buffer, pH 8.0, for at least 40 min on
153 ice. After the rehydration step, samples were digested overnight at 37 °C and peptides
154 were then cleaned-up by using ZipTip C18 according to manufacturer's instructions. The
155 peptide mixture was dried in vacuum centrifugation, and dissolved again in 15 μ L of 1%
156 formic acid prior to mass analysis.

157 Trypsin-digested proteins were analysed using a Dionex UltiMate 3000 RSLCnano
158 system (Thermo Fisher Scientific) coupled to a LTQ Velos-Pro mass spectrometer
159 (Thermo Fisher Scientific). The separation of the peptides was done on an Acclaim
160 PepMap100 Nano Trap Column, C18, 5 μ m, 100 Å, 100 μ m \times 1 cm (Thermo Fisher
161 Scientific) coupled to an RP column Acclaim PepMap RSLC 75 μ m \times 150 mm, C18, 2

162 μm , 100 Å (Thermo Fisher Scientific). Mobile phases A and B were respectively 0.1%
163 formic acid in water and in 100% acetonitrile. A 90 min linear gradient from 5% to 35%
164 B, at a flow rate of 300 nL/min, was used. For ionization, a spray voltage of 2.10 kV and
165 a capillary temperature of 200 °C were used. Peptide detection was performed by using
166 survey scans from 350 to 1600 Da (2 μscans), followed by MS/MS scans (2 μscans) of
167 the six more intense peaks using an isolation width of 1 Da and a normalised collision
168 energy of 35%. Singly charged ions were excluded from MS/MS fragmentation and a
169 dynamic exclusion enabled, with repeat count set to 2 and exclusion duration of 30 s.
170 Protein identification was performed using the database searching function of the PEAKS
171 7 software (Ma et al., 2003; Zhang et al., 2012) (Bioinformatics Solutions Inc, Waterloo,
172 Ontario, Canada), to compare experimental MS/MS spectra against reference MS/MS
173 spectra from the UniProtKB/TrEMBL database (release 2017_04; 84827567 sequence
174 entries), which also included their respective decoy sequences. The following limitations
175 were used for the searches: tryptic cleavage, up to 2 missed cleavage sites, and tolerances
176 ± 1.0 Da for precursor ions and ± 0.8 Da for MS/MS fragments ions. The FDR was kept
177 below 1% and only proteins with at least one unique peptide were considered. When
178 protein identification failed, the PEAKS algorithm was used to perform *de novo*
179 sequencing for each input spectrum. The same parameters (mass error tolerance and
180 PTMs) specified for database search are also used for *de novo* sequencing. Average Local
181 confidence (ALC) for each *de novo* sequence was set at $\geq 60\%$.

182 2.4. Immunohistochemistry

183 Thin sections (3 μm thick) were obtained from all the sampled organs. The sections were
184 placed on slides treated with silane to improve section adherence and dried overnight at
185 37 °C. After deparaffination with xylene (two 5-min washes) and hydration through a
186 graded ethanol series, slides were incubated with a peroxidase-blocking solution (Dako,

187 Denmark) for 30 minutes to quench endogenous peroxidase activity. Antigen retrieval
188 was performed using high pH antigen retrieval buffer (Dako, Denmark), following the
189 manufacturer instruction. The optimal working dilution for the monoclonal antibody anti-
190 human E-cadherin was determined to be 1:50 with an incubation time of 2 h at room
191 temperature. Then, slides were incubated during 30 min with horseradish peroxidase
192 (HRP)-labelled polymer conjugated to rabbit secondary antibody and peroxidase reaction
193 was developed using a diaminobenzidine-positive chromogen (EnVision+ System-HRP
194 kit, K4007, Dako). All incubations were performed in a humid chamber at room
195 temperature, and the sections were washed three times for 5 min in 0.1 M phosphate
196 buffered saline containing 0.05% Tween-20 between all subsequent steps. Sections were
197 finally counterstained with haematoxylin, dehydrated and coverslipped with DePeX
198 mounting medium for microscopic observation. Tissue sections from formalin-fixed
199 paraffin-embedded human intestine were used as positive controls. In sections included
200 as negative controls, primary antibody was replaced with either PBS or antibody diluent.

201 **3. Results**

202 *3.1. Histological analysis*

203 The histological evaluation of the sampled tissues did not show any significant
204 pathological alteration or presence of pathogenic agents.

205 *3.2. Western blotting*

206 In human tissues, an expected signal corresponding to a molecular weight of
207 approximately 97 kDa was detected in both the skin and intestinal extracts. Furthermore,
208 an additional intensely labelled band of about 38 kDa was observed for the skin. In turbot,
209 the band corresponding to the mature form of E-cadherin reached approximately 110

210 kDa, identical for the anterior and posterior intestine. This result was consistent with the
211 theoretical molecular weight indicated by ExPASy Compute pI/Mw tool for the protein.
212 As well, additional 38-kDa labelled bands were observed in both intestinal regions (Fig
213 1).

214 The alignment performed by BLASTP 2.6.1 showed that turbot and human E-cadherin
215 (Uniprot code P12830) amino acid sequences exhibited more than 53% of identity (Fig 2
216 A). Furthermore, the alignment of the human 38 kDa C-terminal fragment of E-cadherin,
217 known as CTF1 (Marambaud et al., 2002; Marezky et al., 2005), with the correspondent
218 last 182 amino acids of turbot protein resulted in more than 75% of identity (Fig 2 B).

219 3.3. Validation of western blot results by MS-proteomics analysis

220 Proteomics data supported the presence of E-cadherin in both 110 kDa and 38 kDa bands
221 (Table 1) by finding a unique peptide sequence which matched with the E-cadherin
222 protein sequence of *Fundulus heteroclitus*. That fish sequence, which is currently
223 available in the UniProtKB/TrEMBL database, exhibited more than 45% of identity with
224 the human E-cadherin (Uniprot code P12830), and more than 57% with turbot's, after
225 their sequence alignment. The further alignment of that unique peptide sequence with the
226 E-cadherin protein sequence of *S. maximus* resulted in 100% of identity (Fig 2 B).
227 Moreover, in the 110 kDa band, *de novo* PEAKS data analysis found an additional peptide
228 sequence (NQGLSQDNTVQTK) in the sample which also exhibited 100% of identity
229 with the E-cadherin protein sequence of turbot.

230 3.4. Immunohistochemistry

231 The expected negative or positive reactions were observed in the negative or positive
232 controls, respectively. E-cadherin was detected in all the studied tissues of turbot, with
233 the exception of the brain and heart, in both formalin- and Bouin-fixed samples. As

234 expected, the immunostaining was mostly observed in the epithelia, located at the cell–
235 cell junction area with a basolateral position. The epidermis and the epithelium covering
236 the gill *lamellae* showed this kind of immunoreactivity (Fig 3 A, B), as well as the lining
237 epithelium of the digestive tract (Fig 3 C) from the oesophagus to the anus. In the stomach
238 the gastric pits were also labelled, but not the entire epithelium of the gastric glands (Fig
239 3 D). As well, in the kidney the expression of E–cadherin was observed in the epithelia
240 of the tubules, ducts and Bowman’s capsule (Fig 4 A). In this organ and in the spleen, the
241 melano–macrophage centres (MMCs) also showed immunoreactivity, which appeared
242 located at the contact area between the cells constituting the centres (Fig 4 B). E–cadherin
243 immunostaining was also noticed in the opercular epithelium that separates the thymus
244 from the gill chamber, as well as drawing a reticular pattern in the thymus parenchyma,
245 with a stronger intensity in the inner part of the organ (Fig 4 C). Finally, E–cadherin was
246 immunolocalised at the intercellular junction between the hepatocytes in the liver and the
247 acinar cells in the pancreas, as well as in the epithelium of the biliar and pancreatic ducts.
248 On contrary, no immunostaining was observed in the endocrine components of the
249 pancreas, the islets of Langerhans (Fig 5).

250 **4. Discussion**

251 To our knowledge, this is the first work addressing the immunolocalisation of E–cadherin
252 in different tissues of a teleost fish. The commercial antibody employed was thoroughly
253 evaluated to test its specificity in recognizing the protein in turbot by western blot and
254 MS–based proteomic analyses.

255 The results suggest that the monoclonal antibody recognizes the C–terminal region of the
256 protein, corresponding to the cytoplasmic domain (CD). In fact, the CD of E–cadherin
257 remained quite conserved from placozoa to man (Hulpiau and van Roy, 2011) and

258 cleavage fragments of human E-cadherin CD have been reported. Particularly, the
259 intramembrane protease complex gamma secretase, through the action of a presenilin-
260 1(PS1), is responsible for the cleavage of the 38 kDa fragment known as CTF1. Upon
261 formation of cell-cell contacts, PS1 is recruited to sites of cell-cell adhesion, where it
262 forms complexes with E-cadherin and b-catenin at the cell surface (van Roy and Berx,
263 2008). There is a lack of information about cleavage sites and fragments of E-cadherin
264 in teleost, however, the observation of 38 kDa labelled bands in turbot tissues indicate the
265 possibility that they might be similar to those described in mammals. As well, a high
266 similarity between the amino acid sequences of the C-terminal region of turbot and
267 human E-cadherin was observed, which would support the cross-reactivity of the
268 antibody employed.

269 The immunohistochemical expression observed in turbot was full consistent with that
270 described in mammalian tissues, being E-cadherin immunolocalised at cell-cell contacts
271 in the lining epithelia covering the body surfaces exposed to the external environment
272 (skin, gills, gastrointestinal tract), as well as in the epithelia of the biliar and pancreatic
273 ducts and the collecting duct system of the kidney. In this last organ, also the simple
274 squamous epithelium of the Bowman's capsule was labelled, as previously seen in human
275 kidney (Tsuchiya et al., 2006). Membranous labelling of pancreas acinar cells and of
276 hepatocytes was also observed, in accordance with what described in mammals (Lim et
277 al., 2007; Tsuchiya et al., 2006). Besides, immunostaining of hepatocytes was also
278 reported in the cell line RTL-W1, originated from a primary culture of rainbow trout
279 normal liver (Malhao et al., 2013).

280 The most controversial results were the lack of immunostaining of the gastric glands and
281 islets of Langerhans in the pancreas, where the expression of E-cadherin was described
282 in human by Tsuchiya et al. (2006), who performed an immunohistochemical study on

283 different healthy human tissues. However, the same authors also observed in both cases
284 immunoreactivity to another member of cadherin family, N-cadherin. Particularly, in the
285 stomach, N-cadherin was detected in parietal cells, which are specialized in secreting
286 hydrochloric acid (Tsuchiya et al., 2006). In teleost, on the other hand, only one cell type
287 is observed in gastric glands, secreting both pepsinogen and hydrochloric acid (Grosell et
288 al., 2010). The presence of other cadherins other than E-cadherin in these locations cannot
289 be discarded and the development of molecular markers is needed for a better
290 characterization of the cadherin family in teleost. Concerning the lack of immunostaining
291 for E-cadherin in turbot heart and brain, this is in accordance with that described in
292 human, where N-cadherin was in turn detected in the intercalated discs of cardiac muscle
293 and in the central nervous system (Tsuchiya et al., 2006).

294 E-cadherin was also immunolocalised, as expected, at cell-cell contacts in the epithelium
295 of the gill chamber separating the thymus from the aquatic milieu. In the thymus, the
296 immunostaining corresponded to the stromal tissue, constituted by a framework of
297 reticulo-epithelial cells that support the parenchyma (Vigliano et al., 2011). This staining
298 was more intense in the inner zone of the organ. Zonation of the thymus has been reported
299 in turbot, consisting in an outer part mainly composed by thymocytes and the inner
300 thymus, where mostly lymphoblasts and macrophages are present (Padrós and Crespo,
301 1996). This might resemble the cortical and medullary portions of human thymus, where
302 a strongest staining intensity of E-cadherin was found in the medulla and has been related
303 with mechanisms involved in T cells development and proliferation (Kutlesa et al., 2002).
304 Further research has to be carried out to investigate the functions of fish E-cadherin others
305 than junctional maintenance, but, based on the emerging evidences in mammals (Van den
306 Bossche and Van Ginderachter, 2013), it is plausible a broader implication in the
307 functioning of teleost immune system.

308 In the same way, immunohistochemical localisation of E-cadherin was observed in the
309 MMCs in kidney and spleen. These are nodular accumulations of closely packed
310 pigment-containing cells, mostly macrophages, which play significant roles in
311 physiological processes, such as destruction, detoxification or recycling of endogenous
312 and exogenous materials, and in the immune response, being proposed as the evolutionary
313 precursors of mammalian germinal centres (Agius and Roberts, 2003; Steinel and
314 Bolnick, 2017). MMCs in spleen and kidney increase in size and/or number in response
315 to infection, vaccination, environmental and physiological changes, as well as they have
316 been seen developing elsewhere in fish body, often in association with chronic
317 inflammation (Agius and Roberts, 2003; Bermúdez et al., 2010). A comprehensive
318 understanding of MMCs functions and of the kinetics of the cells that join, leave or form
319 the centres needs further and systematic investigations (Steinel and Bolnick, 2017), but
320 this is the first evidence of E-cadherin involvement in the interaction between the cells
321 constituting MMCs in a teleost.

322 In conclusion, the immunohistochemical technique optimized with a commercial
323 antibody showed a good specificity and allowed the study of E-cadherin distribution in
324 several tissues of turbot. The recognition of the CD of the protein, well evolutionary
325 conserved, points toward a successful employment of this technique in other fish species.
326 This is a useful tool for the investigation of mucosal barriers and their physiological and
327 pathological changes, for ontogeny studies, as well as to explore the occurrence and
328 significance of cell-cell adhesion in the functioning of the immune system.

329 **Acknowledgments**

330 The authors would like to thank María del Carmen Carreira and Sandra Maceiras for
331 technical assistance. This work was supported by the Spanish Ministry of Economy,

332 Industry and Competitiveness through the projects AGL2015–67039–C3–1–R and
333 AGL2015–67039–C3–3–R.

334 **References**

- 335 Agius, C., Roberts, R.J., 2003. Melano-macrophage centres and their role in fish
336 pathology. *J. Fish Dis.* 26, 499-509.
- 337 Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman,
338 D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein
339 database search programs. *Nucleic Acids Res.* 25, 3389-3402.
- 340 Altschul, S.F., Wootton, J.C., Gertz, E.M., Agarwala, R., Morgulis, A., Schaffer, A.A.,
341 Yu, Y.K., 2005. Protein database searches using compositionally adjusted
342 substitution matrices. *FEBS J.* 272, 5101-5109.
- 343 Baniak, N., Senger, J.-L., Ahmed, S., Kanthan, S.C., Kanthan, R., 2016. Gastric
344 biomarkers: a global review. *World J. Surg. Oncol.* 14, 212.
- 345 Bermúdez, R., Losada, A.P., Vázquez, S., Redondo, M.J., Álvarez-Pellitero, P., Quiroga,
346 M.I., 2010. Light and electron microscopic studies on turbot *Psetta maxima*
347 infected with *Enteromyxum scophthalmi*: histopathology of turbot enteromyxosis.
348 *Dis. Aquat. Organ.* 89, 209-221.
- 349 Bondow, B.J., Faber, M.L., Wojta, K.J., Walker, E., Battle, M.A., 2012. E-cadherin is
350 required for intestinal morphogenesis in the mouse. *Dev. Biol.* 371, 1-12.
- 351 Bruser, L., Bogdan, S., 2017. Adherens junctions on the move-membrane trafficking of
352 E-cadherin. *Cold Spring Harb. Perspect. Biol.* 9.
- 353 Christou, N., Perraud, A., Blondy, S., Jauberteau, M.-O., Battu, S., Mathonnet, M., 2017.
354 E-cadherin: A potential biomarker of colorectal cancer prognosis. *Oncol. Lett.* 13,
355 4571-4576.
- 356 Fuertes, L., Santonja, C., Kutzner, H., Requena, L., 2013. Immunohistochemistry in
357 dermatopathology: a review of the most commonly used antibodies (part II). *Actas*
358 *Dermosifiliograf.* (English Edition). 104, 181-203.
- 359 Gassler, N., Rohr, C., Schneider, A., Kartenbeck, J., Bach, A., Obermuller, N., Otto, H.F.,
360 Autschbach, F., 2001. Inflammatory bowel disease is associated with changes of
361 enterocytic junctions. *Am. J. Physiol. Gastrointest. Liver Physiol.* 281, G216-228.
- 362 Grosell, M., Farrell, A.P., Brauner, C.J., 2010. *Fish physiology: The multifunctional gut*
363 *of fish.* Academic Press, San Diego.
- 364 Hu, H., Kortner, T.M., Gajardo, K., Chikwati, E., Tinsley, J., Krogdahl, A., 2016.
365 Intestinal fluid permeability in Atlantic salmon (*Salmo salar* L.) is affected by
366 dietary protein source. *PLoS One.* 11, e0167515.
- 367 Hulpiau, P., van Roy, F., 2011. New insights into the evolution of metazoan cadherins.
368 *Mol. Biol. Evol.* 28, 647-657.
- 369 Jensen, O.N., Wilm, M., Shevchenko, A., Mann, M., 1999. Peptide sequencing of 2-DE
370 gel-isolated proteins by nanoelectrospray tandem mass spectrometry. *Methods*
371 *Mol. Biol.* 112, 571-588.
- 372 Kutlesa, S., Wessels, J.T., Speiser, A., Steiert, I., Muller, C.A., Klein, G., 2002. E-
373 cadherin-mediated interactions of thymic epithelial cells with CD103+
374 thymocytes lead to enhanced thymocyte cell proliferation. *J. Cell Sci.* 115, 4505-
375 4515.

376 Kuwahara, M., Hatoko, M., Tada, H., Tanaka, A., 2001. E-cadherin expression in wound
377 healing of mouse skin. *J. Cutan. Pathol.* 28, 191-199.

378 Lim, Y.-S., Lee, H.C., Lee, H.-S., 2007. Switch of cadherin expression from E- to N-type
379 during the activation of rat hepatic stellate cells. *Histochem. Cell Biol.* 127, 149-
380 160.

381 Ma, B., Zhang, K., Hendrie, C., Liang, C., Li, M., Doherty-Kirby, A., Lajoie, G., 2003.
382 PEAKS: powerful software for peptide de novo sequencing by tandem mass
383 spectrometry. *Rapid Commun. Mass Spectrom.* 17, 2337-2342.

384 Malhao, F., Urbatzka, R., Navas, J.M., Cruzeiro, C., Monteiro, R.A., Rocha, E., 2013.
385 Cytological, immunocytochemical, ultrastructural and growth characterization of
386 the rainbow trout liver cell line RTL-W1. *Tissue Cell.* 45, 159-174.

387 Marambaud, P., Shioi, J., Serban, G., Georgakopoulos, A., Sarner, S., Nagy, V., Baki, L.,
388 Wen, P., Efthimiopoulos, S., Shao, Z., Wisniewski, T., Robakis, N.K., 2002. A
389 presenilin-1/gamma-secretase cleavage releases the E-cadherin intracellular
390 domain and regulates disassembly of adherens junctions. *EMBO J.* 21, 1948-
391 1956.

392 Maretzky, T., Scholz, F., Kotten, B., Proksch, E., Saftig, P., Reiss, K., 2008. ADAM10-
393 mediated E-cadherin release is regulated by proinflammatory cytokines and
394 modulates keratinocyte cohesion in eczematous dermatitis. *J. Invest. Dermatol.*
395 128, 1737-1746.

396 Maretzky, T., Reiss, K., Ludwig, A., Buchholz, J., Scholz, F., Proksch, E., de Strooper,
397 B., Hartmann, D., Saftig, P., 2005. ADAM10 mediates E-cadherin shedding and
398 regulates epithelial cell-cell adhesion, migration, and beta-catenin translocation.
399 *Proc. Natl. Acad. Sci. U. S. A.* 102, 9182-9187.

400 Moen, T., Torgersen, J., Santi, N., Davidson, W.S., Baranski, M., Ødegård, J., Kjølglum,
401 S., Velle, B., Kent, M., Lubieniecki, K.P., Isdal, E., Lien, S., 2015. Epithelial
402 cadherin determines resistance to infectious pancreatic necrosis virus in Atlantic
403 salmon. *Genetics.* 200, 1313.

404 Oda, H., Takeichi, M., 2011. Evolution: structural and functional diversity of cadherin at
405 the adherens junction. *J. Cell Biol.* 193, 1137-1146.

406 Padrós, F., Crespo, S., 1996. Ontogeny of the lymphoid organs in the turbot *Scophthalmus*
407 *maximus*: a light and electron microscope study. *Aquaculture.* 144, 1-16.

408 Peatman, E., Beck, B.H., 2015. 1 - Why mucosal health?, *Mucosal Health in Aquaculture.*
409 Academic Press, San Diego, pp. 1-2.

410 Peatman, E., Lange, M., Zhao, H., Beck, B.H., 2015. Physiology and immunology of
411 mucosal barriers in catfish (*Ictalurus* spp.). *Tissue Barriers.* 3, e1068907.

412 Robledo, D., Ronza, P., Harrison, P.W., Losada, A.P., Bermúdez, R., Pardo, B.G.,
413 Redondo, M.J., Sitjà-Bobadilla, A., Quiroga, M.I., Martínez, P., 2014. RNA-seq
414 analysis reveals significant transcriptome changes in turbot (*Scophthalmus*
415 *maximus*) suffering severe enteromyxosis. *BMC Genomics.* 15, 1149.

416 Sakamoto, A., Murata, K., Suzuki, H., Yatabe, M., Kikuchi, M., 2008.
417 Immunohistochemical observation of co-expression of E- and N-cadherins in rat
418 organogenesis. *Acta Histochem. Cytochem.* 41, 143-147.

419 Schneider, M.R., Dahlhoff, M., Horst, D., Hirschi, B., Trülzsch, K., Müller-Höcker, J.,
420 Vogelmann, R., Allgäuer, M., Gerhard, M., Steininger, S., Wolf, E., Kolligs, F.T.,
421 2010. A key role for E-cadherin in intestinal homeostasis and Paneth cell
422 maturation. *PLoS One.* 5, e14325.

423 Singhai, R., Patil, V.W., Jaiswal, S.R., Patil, S.D., Tayade, M.B., Patil, A.V., 2011. E-
424 Cadherin as a diagnostic biomarker in breast cancer. *N. Am. J. Med. Sci.* 3, 227-
425 233.

- 426 Steinel, N.C., Bolnick, D.I., 2017. Melanomacrophage centers as a histological indicator
427 of immune function in fish and other poikilotherms. *Front Immunol.* 8, 827.
- 428 Sugihara, T., 2016. Loss of adherens junction protein E-cadherin is a biomarker of high-
429 grade histology and poor prognosis in endometrial cancer. *Ann. Clin. Lab. Res.* 4,
430 1.
- 431 Tsuchiya, B., Sato, Y., Kameya, T., Okayasu, I., Mukai, K., 2006. Differential expression
432 of N-cadherin and E-cadherin in normal human tissues. *Arch. Histol. Cytol.* 69,
433 135-145.
- 434 Van den Bossche, J., Van Ginderachter, J.A., 2013. E-cadherin: from epithelial glue to
435 immunological regulator. *Eur. J. Immunol.* 43, 34-37.
- 436 van Roy, F., Berx, G., 2008. The cell-cell adhesion molecule E-cadherin. *Cell Mol. Life*
437 *Sci.* 65, 3756-3788.
- 438 Vigliano, F.A., Losada, A.P., Castello, M., Bermúdez, R., Quiroga, M.I., 2011.
439 Morphological and immunohistochemical characterisation of the thymus in
440 juvenile turbot (*Psetta maxima*, L.). *Cell Tissue Res.* 346, 407-416.
- 441 von Zeidler, S.V., de Souza Botelho, T., Mendonça, E.F., Batista, A.C., 2014. E-cadherin
442 as a potential biomarker of malignant transformation in oral leukoplakia: a
443 retrospective cohort study. *BMC Cancer.* 14, 972.
- 444 Wu, S., Rhee, K.J., Zhang, M., Franco, A., Sears, C.L., 2007. *Bacteroides fragilis* toxin
445 stimulates intestinal epithelial cell shedding and gamma-secretase-dependent E-
446 cadherin cleavage. *J. Cell Sci.* 120, 1944-1952.
- 447 Zbar, A.P., Simopoulos, C., Karayiannakis, A.J., 2004. Cadherins: an integral role in
448 inflammatory bowel disease and mucosal restitution. *J. Gastroenterol.* 39, 413-
449 421.
- 450 Zhang, J., Xin, L., Shan, B., Chen, W., Xie, M., Yuen, D., Zhang, W., Zhang, Z., Lajoie,
451 G.A., Ma, B., 2012. PEAKS DB: De novo sequencing assisted database search
452 for sensitive and accurate peptide identification. *Mol. Cell. Proteomics.* 11.

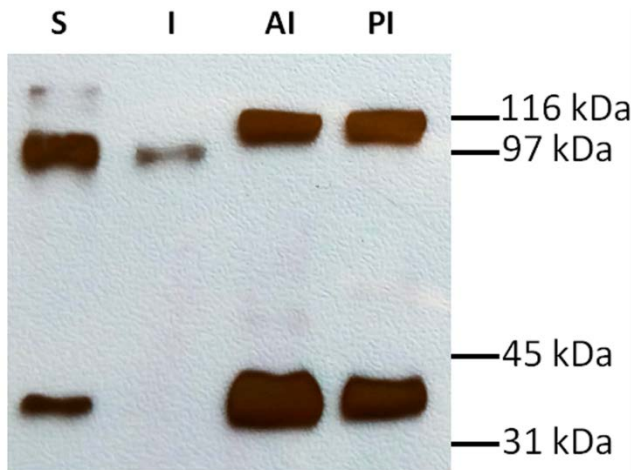
453

454 **Table 1. NanoHPLC-ESI-IT-MS/MS analysis of the 110 kDa and 38 kDa labeled**
 455 **bands found by western blot with an anti-E-cadherin antibody in *Scophthalmus***
 456 ***maximus*.**

Band ID	Presence of E-cadherin	Protein description	Uniprot Code	- 10lgP	#Peptides	#Unique	#Sequences	SC (%)	MWt (kDa)
110 kDa Band	YES	Cadherin-1							
		OS=	A0A146	47,63	1	1	R.PANPD		
		<i>Fundulus heteroclitus</i>	XRX5				ELGNFID	2	83.6
		PE=4 SV=1					DNLK.A		
38 kDa Band	YES	Cadherin-1							
		OS=	A0A146	37,07	1	1	R.PANPD		
		<i>Fundulus heteroclitus</i>	XRX5				ELGNFID	2	83.6
		PE=4 SV=1					DNLK.A		
110 kDa Band	YES	E-cadherin protein	-	-	1	1	D.NQGLS		
		sequence of					QDNTVQ	1.3	110
		<i>S. maximus</i>					TK.V*		

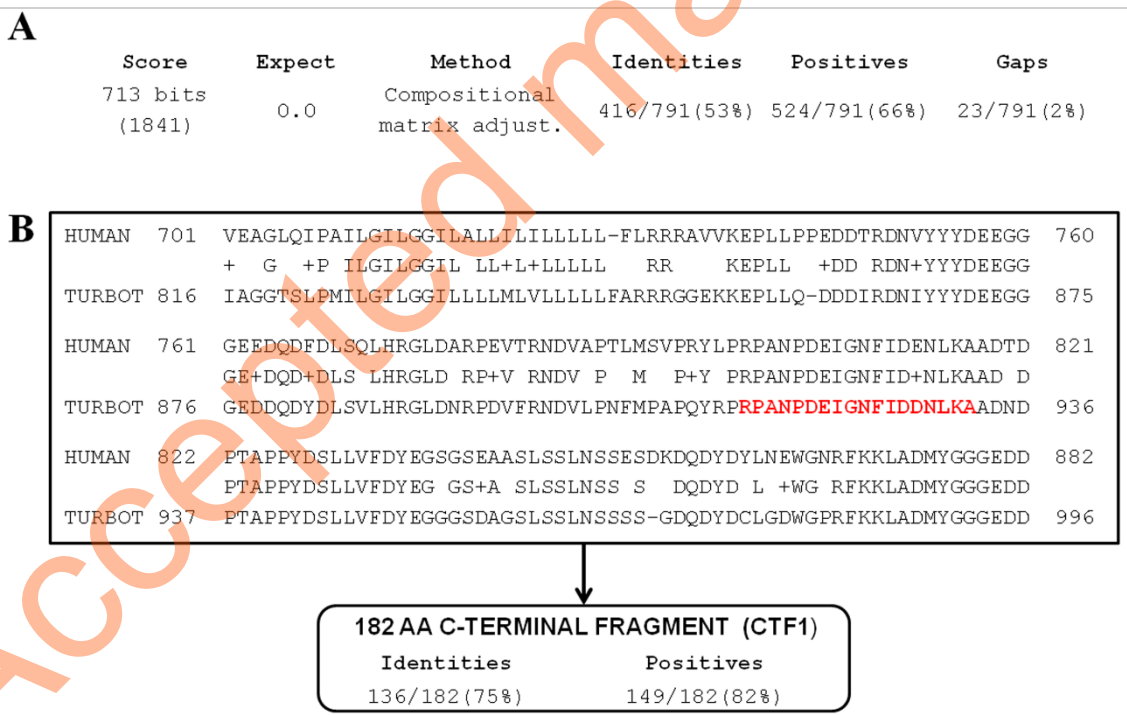
457 Different parameters supporting the successful protein identification of E-cadherin by MS are
 458 indicated: PEAKS protein score (-10lgP), number of matching total and unique peptides (FDR <
 459 1%), peptide sequences, % sequence coverage (SC) and theoretical protein mass (MWt).

460 *Obtained by *de novo* sequencing using PEAKS algorithm and alignment with E-cadherin protein
 461 sequence of *S. maximus* using the BLASTP 2.6.1 (Score: 28.9 bits(63); Expect: 1^{e-06}; Method:
 462 Compositional matrix adjust; Identities: 13/13(100%); Gaps: 0/13(0%)).



463

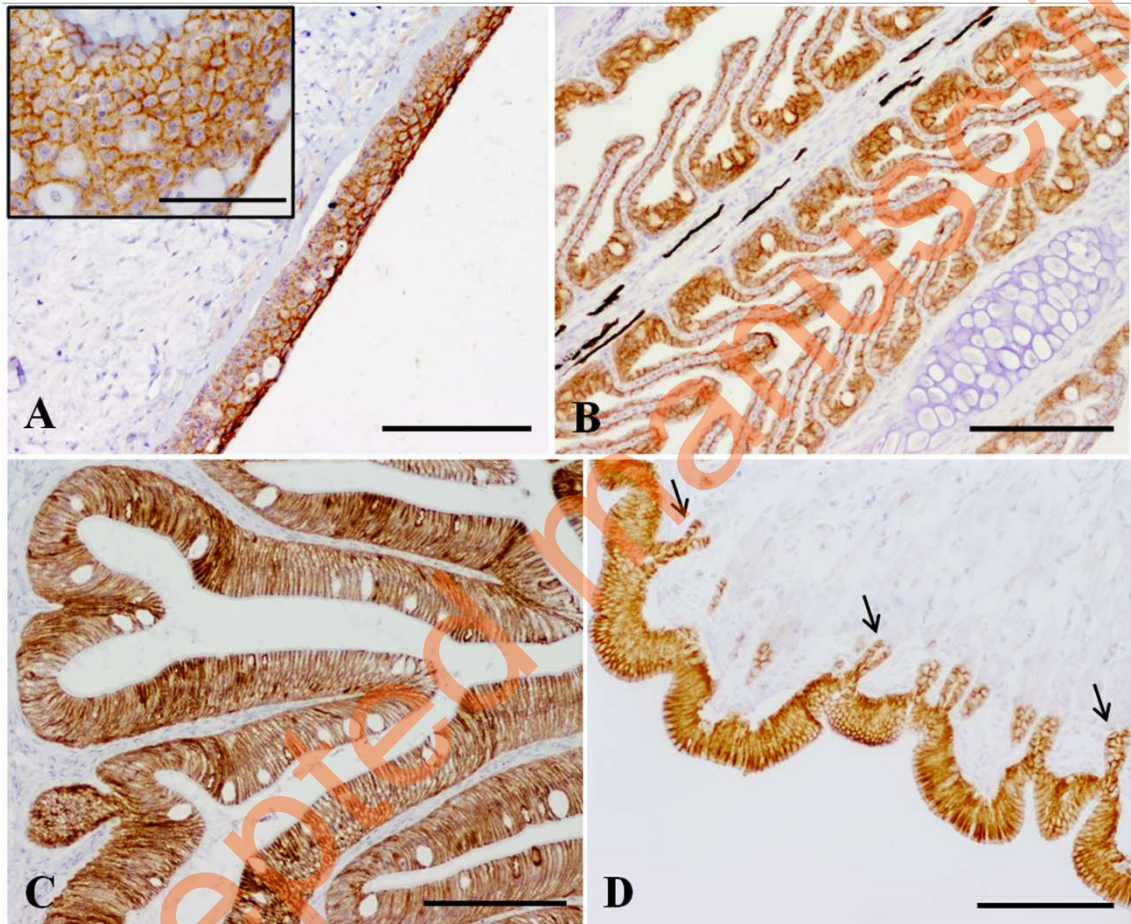
464 **Fig 1. Western blot analysis of E-cadherin expression in protein extracts from**
 465 **human and turbot (*Scophthalmus maximus*) tissues. S = human skin; I = human ileum;**
 466 **AI = turbot anterior intestine; PI = turbot posterior intestine. At the right, the position of**
 467 **the molecular weight standards is indicated.**



468

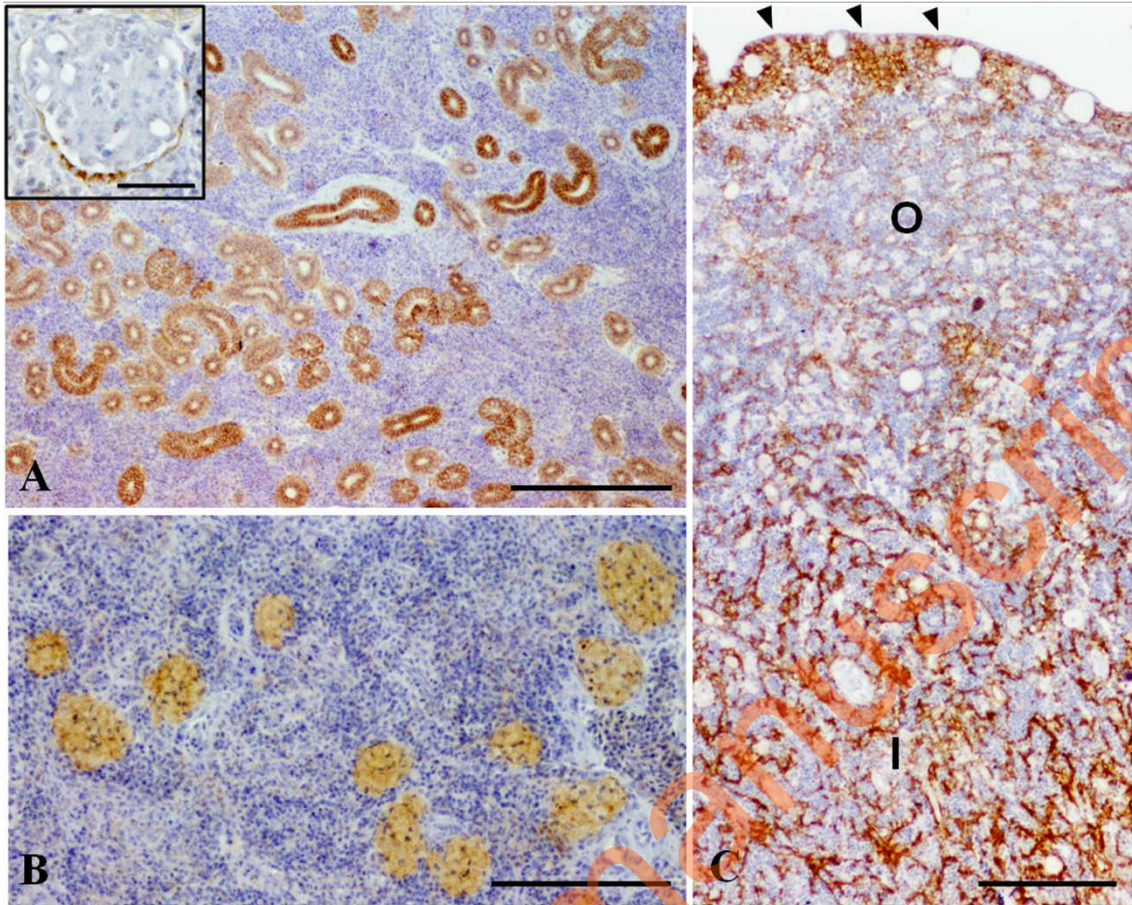
469 **Fig 2. Comparisons between human and turbot E-cadherin amino acid sequences.**
 470 **A) Output of BLASTp alignment of human (P12830) and turbot (*Scophthalmus maximus*)**
 471 **E-cadherin. B) Comparison between the amino acid sequences of the C-terminal fragment**

472 known in human as CTF1 and its counterpart in turbot. The amino acids highlighted in
473 red corresponds to the unique peptide sequence found by nano-LC-MS/MS analysis of
474 the excised bands from Western blot in turbot tissues that matched with the E-cadherin
475 sequence of the teleost *Fundulus heteroclitus*. Conservative amino acid changes are
476 shown by a "+" sign between the aligned residues.



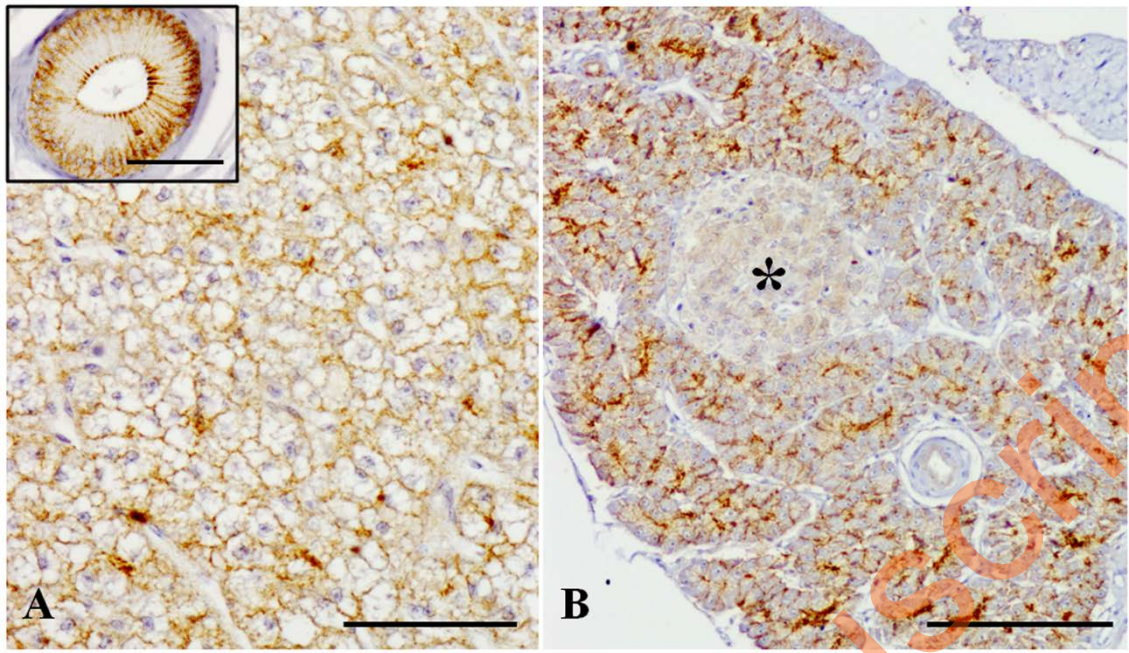
477

478 **Fig 3. E-cadherin immunolocalisation at cell-cell contacts in the lining epithelia of**
479 **turbot skin (A), gills (B), intestine (C) and stomach (D).** Note the immunoreaction of
480 the gastric pits (3D, arrows), while this is not present in the underlying gastric glands.
481 Bars = 100 μ m (Inset 3A= 50 μ m).



482

483 **Fig 4. E-cadherin immunohistochemical expression in turbot (*Scophthalmus***
 484 ***maximus*) kidney (A), spleen (B) and thymus (C).** A) The immunoreaction was
 485 observed in the epithelia of the renal ducts and tubules, as well as in the epithelium of the
 486 Bowman's capsule (inset). Bar = 200 μ m; Inset's bar = 25 μ m. B) Immunostaining of the
 487 splenic melano-macrophage centres. Bar = 100 μ m. C) Immunolocalisation of E-cadherin
 488 in thymus stroma, showing a stronger staining intensity in the inner (I) than in the outer
 489 (O) part of the organ. Note also the immunoreaction of the epithelium separating the
 490 thymus from the gill chamber (arrowheads). Bar = 100 μ m.



491

492 **Fig 5. E-cadherin immunohistochemistry in turbot (*Scophthalmus maximus*) liver**

493 **(A) and pancreas (B).** A) Membranous staining pattern of the hepatocytes and in the

494 epithelium of a biliar duct (Inset). Bars = 50 μ m. B) Immunostaining of E-cadherin at

495 cell-cell contacts between acinar cells, while this is not noticed in a pancreatic islet of

496 Langerhans (star). Bar = 100 μ m.

497