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Abstract: Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) and PACAP-Related Peptide (PRP) are structurally similar peptides encoded in the same transcripts. Their transcription has been detected not only in the brain but also in a wide range of peripheral tissues, even including organs of the immune system. PACAP exerts pleiotropic activities through G-protein coupled membrane receptors: the PACAP-specific PAC-1 and the VPAC-1 and VPAC-2 receptors that exhibit similar affinities for the Vasoactive Intestinal Peptide (VIP) and PACAP. Recent findings added PACAP and its receptors to the growing list of mediators that allow cross-talk between the nervous, endocrine and immune systems in fish. In this study the expression of genes encoding for PACAP and PRP, as well as VIP/PACAP receptors was studied in laboratory-reared brown trout (*Salmo trutta*) after septicaemic infections. Respectively Viral Haemorrhagic Septicaemia Virus (VHSV-1a) or the Gram-negative bacterium *Yersinia ruckeri* (ser. O1 - biot. 2) were used in infection challenges. Kidney and spleen, the teleost main lymphopoietic organs, were sampled during the first two weeks post-infection. RT-qPCR analysis assessed specific pathogens burden and gene expression levels. PACAP and PRP transcription in each organ was positively correlated to the respective pathogen burden, assessed targeting the VHSV-glycoprotein or *Y. ruckeri* 16S rRNA. Results showed as the transcription of PACAP splicing variants and VIP/PACAP receptors is modulated in these organs during an acute viral and bacterial septicaemic infections in brown trout. These gene expression results provide clues as to how the PACAP system is modulated in fish, confirming an involvement during active immune responses elicited by both viral and bacterial aetiological agents. However, further experimental evidence is still required to fully elucidate and characterize the role of PACAP and PRP for an efficient immune response against pathogens.

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Havana, July 23, 2015

Dr. I. Hirono
Editor
Fish and Shellfish Immunology

Dear Dr. Hirono,

It is my pleasure to put down your consideration the manuscript “Viral and bacterial septicaemic infections modulate the expression of PACAP splicing variants and VIP/PACAP receptors in brown trout immune organs” to be reviewed to publish in Fish and Shellfish Immunology.

The manuscript is original; it is not being considered for publication in other sources and had been written according to the journal’s format. In the present study the expression of genes encoding for PACAP and PRP, as well as VIP/PACAP receptors was studied in laboratory-reared brown trout (*Salmo trutta*) after septicaemic infections. Kidney and spleen, the teleost main lymphopoietic organs, were sampled during the first two weeks post-infection and RT-qPCR analysis assessed specific pathogens burden and gene expression levels. Results showed as the transcription of PACAP splicing variants and VIP/PACAP receptors is modulated in these organs during an acute viral and bacterial septicaemic infections in brown trout. This study brings novelty to the fish immunology field. Gene expression results provide clues as to how the PACAP system is modulated in fish, confirming an involvement during active immune responses elicited by both viral and bacterial aetiological agents.

All the authors have revised and approved the manuscript in its present form.

I take advantage of this opportunity to let you know my highest consideration.

Thanks in advance for your time and feel free to contact us if you consider doing any consideration,

Best regards,

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34 **Highlights:**

- 35 • Expression modulation of PACAP gene splicing variants and PAC-1, VPAC-1 and VPAC-2
36 receptors in brown trout *Salmo trutta*
- 37 • RT-qPCR screening adopting new primer sets specifically targeting mRNA
- 38 • Constitutive expression analysed in central and peripheral immune-organs
- 39 • Comparative transcriptomic study during septicaemic infections with VHS and ERM
- 40 • Functional cross-talk between endocrine and immune-systems during infections

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54 **ABSTRACT:**

55 Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) and PACAP-Related Peptide (PRP)
56 are structurally similar peptides encoded in the same transcripts. Their transcription has been
57 detected not only in the brain but also in a wide range of peripheral tissues, even including organs
58 of the immune system. PACAP exerts pleiotropic activities through G-protein coupled membrane
59 receptors: the PACAP-specific PAC-1 and the VPAC-1 and VPAC-2 receptors that exhibit similar
60 affinities for the Vasoactive Intestinal Peptide (VIP) and PACAP. Recent findings added PACAP
61 and its receptors to the growing list of mediators that allow cross-talk between the nervous,
62 endocrine and immune systems in fish. In this study the expression of genes encoding for PACAP
63 and PRP, as well as VIP/PACAP receptors was studied in laboratory-reared brown trout (*Salmo*
64 *trutta*) after septicaemic infections. Respectively Viral Haemorrhagic Septicaemia Virus (VHSV-Ia)
65 or the Gram-negative bacterium *Yersinia ruckeri* (ser. O1 – biot. 2) were used in infection
66 challenges. Kidney and spleen, the teleost main lymphopoietic organs, were sampled during the first
67 two weeks post-infection. RT-qPCR analysis assessed specific pathogens burden and gene
68 expression levels. PACAP and PRP transcription in each organ was positively correlated to the
69 respective pathogen burden, assessed targeting the VHSV-glycoprotein or *Y. ruckeri* 16S rRNA.
70 Results showed as the transcription of PACAP splicing variants and VIP/PACAP receptors is
71 modulated in these organs during an acute viral and bacterial septicaemic infections in brown trout.
72 These gene expression results provide clues as to how the PACAP system is modulated in fish,
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74 aetiological agents. However, further experimental evidence is still required to fully elucidate and
75 characterize the role of PACAP and PRP for an efficient immune response against pathogens.

76

77 **Keywords:**

78 PACAP, PRP, VIP/PACAP receptors, VHS, ERM, *Salmo trutta*, Functional cross-talk

79 **Abbreviations:**

80 PACAP, Pituitary Adenylate Cyclase-Activating Polypeptide; PRP, PACAP-Related Peptide; VIP,
81 Vasoactive Intestinal Peptide; PAC-1, PACAP receptor; VPAC-1, VIP/PACAP receptor subtype 1;
82 VPAC-2, VIP/PACAP receptor subtype 2; VHS, Viral Haemorrhagic Septicaemia; ERM, Enteric
83 Red Mouth disease; SPF, Specific-pathogen-free; p.i., post infection; RT-qPCR, Reverse
84 Transcription quantitative polymerase chain reaction.

1. Introduction

86 Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) is a pleiotropic neuropeptide
87 associated with a number of physiological processes. Endocrine, metabolic, gastrointestinal and
88 immune modulatory effects are known in mammals [1–3]. It was discovered from ovine
89 hypothalamus due to its ability to stimulate cyclic Adenosine Mono Phosphate (cAMP) synthesis in
90 rat anterior pituitary cells [4]. PACAP belongs to the secretin/glucagon family of peptides. PACAP
91 has two molecular forms resulting from post-translational process, PACAP of 38 amino acids
92 (PACAP-38) and the shorter peptide of 27 residues (PACAP-27) [4,5]. PACAP was recently
93 identified in a wide range of chordates, including protochordates, fish, amphibians, birds, and
94 mammals [6–9]. The sequence of PACAP has been remarkably well preserved from tunicate to
95 human; by contrast, PRP is moderately conserved. The high conservation of PACAP nucleotide
96 sequence indicates this peptide fulfils an important biological function in a broad spectrum of
97 organisms [1]. In fish, PRP and PACAP are encoded on the same gene, but alternative splicing
98 processes render a full length mRNA containing both genes isoforms (PRP/PACAP) together with a
99 transcript in which exon 4 (PRP) is lacking [6,10–12]. As in mammals, in teleost fish mediates
100 Growth Hormone (GH) and Gonadotropin release in the pituitary gland [13], and is an important
101 hypophysiotropic factor [14]. The majority of studies on the biological functions of PACAP in fish
102 have mainly focused on growth or reproduction among the pleiotropic physiological functions of
103 PACAP, for example, its growth-promoting effects in African catfish *Clarias gariepinus* [15] and
104 Atlantic cod *Gadus morhua* [16]. Fish PRP, formerly known as GH-Releasing Hormone (GHRH)-
105 like, are less studied and research has been focus mainly in PRP role in GH-releasing activity in the
106 pituitary gland [10]. More recent findings explore PRP roles also in growth [15] and reproduction
107 [9,17]. In fish, PACAP and PRP were initially isolated from sockeye salmon *Oncorhynchus nerka*
108 [11] and subsequently from many other species, including Atlantic cod [18], catfish *Ictalurus*
109 *punctatus* [19,20], and *C. gariepinus* [15], zebrafish *Danio rerio* [21], sturgeon *Acipenser*
110 *transmontanus*, whitefish *Coregonus clupeaformis*, arctic grayling *Thymallus arcticus*, Yellowtail
111 flounder *Pleuronectes ferrugineus*, Atlantic halibut *Hippoglossus hippoglossus* [22] and several
112 other salmonids [12].

113 PACAP exerts multiple activities through G-protein-coupled receptors composed of several
114 transmembrane domains. It binds to a specific receptor called PAC-1 and additionally to VPAC-1
115 and VPAC-2 receptors, which also bind Vasoactive Intestinal Peptide (VIP) with an equal affinity.
116 These receptors are widely distributed in the organism, a feature allowing PACAP to exert a wide
117 range of effects [23]. On the other hand, PRP receptor, formerly referred to as GHRH-receptor, has

118 been isolated only in goldfish *Carassius auratus*, zebrafish, Fugu and blue gourami *Trichogaster*
119 *trichopterus* [13,24,25].

120 Little is known of the expression pattern of PACAP alternative splicing variants in different fish
121 tissues. Even less is known about the expression of PACAP ligands and receptors, or their function,
122 in the fish immune system. The different anatomical distribution found for PACAP transcriptional
123 splicing variants and VIP/PACAP receptors in rainbow trout *Oncorhynchus mykiss* lymphoid
124 tissues suggests the existence of diverse mechanisms of regulation of immune functions in fish
125 mediated by the VIP/PACAP system [26]. Recent discoveries, using immune reactivity assays and
126 recombinant proteins have added PACAP and its receptors to the growing list of mediators allowing
127 a functional cross-talk between the nervous, endocrine and immune system in fish [27–29]. Fish
128 PACAP modulate cytokine synthesis in carp *Ctenopharyngodon idella* [30], japanese flounder
129 *Paralichthys olivaceus* [29] and rainbow trout [31]. Such evidence suggests that PACAP might
130 directly regulate fish immune cells, acting to promote antiviral/antibacterial immunity. Treatments
131 with recombinant PACAP (isolated from African catfish) improve salmonids survival after viral
132 infections. An even better protection is conferred when administered in conjunction with antiviral
133 drugs (e.g. Ribavirin) [32]. Further experimental data are required to fully characterise the role of
134 PACAP during immune responses to heterologous pathogens in fish.

135 Viral Haemorrhagic Septicaemia (VHS), an OIE notifiable listed disease caused by a
136 *Novirhabdovirus* (VHSV), is world-wide regarded as one of the most economically important threat
137 for wild and cultured fish, resulting in high morbidity and mortality [33–35]. Despite decades of
138 effort vaccines against VHSV are not yet available for commercial use. Fish Yersiniosis or Enteric
139 Red Mouth disease (ERM), caused by the Gram negative bacteria *Yersinia ruckeri*, is a major
140 bacterial disease posing a threat to trout aquaculture [36,37]. ERM vaccines are commercially
141 available for aquaculture use, but have limited effectiveness to the motile serovar O1 biotype 1, in
142 development for other biotypes [38,39]. Due to the recent emergence of new bacterial strains the
143 disease is still widespread in many countries from Europe to North America, as well as South
144 Africa and Australia [40,41]. Biotype 2 variants of the bacterium, characterized by the concomitant
145 loss of motility and secreted lipase activity [41–43], were considered an emergent disease for both
146 American and European salmonid aquaculture, causing outbreaks even in previously vaccinated fish
147 [41,44].

148 In this study, the expression of genes encoding for PACAP splicing variants and VIP/PACAP
149 receptors, was studied in laboratory-reared specific-pathogen-free (SPF) brown trout *Salmo trutta*,
150 the European native trout species. New exon-skipping primers were developed and RT-qPCR

151 assays compared the specific gene transcription levels. Transcriptomic analysis revealed their
152 physiologic expression in central and peripheral immune-organs. Their expression modulation was
153 studied during VHS and Yersiniosis, known to typically elicit quick T_H1-like responses [45], in
154 kidney and spleen, sampled from infected fish during the first two weeks of infection. The
155 respective virus or bacteria burden was correlated to the PACAP splicing variants and affine
156 receptors expression modulation (using GLM analysis). Results from this study provide further
157 evidence to support the theory of an active involvement of PACAP pathways during an efficient
158 immune response.

159

160 **2. Materials and Methods**

161 **2.1 Fish maintenance and experimental design**

162 Triploid brown trout *Salmo trutta*, were kept under SPF conditions in a recirculation system at 16°C
163 (CEFAS, Weymouth, England). Routine bacteriological, virological and parasitological
164 examinations confirmed the absence of known pathogens. Fish, kept in a 12 h light/ 12 h dark
165 photoperiod (~ 200 lux at water surface, with 30 min dusk and dawn), were fed 1% bodyweight/day
166 with a standard commercial trout pellet diet (No. 45 Elite Trout Slow Sinking Food, Skretting).
167 Twenty brown trout (mean weight 69.5 g ± 16.4 g) were randomly allocated to 30 l tanks, with
168 three replicate tanks for each treatment group. The water temperature was gradually reduced to
169 12°C (5 days prior to pathogen challenge), to increase fish susceptibility to VHSV [46], but was
170 maintained at 16°C for the bacterial challenge, a temperature considered optimal for ERM
171 pathogenesis [43].

172

173 **2.2 Infection challenge assessment of pathogen burden**

174 VHSV infection challenge was carried out as previously described [45], with VHSV-Ia (isolate UK-
175 J167, highly pathogenic for rainbow trout [47]) at 5.56x10⁵ TCID₅₀/ml for 4 h at 12°C. The water
176 volume was halved and flow suspended for 4 h, keeping oxygen saturation at > 80%. For the mock-
177 exposed group the same procedure was used but with an equal amount of sterile transport medium
178 used to suspend the virus (Glasgow minimum essential medium, SAFC Biosciences), added to the
179 tanks.

180 *Yersinia ruckeri* infection was carried out as for the viral challenge, but with a suspension of
181 serotype O1 biotype 2, isolate UK-06041 (RD6), at 2.7×10^7 CFU/ml. This isolate is highly
182 pathogenic for Atlantic salmon and rainbow trout, and was used to characterize the new isolates
183 virulence [43,48]. The mock-exposed group had instead added sterile Dulbecco's phosphate
184 buffered saline (Sigma-Aldrich) added, used to suspend the bacteria.

185 Five brown trout were sampled at days 1, 3, 7 and 14 p.i. from each of the triplicate treatment tanks.
186 Kidney and spleen tissues were dissected aseptically and stored in RNAlater (Ambion). Additional
187 healthy tissues, including gills, thymus, mid-gut and liver were sampled from mock-exposed groups
188 after 1 day.

189 VHSV was confirmed by virology screening on monolayers of *Epithelioma papulosum cyprini*
190 (EPC) cells as described previously [49]. The growth of any bacteria was checked with kidney and
191 spleen swabs onto Tryptone Soya Agar (TSA, Oxoid) plates. *Y. ruckeri* was confirmed with
192 bacteriology screening and by means of a commercial monoclonal antibody agglutination test
193 (Mono-Yr, Bionor, Norway). Experimental infection experiments were conducted in accordance
194 with the current UK animal-welfare regulations.

195

196 **2.3 Total RNA extraction and cDNA synthesis**

197 Total RNA was extracted using TRI-reagent (Sigma-Aldrich), as described previously [45]. Tissues
198 were lysed with two 3 mm diameter Tungsten Carbide Beads (Qiagen), for 3 min at 30 Hz, in a
199 bench mixer TissueLyserII (Qiagen). No DNase treatment was applied. The RNA pellet was diluted
200 in TE buffer (10 mM Tris HCl; 1 mM EDTA, pH 8.0). RNA purity and concentration were
201 determined using a NanoDrop ND-1000 (Thermo Scientific). RNA was stored at -80°C until use. 5
202 μg of total RNA were converted into cDNA using Oligo-dT28VN (Sigma-Aldrich) primers and
203 RevertAid Reverse Transcriptase (Fermentas), following the manufacturer's instructions. cDNA
204 was diluted with TE buffer and stored at -20°C until use.

205

206 **2.4 RT-qPCR assays**

207 For each tissue sample RT-qPCR was performed by adding to 4 μl cDNA template 1 μl of each
208 forward and reverse primer (10 μM) and 14 μl of master mix. The latter was made with Immolase

209 (Bioline), SYBR Green fluorescent tag (Invitrogen), dNTPs mix (Bioline), ImmoBuffer (Bioline),
210 following the manufacturer's instructions. Assays were performed in technical duplicates in 96-well
211 plates (Roche), using a LightCycler 480 Real-Time PCR System (Roche). General cycling
212 conditions were: 10 min at 95°C, followed by 45 cycles with denaturation (94°C, 30 s), annealing
213 (62°C (*Ta*), 30 s) and elongation (72°C, 20 s (*Te*)); 1 min of melting temperature (*Tm*) recording at
214 84°C. RT-qPCR assays also measured specific pathogen burdens, viral or bacterial, in tissues (Tab.
215 1). VHSV-Ia burden was measured by targeting the transmembrane glycoprotein (G) gene (F/R-
216 1028 [50]), while bacterial burden targeted the *Y. ruckeri* 16S rRNA [51].

217

218 **2.5 Optimization of gene transcription detection**

219 Available rainbow trout *Oncorhynchus mykiss* PACAP splicing variants and VIP/PACAP receptor
220 nucleotide sequences were retrieved from the National Centre for Biotechnology Information
221 (NCBI) (www.ncbi.nlm.nih.gov/). Multiple sequence alignments were generated using
222 CLUSTALW2 (www.ebi.ac.uk/Tools/msa/clustalw2/). Intron-exon boundaries for brown trout
223 PACAP and PRP genes was predicted aligning rainbow trout PACAP (AF343977, lacking exon 4)
224 and PRP (AF343976) to the Atlantic salmon *Salmo salar* whole genome shotgun (WGS) contigs
225 AGKD01043834. WGS contigs were obtained with the BLAST/BLAT search tool, with default
226 options, from Ensembl Genome Browser (<http://www.ensembl.org/>). A similar approach was used
227 for the VIP/PACAP receptors, retrieved by aligning the *O. mykiss* sequences, PAC-1 (AY706216),
228 VPAC-1 (AY706218) and VPAC-2 (AY706217), respectively to contigs CAAK05042347,
229 CABZ01001604 and CABZ01032844 from zebrafish *Danio rerio* WGS. Exon-skipping primers
230 were specifically designed using Primer3Plus (www.bioinformatics.nl/primer3plus/). Genomic
231 DNA amplification was excluded by designing at least one primer crossing an intron-exon
232 boundary. Primer sequences were analysed for hairpin structures, self and hetero-dimers using the
233 online oligonucleotide properties calculator OligoCalc
234 (www.basic.northwestern.edu/biotools/OligoCalc.html). Conventional PCR tests with brown trout
235 genomic DNA and cDNA templates verified that no genomic amplification occurred. Amplicons
236 were purified with Gel/PCR Purification Kit (Biomiga), following the manufacturer's instructions.
237 Sequences were obtained from Eurofins MWG Operon's sequencing service (Ebersberg
238 Laboratories, Germany). Product specificity was assured by BLAST search
239 (www.blast.ncbi.nlm.nih.gov/Blast.cgi). The efficiency of each primer pair was calculated by
240 means of a calibration curve, using 10-fold serial dilutions in TE buffer from a 1 nM solution of

241 purified amplicon. Amplification efficiency was calculated with the LightCycler Software (Roche).
242 Each selected primer pair had specifically optimised T_a , T_e , and T_m for RT-qPCR (Tab. 1).

243

244 **2.6 Gene expression data analysis**

245 Tissues from 6 mock-exposed fish, sampled at day 1 p.i., were used to assess the constitutive
246 transcription of PACAP splicing variants and VIP/PACAP receptors. Their transcription
247 modulation was assessed in kidney and spleen tissues from 6-7 fish from each treatment group at
248 each time point. RT-qPCR assays were always performed in duplicate for each sample. The
249 inclusion of two dilutions enabled the relative quantification, and transcript level was calculated
250 using the LightCycler 480 (Roche) integrated software. Specific gene expression was normalised to
251 the expression of a reference gene, *S. trutta* Elongation Factor-1 α (EF-1 α). The fold change
252 between each infected group and respective unexposed control was calculated at each time point.
253 Normalised individual fold change values were anchored to the lowest value recorded in each data
254 set and then Log₂ transformed, as described previously [52]. Specific pathogen burden was
255 similarly assessed, from each kidney and spleen cDNA sample (Fig. 2), as described previously
256 [53].

257

258 **2.7 Statistical analysis**

259 The significance of the average fold change between uninfected and infected groups was analysed
260 by one-way analysis of variance (ANOVA) and LSD post hoc test for comparison of group means.
261 Differences were considered statistically significant when $p < 0.05$. The degree of the correlation
262 between specific pathogen burden and host gene expression was calculated by parametric
263 correlation analysis, as approached in previous fish infection studies [53]. The Pearson product-
264 moment correlation coefficient r was considered significant at $p < 0.05$ (2-tailed). A General Linear
265 Model (GLM) for analysis of the covariance assessed the significance of the factorial interaction
266 between individual gene expression and individual pathogen burden in relation to day post infection
267 [Time], used as covariate. The interaction was considered significant at $p < 0.05$. Statistical
268 analyses were performed with SPSS[®] Statistics package version 20.0 (IBM Corporation) and
269 graphically represented using GraphPad Prism version 5.04 (GraphPad Software Inc.).

270

271 **3. Results**

272 **3.1 Brown trout gene confirmation**

273 Each amplicon was sequenced to confirm primers specificity to target mRNA. Sequences were
274 verified as the brown trout homologue. All selected primers had amplification efficiency ≥ 1.91 and
275 gave no sensitive amplification when used on genomic DNA. For this purpose primers were tested
276 on gDNA from brown trout, giving same results also on gDNA from rainbow trout and Atlantic
277 salmon. Sequences were deposited in the GenBank database under Accession Numbers: HG000281
278 for PACAP, HG000280 for PRP/PACAP, HG000282 for PAC-1, HG000283 for VPAC-1 and
279 HG000284 for VPAC-2. Additionally, PRP intron 4 sequence was obtained and deposited in the
280 GenBank database under Acc. No. HG000279. A common reverse primer was designed to both
281 PACAP and PRP splicing variants, named as “PRP/PACAP-R”, but exclusively used for PACAP
282 detection. The reverse primer for specific PRP transcription detection (named as “PRP-R2”) was
283 instead designed between exon 4 and 5 boundaries, thus skipping intron 4. A summary of optimised
284 primers and RT-qPCR conditions for genes screened during this study is provided in Table 1.

285

286 **3.2 Constitutive expression of PACAP splicing variants and PAC-1, VPAC-1 and**
287 **VPAC-2 receptors**

288 The study of PACAP ligands constitutive expression showed a detectable transcription in all the
289 organs tested (Fig. 1.A). Generally a higher constitutive transcription was found for PRP when
290 compared to PACAP. Both PACAP splicing variants showed their highest physiologic expression
291 in the gut. The expression of PACAP and PRP receptors was instead more variable. Whilst PAC-1,
292 VPAC-1 and VPAC-2 constitutive transcription was also detectable in all the organs tested (Fig.
293 1.B), a relatively high transcript level was seen for PAC-1 in immune tissues but not in liver which
294 in contrast had highest VPAC-1 expression. VPAC-2 had the highest expression in the mid-gut,
295 spleen and kidney (average Cq in control group ranging between 22.5 to 24.1).

296

297

298 **3.3 Experimental infection and assessment of pathogen burdens**

299 Experimental infection with VHSV-Ia was confirmed by the appearance CPE during virology tests,
300 indicating a successful experimental infection of the SPF brown trout. Fish from the mock-
301 challenged group showed no mortality or disease symptoms, no appreciable viral titres and no fish
302 had detectable bacteria. VHS pathognomonic clinical signs were observed, including acute
303 petechial haemorrhages in many organs, including skin, brain, heart, liver, spleen, kidney
304 (pronephros and mesonephros), trunk muscle, intestine and perivisceral abdominal adipose tissue.
305 VHS gave sporadic mortalities, reaching a total of 4.9% at 2 weeks p.i., with the first event recorded
306 at day 8. VHSV-Gp detection in kidney and spleen showed a higher burden at day 3 p.i., gradually
307 decreasing thereafter (Fig. 2.A). Cross-correlation of the viral burden in kidney and spleen
308 suggested a quick and even distribution of the virus during the septicaemic infection ($r = 0.927$).

309 Experimental infection with *Yersinia ruckeri* (serotype O1 biotype 2) was confirmed with
310 diagnostic tests, indicating a successful experimental infection of the SPF brown trout. No other
311 bacterial infections were detected in any experimental fish. All mock-infected fish were negative.
312 ERM pathognomonic clinical signs were observed, including lethargy, skin darkening, and
313 anorexia. Erythema at the base of the pectoral fins, spiralling within the tanks, strong darkening
314 followed by mortality were also seen in the most advanced stages. The ERM typical reddening
315 around the mouth was only sporadically observed in brown trout. At the necropsy, fish showed
316 typical lesions due to bacterial haemorrhagic septicaemia, with a marked inflammatory response in
317 all tissues. However, mortality was low, with a cumulative mortality of 12.1% during the 2 weeks
318 trial, with the first death recorded at day 6 p.i.. The bacterial septicaemic spreading was confirmed
319 by the detection of *Y. ruckeri* 16S rRNA at each time point in both tissues (Fig. 2.B), with a peak at
320 day 7 p.i., and a rapid decrease thereafter.

321

322 **3.4 Comparative analysis of PACAP splicing variants during septicaemic infection**

323 The expression of PACAP was rapidly (within 1 day) modulated in both kidney and spleen in
324 response to VHSV-Ia infection (Fig. 3.A). The highest level of induction was found in spleen, with
325 a 143 fold-increase at day 3 p.i.. A gradual decrease in transcript level followed to the end of the
326 experiment at day 14 p.i. but it remained significantly upregulated in spleen. PRP showed a
327 radically differential response to VHS (Fig. 3.C), where a delayed and selective induction was seen
328 in spleen. In contrast ERM stimulated the transcription of both PACAP and PRP. By day 1 p.i. both

329 transcripts were strongly upregulated in spleen, reaching a 132 and 29 fold-increase respectively
330 (Fig. 3.B). PACAP transcription was more sustained in the spleen at all sampling points, when
331 compared to kidney. A similar selective induction was seen for PRP in spleen during the early
332 stages of the infection (Fig 3.D). Cross correlation analyses between the expression of PACAP and
333 PRP showed a significant positive relationship, with a higher coefficient for spleen in both diseases:
334 during VHS, in kidney $r = 0.464$ while in spleen $r = 0.606$; during ERM, in kidney $r = 0.553$ while
335 in spleen $r = 0.727$. PACAP and PRP expression in kidney and spleen was positively correlated to
336 the VHSV infection marker. However their upregulation was significantly correlated to time p.i.
337 only in spleen (Table 2). PACAP expression was also positively correlated to the bacterial burden in
338 both tissues and a significant interaction with time p.i. was found. PRP expression had a significant
339 correlation with time p.i. in spleen only.

340

341 **3.5 Comparative analysis of VIP/PACAP receptors expression during septicaemic**

342 **infections**

343 Comparative expression profiles of PACAP receptors showed that the three receptors were
344 differentially regulated, but mainly in the kidney (Fig. 4). A high individual variance confounded
345 the analysis due to private response patterns. The expression of PAC-1, the PACAP and PRP
346 specific receptor, showed a strong down-regulation at day 1 p.i. in spleen during ERM. Indeed
347 PAC-1 expression was negatively correlated to bacterial burden in spleen, confirmed by the co-
348 variance analysis (Table 2). However, PAC-1 showed a delayed upregulation in kidney during VHS
349 and in both kidney and spleen during ERM (Fig 4.A and .B). During VHS PAC-1 expression was
350 influenced (significantly) by time in kidney. Few changes were detected for VPAC-1 expression
351 between infected and non-infected brown trout, despite some significant differences being apparent
352 between kidney and spleen at day 1 p.i. during both infections (Fig. 4.C and .D). However, a
353 significant correlation of VPAC-1 expression with time p. i. was found for both tissues during VHS
354 (Table 2). In contrast, VPAC-2 showed an initial downregulation in both kidney and spleen 1 day
355 post VHSV-Ia infection, followed by a small increase in kidney at day 3 and day 14 p.i. (Fig. 4.E).
356 VPAC-2 was also negatively correlated to VHSV burden in spleen and its expression was
357 significantly correlated to time p.i. (Table 2). During ERM, VPAC-2 showed a large induction in
358 kidney from day 1 to day 14 p.i., with no significant modulation occurring in spleen (Fig. 4.F). In
359 agreement with this expression pattern VPAC-2 was positively correlated with bacterial burden and
360 time post-infection in the kidney (Table 2).

361 4. Discussion

362 One characteristic of the salmonid PRP/PACAP precursor gene is the phenomenon of exon-
363 skipping, often observed in non-mammalian vertebrates such as teleosts but not in mammals [22].
364 In this study, we investigated the differential expression of the two PACAP splicing variants by RT-
365 qPCR in two brown trout immune tissues post-infection. Specific oligonucleotides were optimised
366 to independently amplify both PACAP and PRP, and showed that the highest expression of both
367 transcripts was found in the mid-gut. The expression of these transcripts in the intestine and pyloric
368 caecum has been previously shown for sockeye salmon [12] and rainbow trout [26]. It is known in
369 mammals that PACAP stimulates histamine release from enterochromaffin cells through activation
370 of L-type calcium channels in the gastric mucosa [1,54]. Additionally, the PACAP involvement in
371 the control of gut mobility and secretion was also demonstrated in Atlantic cod [18], and recent
372 studies have established a connection between PRP/PACAP mRNA expression in the intestine of *P.*
373 *olivaceus* and possible immune functions in the gut [29]. Interestingly, the entire PRP/PACAP
374 precursor mRNAs are induced in the intestine and pyloric caecum of olive flounder within 1 h post-
375 challenge with challenge with *Edwardsiella tarda*, reaching their highest level 24 h post-challenge,
376 suggesting that PRP and PACAP may act as regulators of the immune system, especially in the
377 gastrointestinal tract.

378 In this study, PRP and PACAP mRNA was detected in both central (thymus) and peripheral
379 (spleen) lymphoid organs. In mammals, PACAP and its receptors are expressed by immune cells
380 [55,56]. PACAP positive cells and PACAP mRNA expression is detected in the thymus, spleen and
381 lymph nodes of rats [56]. In teleosts, PACAP splicing variants are present in the spleen of tilapia
382 *Oreochromis mossambicus* [57], rainbow trout [26] and darkbarbel catfish *Pelteobagrus vachelli*
383 [58]. The spleen is one of the major lymphoid organs of teleosts, also involved in hematopoiesis,
384 although its role is generally limited to erythropoiesis and thrombopoiesis [59,60].

385 PACAP and PRP were also detected in gills. It is well known in fish that the mucous surfaces of
386 skin and gills constitute a first barrier against pathogens. In zebrafish using *in situ* hybridization, the
387 first T cell receptor (TcR α) positive cells outside the thymus are found in the intestine and gills at
388 day 9 post fertilization [61]. In rainbow trout, only PACAP was detected in the gills [26], and both
389 were absent from kidney and liver unlike the results of the present study. Indeed, PACAP
390 expression has not been previously detected in kidney [20,21,26,28,58,62]. Then again, mRNA
391 expression in the liver was detected before in darkbarbel catfish [58], but not in channel catfish
392 [20], tilapia [57] and rainbow trout [26]. These differences could be explained because of the lower

393 sensitivity of conventional PCR [20,28,62] or the exon-skipping phenomenon of the PRP/PACAP
394 precursor gene, which some evidence suggests is a regulatory mechanism that may differ by
395 species, even in the same lineage. The promoter region possessing regulatory elements for gene
396 expression is quite divergent between species, suggesting that while the coding region of the
397 PACAP gene is well-conserved throughout vertebrates, the transcriptional regulation has evolved
398 and developed in a species-specific manner [29].

399 Assessment of the constitutive expression of teleost VIP/PACAP receptors has been performed
400 previously and showed a widespread distribution throughout the body [26,58,63–66]. For example,
401 in the darkbarbel catfish PAC-1 was detectable at various levels in all tissues studied (including
402 brain, gill, intestine, liver, muscle, ovary, testis, stomach, spleen) except the gills [58]. Recently, the
403 expression of rainbow trout PAC-1, VPAC-1 and VPAC-2 receptors was assessed by RT-qPCR
404 [27]. PAC-1 receptor mRNA was detected in brain, gills, intestine, head kidney, spleen, blood and
405 skin. VPAC-1 receptor mRNA was detected in brain, intestine, spleen and blood but not in gills,
406 head kidney or skin, and lastly, VPAC-2 receptor mRNA was detected in brain, gills, intestine,
407 spleen and skin but not in head kidney or blood. No mRNAs for any of the genes were detectable in
408 liver and gonads [26]. The latter contrasts with our study in brown trout, where a clear expression of
409 VPAC-1 and VPAC-2 was seen in liver.

410 The wide distribution of PACAP splicing variants and VIP/PACAP receptors in various tissues in
411 brown trout is similar to that in mammals [1,26], suggesting an involvement of PACAP and VIP
412 peptides in a broad spectrum of biological functions in these organisms. Besides the presence of
413 PACAP and its receptors in fish immune-related tissues, the immunoregulatory role of PACAP in
414 teleosts has been investigated recently in catfish, tilapia, rainbow trout, olive flounder and grass
415 carp [26–32] but further studies are warranted. In contrast to PACAP, the biological role of PRP has
416 still to be fully elucidated. PRP was previously termed GHRH or GHRH-like peptide, and was
417 considered the mammalian GHRH homolog in non-mammalian vertebrates due to its ability to
418 stimulate GH release from pituitary cells in several fish species. However subsequently the
419 authentic GHRH genes were discovered in teleosts and amphibians [13]. Unlike PACAP, *P.*
420 *olivaceus* PRP fails to regulate intracellular cyclic adenosine monophosphate (cAMP) production in
421 Hirame natural embryonic cells (HINAE) transfected to over express the PAC-1 mRNA, and does
422 not up-regulate PAC-1 transcript expression [29]. A PRP-specific receptor has yet to be cloned in
423 that specie; in fact, PRP receptor has been isolated only from few species [13,24,25] and therefore,
424 the biological activity of PRP in many aspects remains to be fully elucidated [29].

425 In this study, comparative expression profiles of the two PACAP splicing variants and VIP/PACAP
426 receptors were studied after viral (VHSV-Ia) and bacterial (*Y. ruckeri*) infection. VHSV is a
427 negative single stranded RNA virus that causes a serious systemic hemorrhagic septicaemia in a
428 wide variety of wild and cultured fish species, posing a major threat to the development of salmonid
429 aquaculture [33–35]. PACAP was predominantly found to be induced during the early stages of the
430 viral infection, while irregular expression patterns were observed for the receptors. The PACAP
431 receptors expression modulation was mainly limited to the kidney, but a high individual variance
432 confounded the analysis due to private response patterns.

433 In mammals, VIP and PACAP have well-characterized effects on the immune system and anti-
434 inflammatory properties, including inhibition of macrophage adherence and down-regulation of
435 inflammatory cytokines and production of reactive oxygen species [55]. Moreover, they can induce
436 the production of anti-inflammatory cytokines such as IL-10 and β -chemokines, indeed upregulated
437 during the inflammation playing a protective action by downregulating of pro-inflammatory
438 cytokines [67–70]. Due to their immunomodulatory properties, both neuropeptides have been
439 considered as promising therapeutic agents for a range of pathologies [71–74]. Their involvement in
440 heterogeneous viral infections has been demonstrated [75–78]. For example, PACAP has potent
441 regulatory activity on Herpes simplex virus activation [75], with increased plasma PACAP-38
442 levels observed in patients with chronic hepatitis B following lamivudine-induced elimination of
443 viraemia [76], and increased antiviral immunity was seen in the absence of VIP [77]. Treating HIV-
444 1-infected macrophages with VIP and PACAP diminished viral production, and treatment with
445 specific agonists of the neuropeptide receptors VPAC-2 and PAC-1 showed similar effects. VIP
446 promoted HIV-1 inhibition through stimulation of the receptors VPAC-1 and VPAC-2 but not
447 through stimulation of PAC-1. The ability of PACAP to diminish HIV-1 replication, on the other
448 hand, resulted from its ligation of all three receptors since its effect was only abrogated when all
449 three were blocked. The mechanisms underlying these effects seem to be related to the regulation of
450 the chemokine axis but also implicate induction of IL-10 secretion by macrophages [70,78].

451 The expression of genes encoding for PACAP and PRP, as well as for their affine receptors, was
452 also studied after infection with *Y. ruckeri*. This Gram-negative bacterium is the aetiological agent
453 of Yersiniosis or ERM, that still causes significant economic losses in salmonid aquaculture
454 worldwide [79]. Although PACAP had higher levels of expression, both splicing variants were
455 found to be consistently and highly induced, peaking during the early stages of the bacterial
456 infection, with a predominant induction in the spleen and, as occurs after viral challenge, irregular
457 expression patterns were observed for the receptors. Transcriptional regulation of PACAP splicing

458 variants after bacterial infection has been rarely studied and no previous reports exist on
459 VIP/PACAP receptors transcriptional regulation after bacterial challenge in teleost fish. Only one
460 report studied transcriptional regulation of the PRP/PACAP precursor in *P. olivaceus* challenged
461 with the bacterial pathogen *E. tarda* [29]. PRP/PACAP precursor expression appeared in gut tissue,
462 such as the intestine and pyloric caecum, in challenged but not in healthy fish. This pathogenic
463 bacterium commonly resides in the fish intestine, where the epithelium of the gastrointestinal tract
464 is the main site of attachment. Expression of the longer (PRP-containing) transcript gradually
465 increased in the intestine of the bacteria-challenged flounder, suggesting an immunological role for
466 these molecules, especially in the gastrointestinal tract [29]. Recently, the possible role of PACAP
467 under LPS challenge was studied in grass carp [30]. The results showed that PACAP stimulated
468 synthesis of pro-inflammatory cytokines (TNF- α and IL-1 β) in head kidney, but affected neither
469 expression of anti-inflammatory cytokine (IL-10) nor immune stimuli-induced expression (LPS or
470 *A. hydrophila* stimuli) of the cytokines, implying that PACAP may not play an anti-
471 proinflammatory role in grass carp unlike in mammals. This discrepancy reflects the different roles
472 of PACAP in mammals and fish with LPS challenge, but the possibility that it may be caused by
473 different cell models and species variability is not excluded [30], thus further experiments are still
474 required to clarify the involving of PACAP in inflammatory response in teleost fish.. In terms of the
475 receptor expression, in our study VPAC-2 seems to be more actively regulated by bacterial
476 infection compared with PAC-1 and VPAC-1, in agreement with results in mammals where
477 expression of VPAC-2 in macrophages was reported to be up regulated by components of Gram-
478 negative bacteria such as TLR-4 ligands [80].

479

480 **5. Conclusions**

481 This study reports for the first time the transcriptional modulation of PACAP gene splicing variants
482 and VIP/PACAP receptors in brown trout (*Salmo trutta*). The expression of PACAP and PRP, as
483 well as for the receptors PAC-1 (PACAP affinity), VPAC-1 and VPAC-2 (VIP/PACAP affinity),
484 was comparatively studied in 6 tissues to assess their constitutive expression levels in this species.
485 Subsequently the kidney and spleen were screened for the relative expression of these PACAP-
486 system genes over the course of a viral (VHSV-Ia) and bacterial (*Y. ruckeri*, serotype O1 biotype 2)
487 septicaemic infections. Both PACAP and PRP were strongly induced during the pathogenesis of
488 both diseases, although with specific patterns. A high individual variance confounded the
489 transcriptomic analysis of PAC-1, VPAC-1 and VPAC-2 receptors, due to the presence of private

490 response patterns. Nevertheless, PAC-1 was found to have a late upregulation (after 2 weeks p.i.) in
491 both disease states, while VPAC-2 showed a selective modulation in the kidney. Overall the results
492 obtained from brown trout during this study provide more data as to how and when the PACAP
493 system is modulated in teleosts, in this case during immune responses elicited by septicaemic
494 infections. Nevertheless, further experimental evidence is still required to more fully characterize
495 their involvement and precise role in protective immune responses to viral and bacterial pathogens.

496

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501

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736 **Tables**

737 **Table 1. Brown trout *Salmo trutta* sequence references, primers, amplicon sizes, specific RT-**
738 **qPCR conditions for genes expression screened during this study.** Additional PRP intron 4
739 sequence obtained with Accession Number HG000279. *Note: a common reverse primer to both
740 splicing variants is used for PACAP detection.

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743 **Table 2. PACAP and PRP ligands and VIP/PACAP receptors gene transcription correlation**
744 **to infection markers.** Pearson product-moment correlation coefficients (r), with their respective p
745 values (2-tailed) given for correlations between individual gene expression and individual pathogen
746 burden. Significant correlations are shown in bold; *p < 0.05; **p < 0.01 (2-tailed). Time*pathogen
747 burden = p value based on Fisher's F-distributions using GLM analysis (ANCOVA) of specific gene
748 expression to assess the significance of the interaction between days p.i., with the effect of
749 individual pathogen burden as covariate.

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760 **Figures**

761 **Fig. 1. Constitutive expression profiles of brown trout PACAP and PRP ligands and**
762 **VIP/PACAP receptors genes, detected by RT-qPCR:** (A) PACAP system ligands: PACAP and
763 PRP; (B) VIP/PACAP receptors: PAC-1, VPAC-1 and VPAC-2. Tissues were sampled from 6
764 healthy fish. Transcript concentrations were calculated using a standard curve specifically obtained
765 for each primer pair. Data are normalised to the expression of a reference gene, EF-1 α , and
766 presented as group means + SEM.

767

768 **Fig. 2. Pathogen burden assessed on cDNA samples from brown trout kidney and spleen**
769 **tissues.** (A) Viral burden assessed by RT-qPCR targeting the VHSV transmembrane glycoprotein
770 gene; (B) Bacterial burden assessed by RT-qPCR targeting the *Y. ruckeri* 16S rRNA. Results are
771 obtained from individual kidney and spleen cDNAs and presented as mean fold change + SEM
772 normalised to a *Salmo trutta* reference gene, EF-1 α .

773

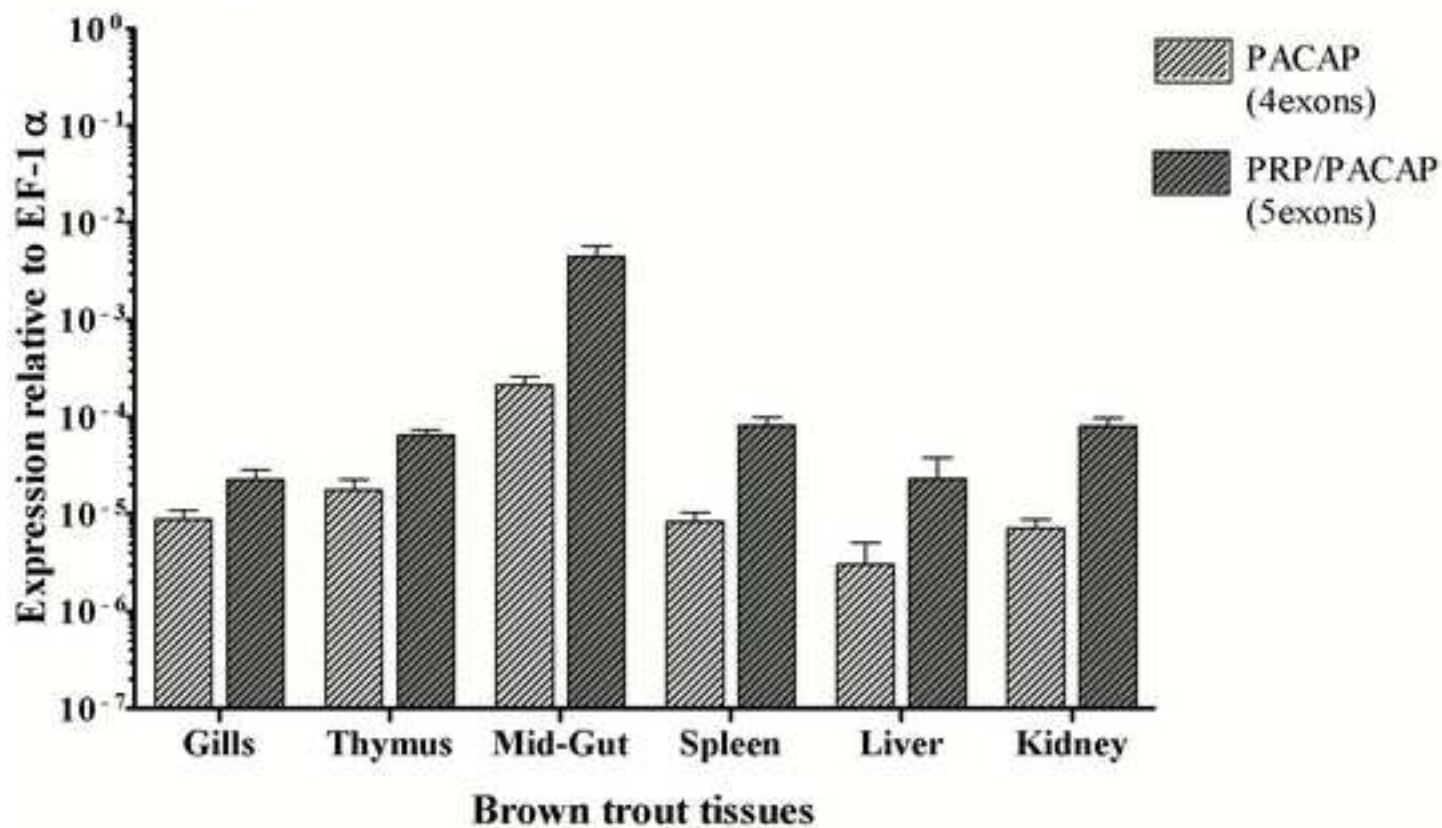
774 **Fig. 3. Expression kinetics of PACAP and PRP genes in kidney and spleen of VHSV-Ia and**
775 ***Y. ruckeri* infected brown trout:** (A; B) PACAP; (C; D) PRP. RT-qPCR detected transcript levels
776 were normalised to the expression of a reference gene, EF-1 α , and presented as group means +
777 SEM. The p value of a LSD post hoc test between the pathogen infected group and the
778 corresponding control (not shown in the graph) is shown above the bars as: *p < 0.05; **p < 0.01.

779

780 **Fig. 4. Expression kinetics of VIP/PACAP receptor genes in kidney and spleen of VHS-Ia and**
781 ***Y. ruckeri* infected brown trout:** (A; B) PAC-1; (C; D) VPAC-1; (E; F) VPAC-2. RT-qPCR
782 detected transcript levels were normalised to the expression of a reference gene, EF-1 α , and
783 presented as group means + SEM. The p-value of a LSD post hoc test between the VHS infected
784 group and the corresponding control (not shown in the graph) is shown above the bars as: *p < 0.05;
785 **p < 0.01.

Figure 1
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A - Ligands



B - Receptors

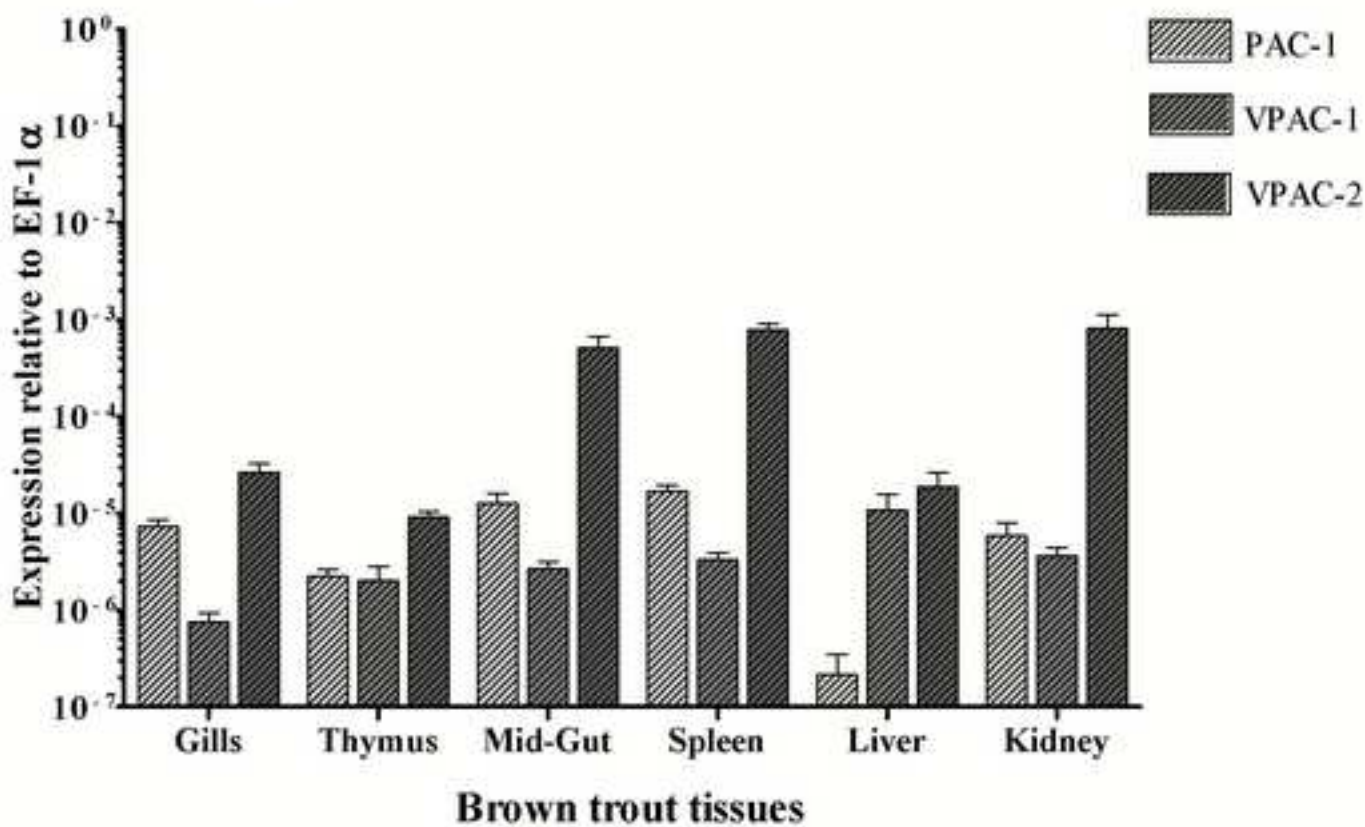


Figure 2

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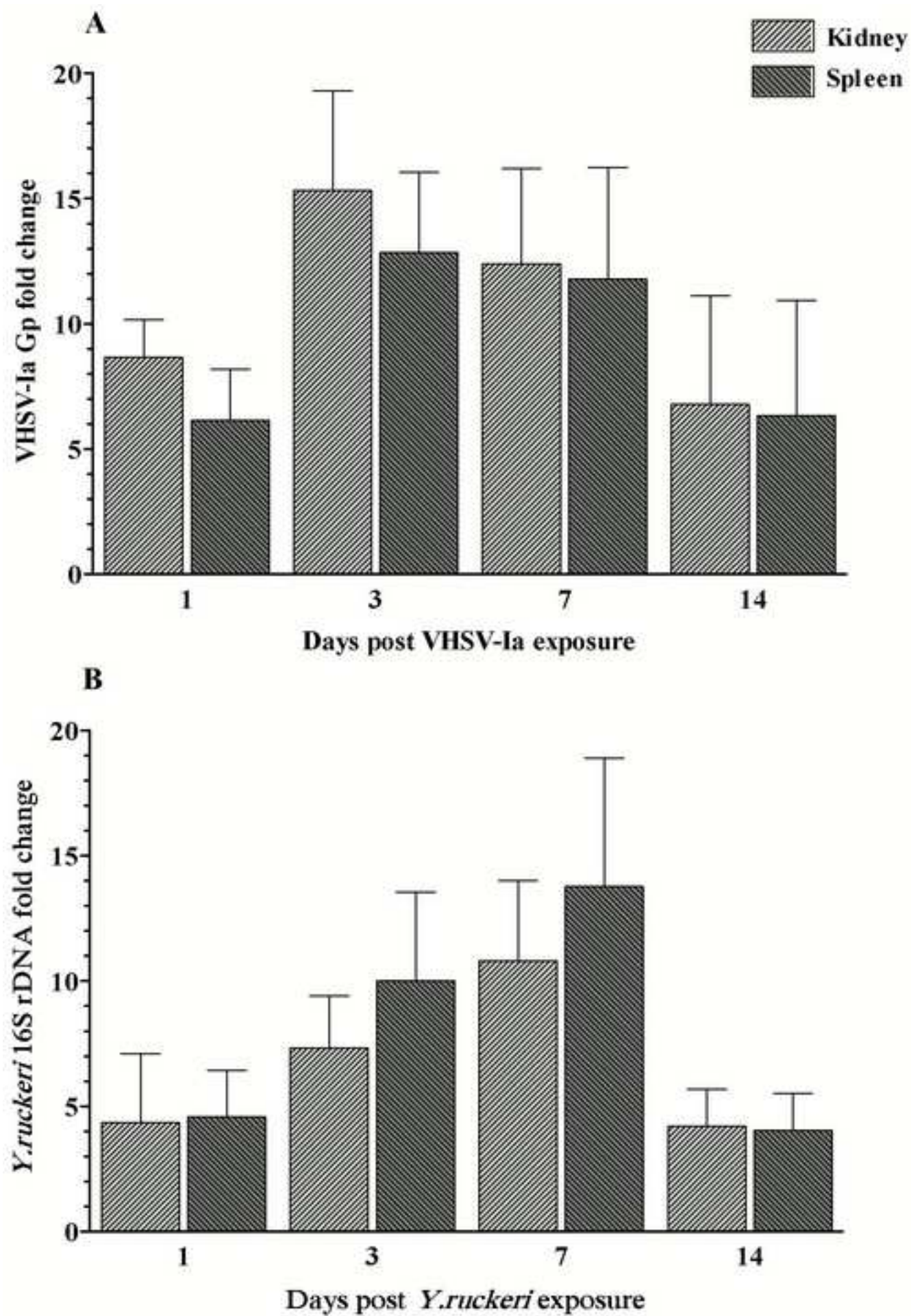


Figure 3
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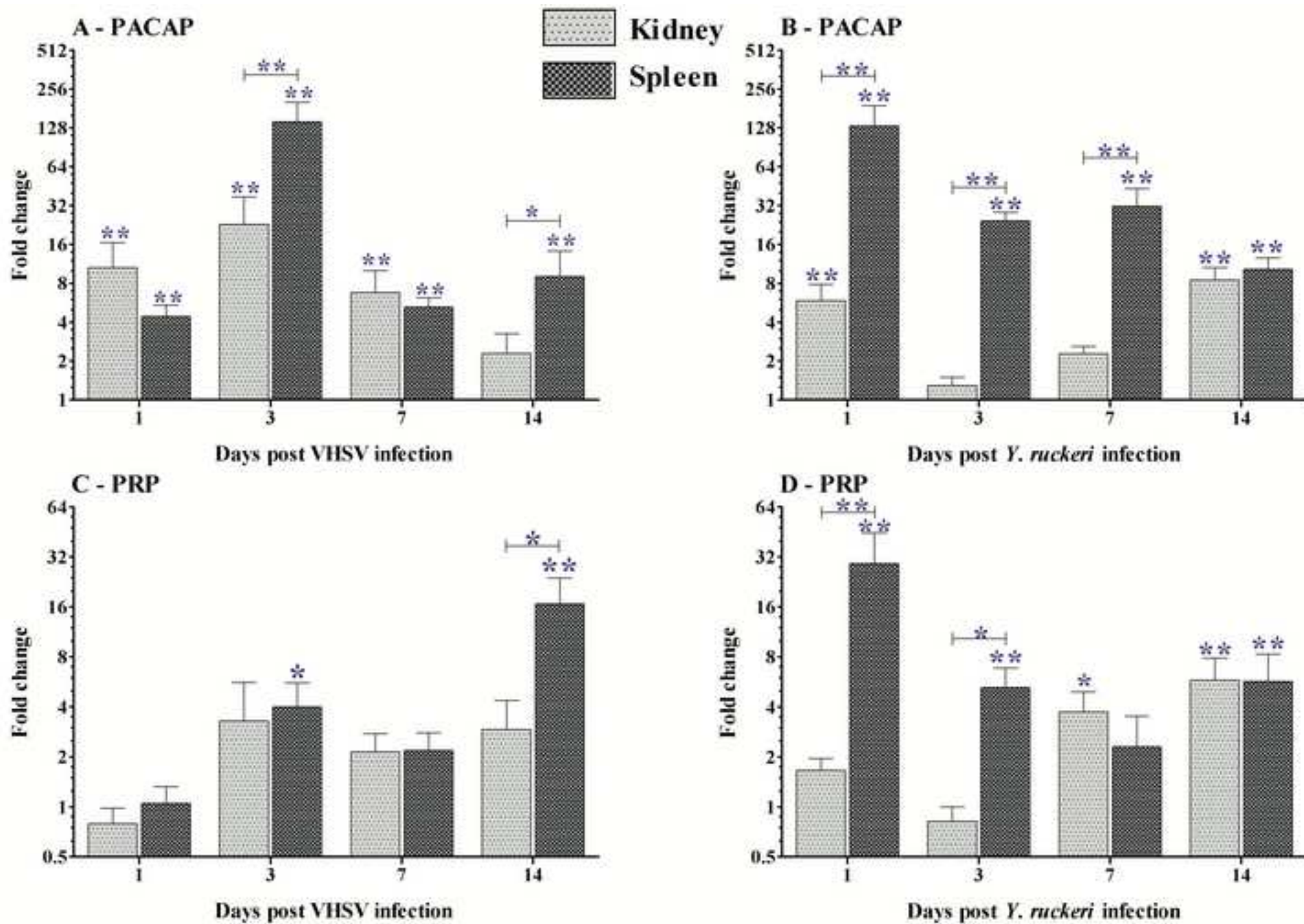


Figure 4

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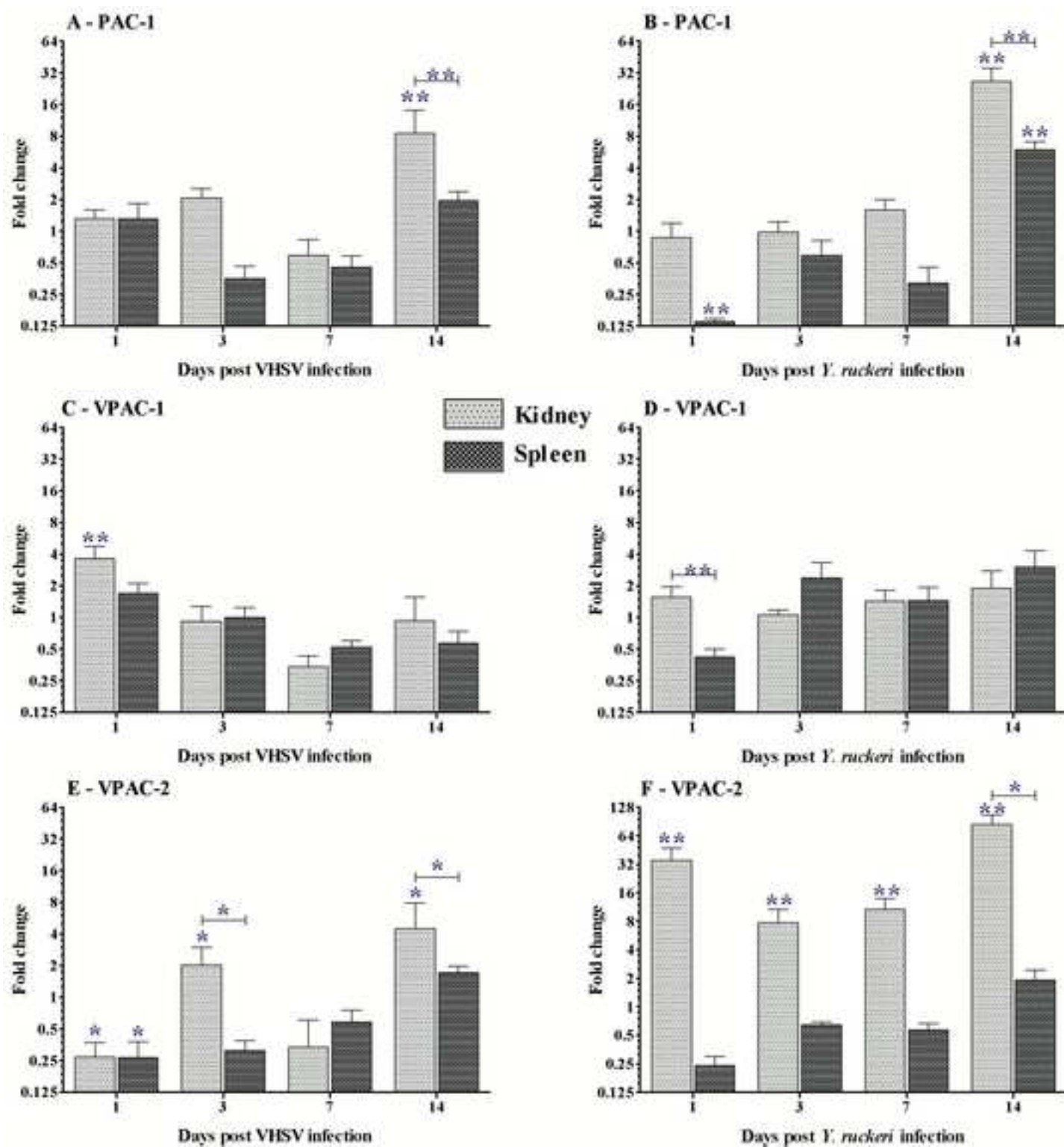


Table 1. Brown trout *Salmo trutta* sequence references, primers, amplicon sizes, specific RT-qPCR conditions for genes expression screened during this study.

Gene	GenBank Accession number	Forward primer (5' to 3')	Reverse primer (5' to 3')	Amplicon size (bp)	RT-qPCR conditions				
					Ta	Te	Mt		
EF-1α	HF563594	EF-1 α -F	CAAGGATATCCGTCGTGGCA	EF-1 α -R	ACAGCGAAACGACCAAGAGG	327	63	30	88
PACAP	HG000281	PACAP-F	GGAGAAAAGTGGAGGGAGCA	PRP/PACAP-R *	TGTCTATACCTTTCCCAAGGACTG	153	62	18	85
PRP	HG000280	PRP-F2	CCACCGGAGAAAAGAACGGA	PRP-R2	GCTTTGCCATCAGAGAATGGAG	112	64	18	82
PAC-1	HG000282	PAC1-F2	CTGCTTCTTACACACTGTGGAGTG	PAC1-R2	CATATCCCAACACCCTATGTCA	244	62	22	83
VPAC-1	HG000283	VPAC1-F2	GTCCAACCAGTACTTGAGGCTG	VPAC1-R3	CAACCGCAAATCCCTGGAATG	158	64	18	86
VPAC-2	HG000284	VPAC2-F2	GTGCTGGGAGAGGAATGACATC	VPAC2-R2	GACTTAGCCAGACGCCTGTATTG	177	63	18	84
VHSV-Gp	JN180851	VHSV G 1018 F	CTCATTTCTCCTCTCAAAGTTTCG	VHSV G 1018 R	CCGTCTGTGTTGTTGTCTACC	192	60	18	86
<i>Y. ruckeri</i> 16S rRNA	EU401667	16S F	GCGAGGAGGAAGGGTTAAGTG	16S R	GAAGGCACCAAGGCATCTCT	589	63	30	87

Additional PRP intron 4 sequence obtained with accession number HG000279. *Note: a common reverse primer to both splicing variants is used for PACAP detection.

Table 2. PACAP system gene transcription correlation to the viral and bacterial burden and influence of time.

Correlations Analysis	Gene Vs Viral burden (Pearson's r)		Time*Viral burden (p value)		Gene Vs Bacterial burden (Pearson's r)		Time*Bacterial burden (p value)	
	Kidney	Spleen	Kidney	Spleen	Kidney	Spleen	Kidney	Spleen
PACAP	.736**	.818**	.822	.000	.397**	.690**	.000	.000
PRP	.342*	.566**	.163	.001	.170	.240	.101	.001
PAC-1	.039	-.227	.007	.304	.032	-.365*	.154	.002
VPAC-1	-.143	-.255	.008	.006	.108	.021	.291	.316
VPAC-2	.103	-.298*	.009	.026	.461**	-.091	.000	.085

Pearson product-moment correlation coefficients (r), with their respective p values (2-tailed) given for correlations between individual gene expression and individual pathogen burden. Significant correlations are shown in bold; * $p < 0.05$; ** $p < 0.01$ (2-tailed). Time*pathogen burden = p value based on Fisher's F-distributions using GLM analysis (ANCOVA) of specific gene expression to assess the significance of the interaction between days p.i., with the effect of individual pathogen burden as covariate.