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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-Ia) 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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*Cover Letter

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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,

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(Title): Viral and bacterial septicaemic infections modulate the expression of PACAP splicing variants and VIP/PACAP receptors in brown trout immune organs.

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Highlights:

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

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-Ia) 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.

Keywords:

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

Abbreviations:

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

1. Introduction

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

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

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

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

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

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

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

2. Materials and Methods

2.1 Fish maintenance and experimental design

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

2.2 Infection challenge assessment of pathogen burden

VHSV infection challenge was carried out as previously described [45], with VHSV-Ia (isolate UK-J167, highly pathogenic for rainbow trout [47]) at $5.56x10^5$ $TCID_{50}$/ml for 4 h at 12ºC. The water volume was halved and flow suspended for 4 h, keeping oxygen saturation at > 80%. For the mock-exposed group the same procedure was used but with an equal amount of sterile transport medium used to suspend the virus (Glasgow minimum essential medium, SAFC Biosciences), added to the tanks.

Yersinia ruckeri infection was carried out as for the viral challenge, but with a suspension of serotype O1 biotype 2, isolate UK-06041 (RD6), at $2.7x10^7$ CFU/ml. This isolate is highly pathogenic for Atlantic salmon and rainbow trout, and was used to characterize the new isolates virulence [43,48]. The mock-exposed group had instead added sterile Dulbecco's phosphate buffered saline (Sigma-Aldrich) added, used to suspend the bacteria.

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

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

2.3 Total RNA extraction and cDNA synthesis

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

2.4 RT-qPCR assays

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

(Bioline), SYBR Green fluorescent tag (Invitrogen), dNTPs mix (Bioline), ImmoBuffer (Bioline), following the manufacturer's instructions. Assays were performed in technical duplicates in 96-well plates (Roche), using a LightCycler 480 Real-Time PCR System (Roche). General cycling conditions were: 10 min at 95°C, followed by 45 cycles with denaturation (94°C, 30 s), annealing (62°C (*T*a), 30 s) and elongation (72°C, 20 s (Te)); 1 min of melting temperature (*T*m) recording at 84°C. RT-qPCR assays also measured specific pathogen burdens, viral or bacterial, in tissues (Tab. 1). VHSV-Ia burden was measured by targeting the transmembrane glycoprotein (G) gene (F/R-1028 [50]), while bacterial burden targeted the *Y. ruckeri* 16S rRNA [51].

2.5 Optimization of gene transcription detection

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

purified amplicon. Amplification efficiency was calculated with the LightCycler Software (Roche). Each selected primer pair had specifically optimised *T*a, Te, and *T*m for RT-qPCR (Tab. 1).

2.6 Gene expression data analysis

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

2.7 Statistical analysis

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

3. Results

3.1 Brown trout gene confirmation

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

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

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

3.3 Experimental infection and assessment of pathogen burdens

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

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

3.4 Comparative analysis of PACAP splicing variants during septicaemic infection

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

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

3.5 Comparative analysis of VIP/PACAP receptors expression during septicaemic infections

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

4. Discussion

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

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

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

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

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

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

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

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

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

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

5. Conclusions

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

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

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Tables

Table 1. Brown trout *Salmo trutta* sequence references, primers, amplicon sizes, specific RT-qPCR conditions for genes expression screened during this study. 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 and PRP ligands and VIP/PACAP receptors gene transcription correlation to infection markers. 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.

Figures

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

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

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

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

Figure 1
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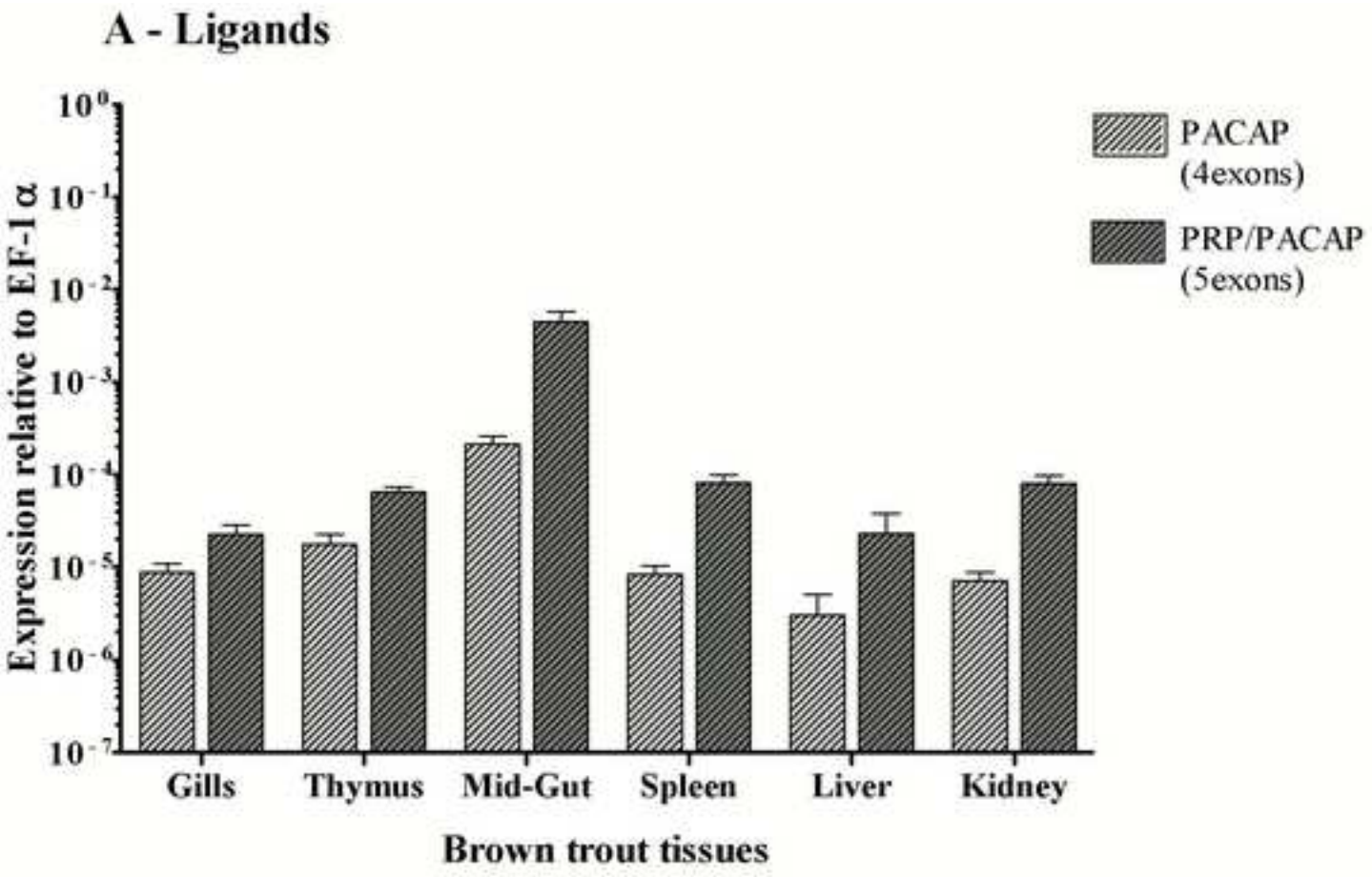

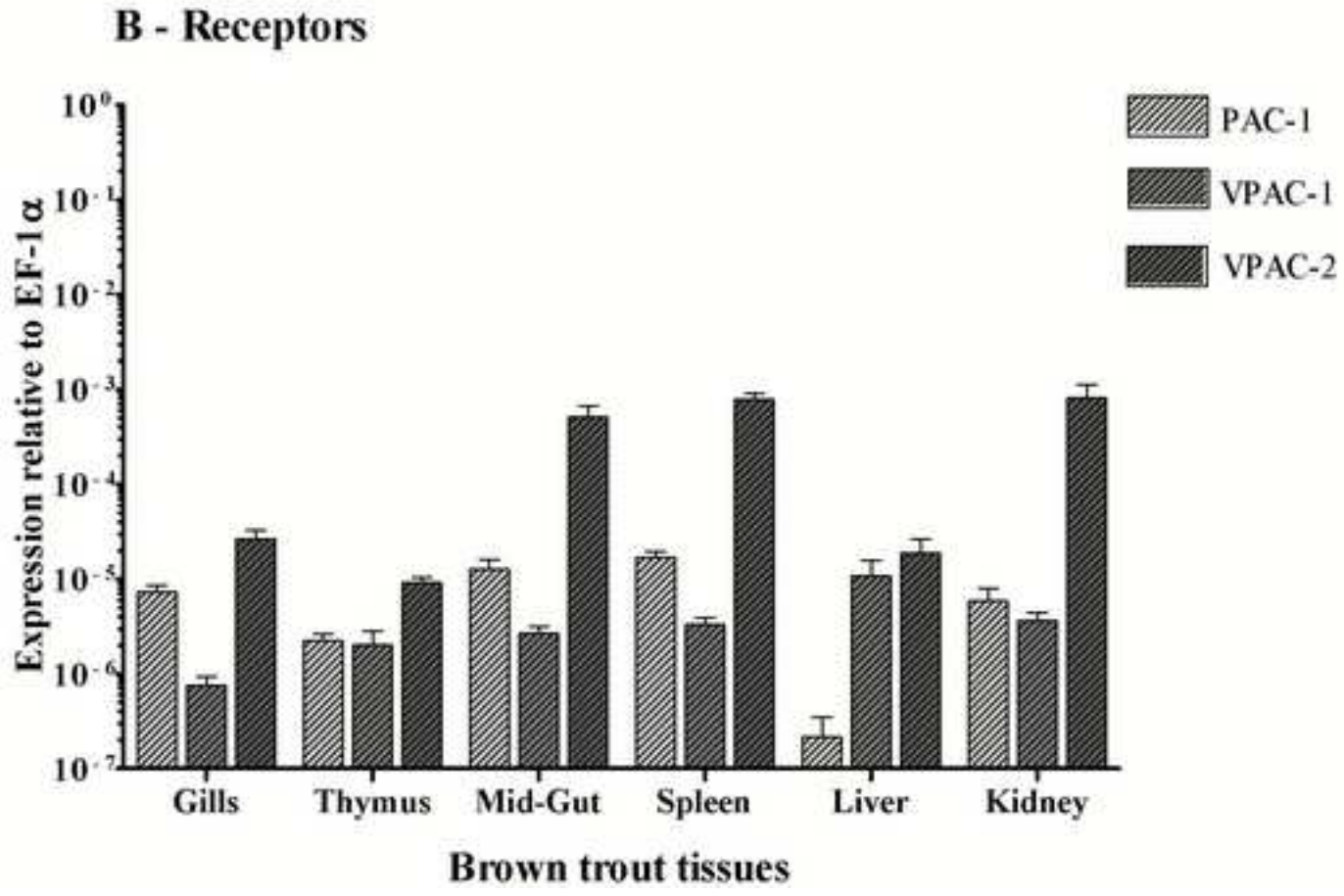

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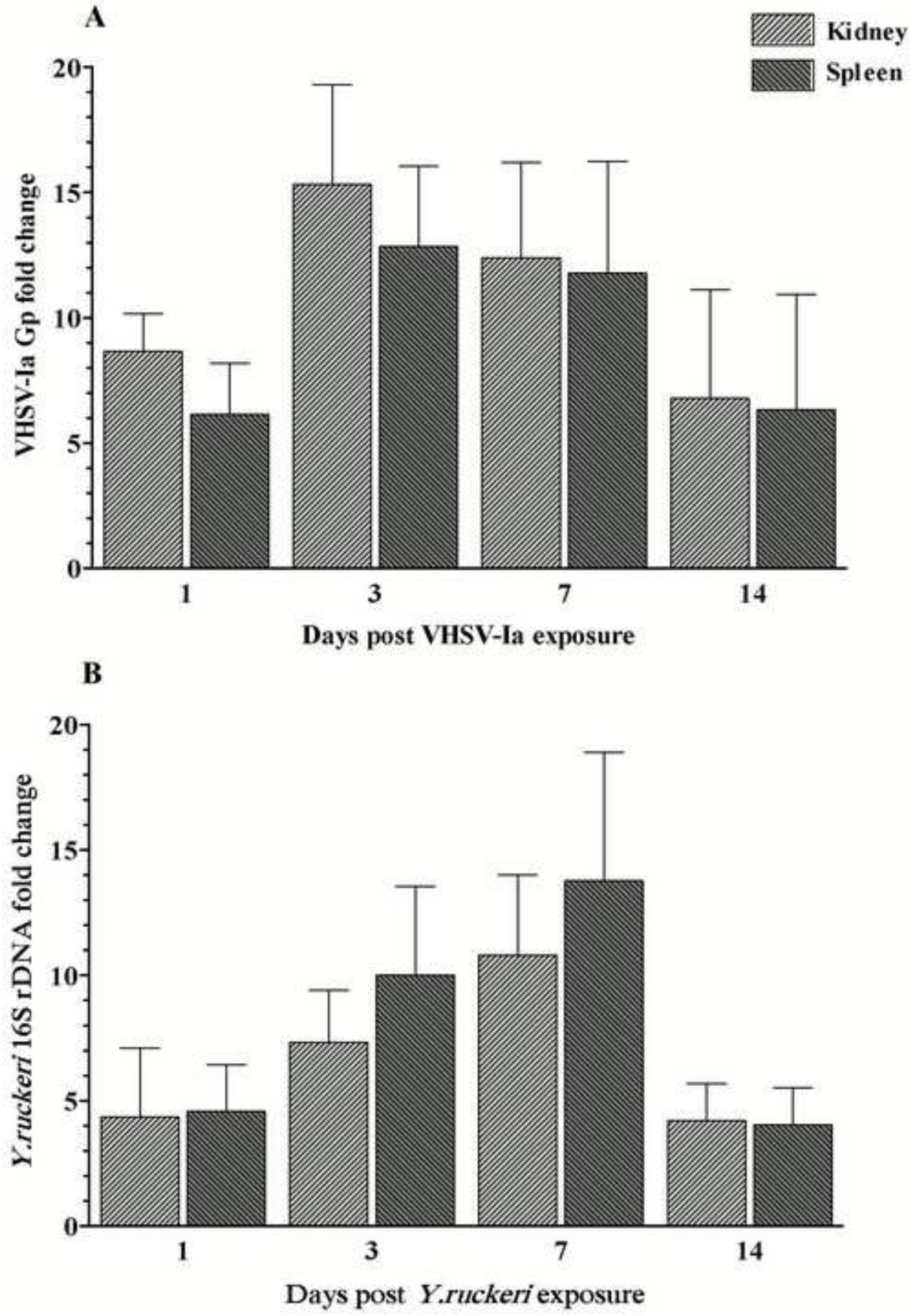

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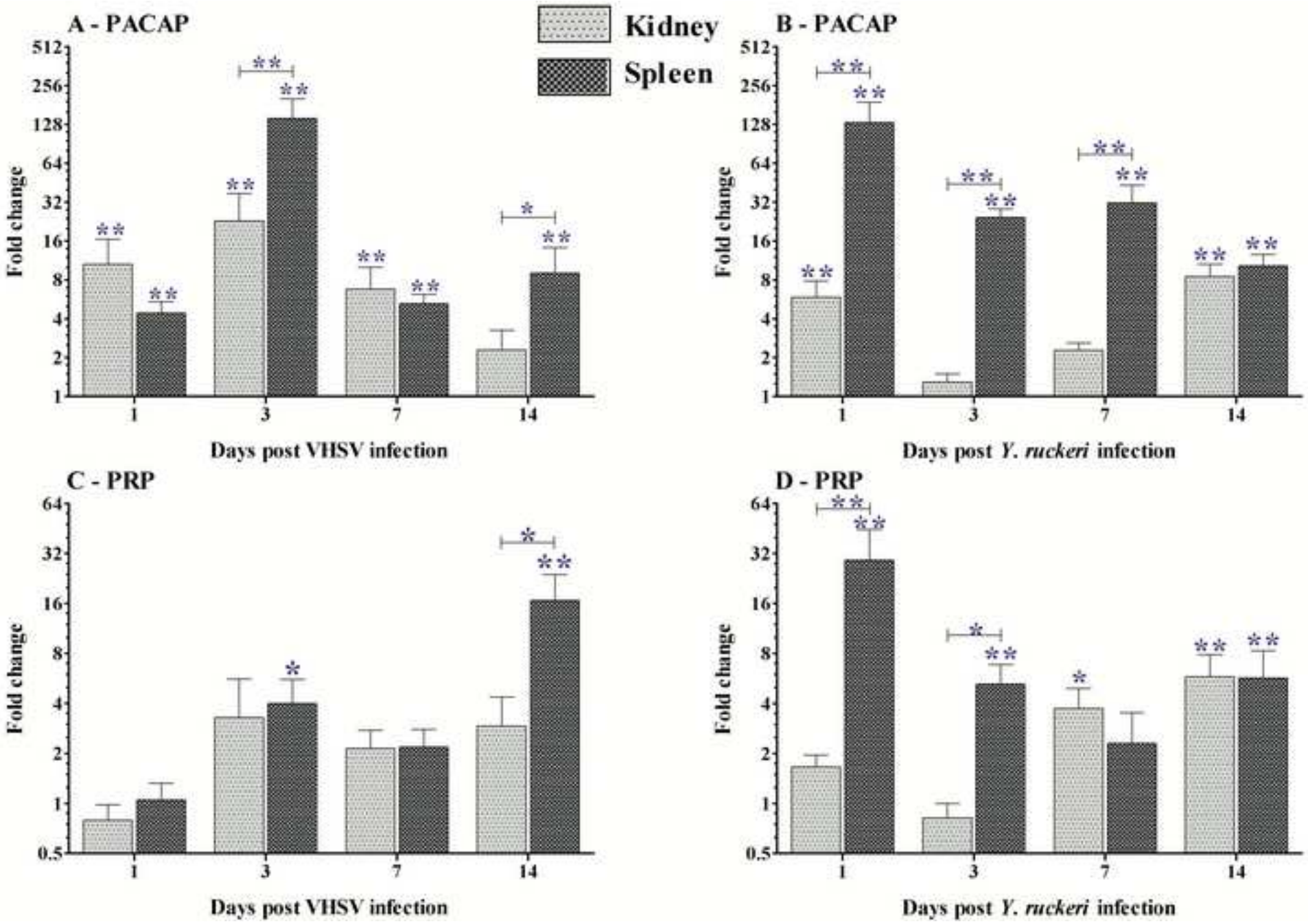

Figure 4

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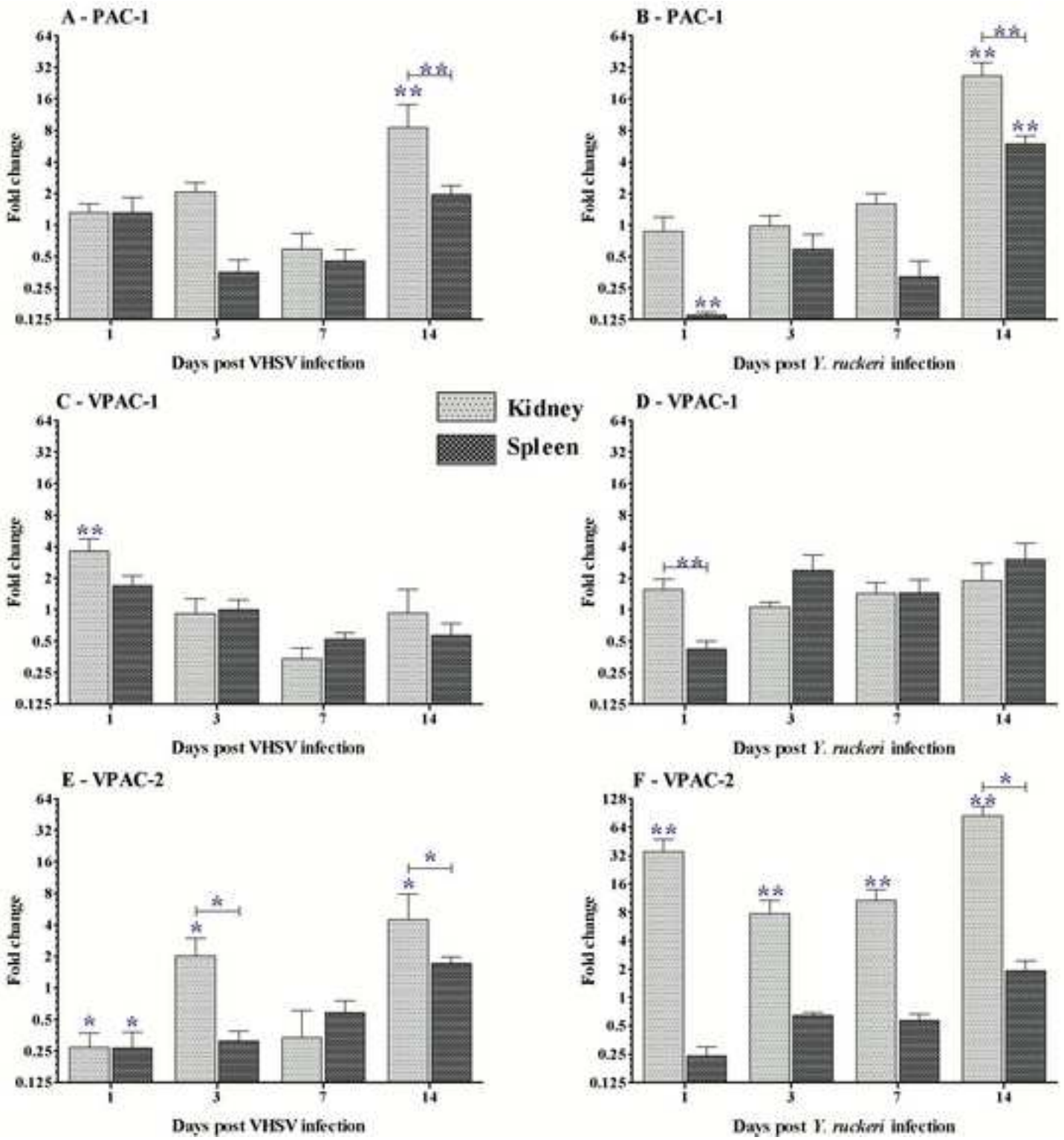

Table 1

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-1α-F	CAAGGATATCCGTCGTGGCA	EF-1α-R	ACAGCGAAACGACCAAGAGG	327	63	30	88
PACAP	HG000281	PACAP-F	GGAGAAAAGTGGAGGGAGCA	PRP/PACAP-R *	TGTCTATACCTTTTCCCAAGGACTG	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
***Y. ruckeri* 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.