

New Evidence for the Role of PACAP as an Antimicrobial Peptide in Teleost Fish

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Supplementary Table S1. Molecular identification of *Yersinia ruckeri* 16S rRNA gene in rainbow trout (*Oncorhynchus mykiss*) by qRT-PCR

Control Group		Infected Group	
Sample	Mean of CT value	Sample	Mean of CT value
Control 3	31.46	Infected 1	13.68
Control 4	32.91	Infected 2	14.24
Control 5	32.87	Infected 3	13.40
Control 6	31.35	Infected 4	14.39
Control 7	33.03	Infected 5	12.30
Control 8	30.60	Infected 6	13.61
Control 9	30.99	Infected 7	13.33
Control 10	33.28	Infected 8	15.47
Control 11	31.69	Infected 9	15.27
Control 12	31.61	Infected 10	13.68
Control 13	32.13	Infected 11	17.52
Control 15	33.66	Infected 12	13.49
Control 16	31.06	Infected 13	13.69
Control 17	32.28	Infected 14	13.39
Control 18	33.32	Infected 15	15.56
Control 19	32.40	Infected 16	14.85
Control 20	31.38	Infected 17	20.57
Control 21	32.58	Infected 18	15.30
Control 22	32.91	Infected 19	15.44
Control 23	32.24	Infected 20	13.17
Control 24	31.33	Infected 21	18.51
Control 25	31.68	Infected 22	17.46
Control 26	34.84	Infected 23	23.98
Control 27	35.13	Infected 24	21.56
Control 34	32.68	Infected 25	16.86
Control 35	30.98	Infected 26	20.55
Control 37	30.72	Infected 27	28.12
		Infected 28	13.15
		Infected 29	15.11
		Infected 30	19.68
		Infected 31	16.87
		Infected 32	21.77
Average	32.26	Average	16.44
SD	1.16	SD	3.70

Supplementary Table S2. Statistical analyzed performed to the direct antimicrobial activity of synthetic *Clarias gariepinus* PACAP-38 against a *Y. ruckeri* isolate by broth microdilution peptide assay in Luria-Bertani broth

Tukey's multiple comparisons test	Summary	Adjusted P Value
0 μM CgPACAP-38		
NaCl 1% vs. NaCl 0.5%	ns	>0.9999
NaCl 1% vs. NaCl 0%	ns	>0.9999
NaCl 0.5% vs. NaCl 0%	ns	>0.9999
2.5 μM CgPACAP-38		
NaCl 1% vs. NaCl 0.5%	*	0.0358
NaCl 1% vs. NaCl 0%	***	0.0007
NaCl 0.5% vs. NaCl 0%	ns	0.1502
12.5 μM CgPACAP-38		
NaCl 1% vs. NaCl 0.5%	**	0.0038
NaCl 1% vs. NaCl 0%	****	<0.0001
NaCl 0.5% vs. NaCl 0%	****	<0.0001
25 μM CgPACAP-38		
NaCl 1% vs. NaCl 0.5%	****	<0.0001
NaCl 1% vs. NaCl 0%	****	<0.0001
NaCl 0.5% vs. NaCl 0%	ns	0.1956
50 μM CgPACAP-38		
NaCl 1% vs. NaCl 0.5%	***	0.0003
NaCl 1% vs. NaCl 0%	***	0.0001
NaCl 0.5% vs. NaCl 0%	ns	0.8931

Supplementary Table S3. Statistical analyzed performed to the direct antimicrobial activity of synthetic human PACAP-38 against a *Y. ruckeri* isolate by broth microdilution peptide assay in Luria-Bertani broth

Tukey's multiple comparisons test	Summary	Adjusted P Value
0 μM hPACAP-38		
NaCl 1% vs. NaCl 0.5%	ns	>0.9999
NaCl 1% vs. NaCl 0%	ns	>0.9999
NaCl 0.5% vs. NaCl 0%	ns	>0.9999
2.5 μM hPACAP-38		
NaCl 1% vs. NaCl 0.5%	ns	0.0569
NaCl 1% vs. NaCl 0%	****	<0.0001
NaCl 0.5% vs. NaCl 0%	****	<0.0001
12.5 μM hPACAP-38		
NaCl 1% vs. NaCl 0.5%	****	<0.0001
NaCl 1% vs. NaCl 0%	****	<0.0001
NaCl 0.5% vs. NaCl 0%	****	<0.0001
25 μM hPACAP-38		
NaCl 1% vs. NaCl 0.5%	****	<0.0001
NaCl 1% vs. NaCl 0%	****	<0.0001
NaCl 0.5% vs. NaCl 0%	****	<0.0001
50 μM hPACAP-38		
NaCl 1% vs. NaCl 0.5%	****	<0.0001
NaCl 1% vs. NaCl 0%	****	<0.0001
NaCl 0.5% vs. NaCl 0%	***	0.0009

Supplementary Table S4. Statistical analyzed performed to the quantification of *Y. ruckeri* by standard plate count (SPC) of cell culture media during live infection (MOI of 0.001) of PACAP-treated RTS11

Uncorrected Fisher's LSD	Summary	Individual P Value
No PACAP vs. hPACAP-38	ns	0.1049
No PACAP vs. HSP70	ns	0.4080
No PACAP vs. CgPACAP-38 0.02 μ M	ns	0.0848
No PACAP vs. CgPACAP-38 0.1 μ M	*	0.0237
hPACAP-38 vs. HSP70	ns	0.3743
hPACAP-38 vs. CgPACAP-38 0.02 μ M	ns	0.8967
hPACAP-38 vs. CgPACAP-38 0.1 μ M	ns	0.2979
HSP70 vs. CgPACAP-38 0.02 μ M	ns	0.3132
HSP70 vs. CgPACAP-38 0.1 μ M	ns	0.0842
CgPACAP-38 0.02 μ M vs. CgPACAP-38 0.1 μ M	ns	0.3502

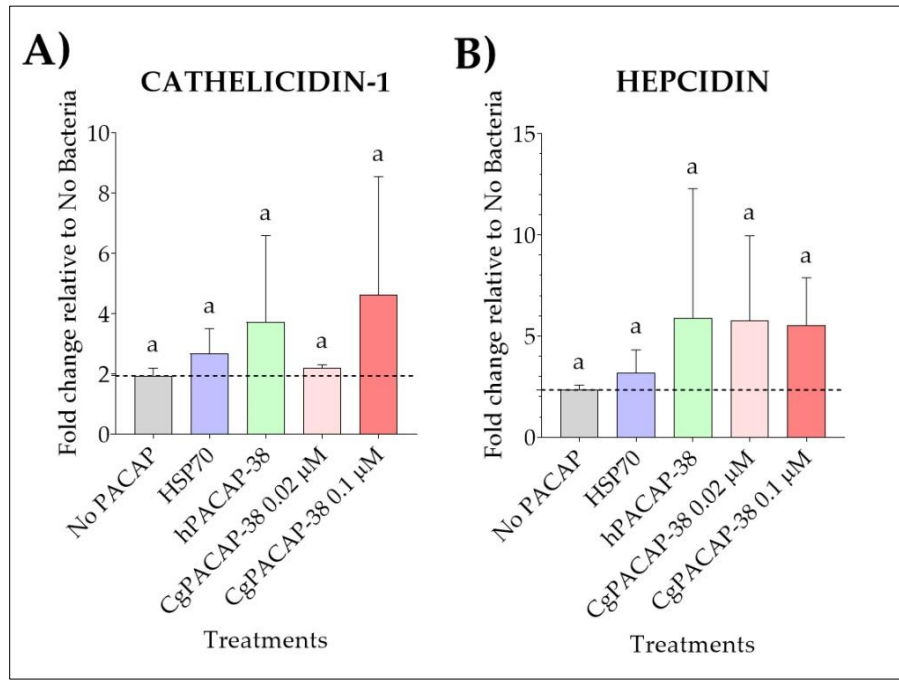
Supplementary Table S5. Statistical analyzed performed to the relative expression levels of pro-inflammatory cytokines in RTS11 cells during live infection with *Y. ruckeri* (MOI of 0.001) after PACAP pre-treatment

Uncorrected Fisher's LSD	Summary	Individual P Value
IL-1β		
No PACAP vs. hPACAP-38	ns	0.3569
No PACAP vs. HSP70	ns	0.6856
No PACAP vs. CgPACAP-38 0.02 μ M	ns	0.8416
No PACAP vs. CgPACAP-38 0.1 μ M	ns	0.3987
hPACAP-38 vs. HSP70	ns	0.1982
hPACAP-38 vs. CgPACAP-38 0.02 μ M	ns	0.2695
hPACAP-38 vs. CgPACAP-38 0.1 μ M	ns	0.1132
HSP70 vs. CgPACAP-38 0.02 μ M	ns	0.8365
HSP70 vs. CgPACAP-38 0.1 μ M	ns	0.6210
CgPACAP-38 0.02 μ M vs. CgPACAP-38 0.1 μ M	ns	0.5004
IL-6		
No PACAP vs. hPACAP-38	ns	0.0952
No PACAP vs. HSP70	ns	0.8464
No PACAP vs. CgPACAP-38 0.02 μ M	ns	0.9911
No PACAP vs. CgPACAP-38 0.1 μ M	ns	0.2870
hPACAP-38 vs. HSP70	ns	0.0691
hPACAP-38 vs. CgPACAP-38 0.02 μ M	ns	0.0935
hPACAP-38 vs. CgPACAP-38 0.1 μ M	*	0.0207
HSP70 vs. CgPACAP-38 0.02 μ M	ns	0.8551
HSP70 vs. CgPACAP-38 0.1 μ M	ns	0.3653
CgPACAP-38 0.02 μ M vs. CgPACAP-38 0.1 μ M	ns	0.2911
TNF-α		
No PACAP vs. hPACAP-38	ns	0.0653
No PACAP vs. HSP70	ns	0.8431
No PACAP vs. CgPACAP-38 0.02 μ M	ns	0.8428
No PACAP vs. CgPACAP-38 0.1 μ M	ns	0.5128
hPACAP-38 vs. HSP70	*	0.0468
hPACAP-38 vs. CgPACAP-38 0.02 μ M	*	0.0468
hPACAP-38 vs. CgPACAP-38 0.1 μ M	*	0.0308
HSP70 vs. CgPACAP-38 0.02 μ M	ns	0.9997
HSP70 vs. CgPACAP-38 0.1 μ M	ns	0.6297
CgPACAP-38 0.02 μ M vs. CgPACAP-38 0.1 μ M	ns	0.6299

IFN-γ		
No PACAP vs. hPACAP-38	ns	0.6968
No PACAP vs. HSP70	ns	0.9985
No PACAP vs. CgPACAP-38 0.02 μ M	ns	0.8738
No PACAP vs. CgPACAP-38 0.1 μ M	*	0.0316
hPACAP-38 vs. HSP70	ns	0.6954
hPACAP-38 vs. CgPACAP-38 0.02 μ M	ns	0.5854
hPACAP-38 vs. CgPACAP-38 0.1 μ M	ns	0.0569
HSP70 vs. CgPACAP-38 0.02 μ M	ns	0.8754
HSP70 vs. CgPACAP-38 0.1 μ M	*	0.0315
CgPACAP-38 0.02 μ M vs. CgPACAP-38 0.1 μ M	*	0.0248

Supplementary Table S6. Statistical analyzed performed to the relative expression levels of anti-inflammatory cytokines and MYD88 signal transduction adaptor gene in RTS11 cells during live infection with *Y. ruckeri* (MOI of 0.001) after PACAP pre-treatment

Uncorrected Fisher's LSD	Summary	Individual P Value
IL-10		
No PACAP vs. hPACAP-38	ns	0.8981
No PACAP vs. HSP70	ns	0.2110
No PACAP vs. CgPACAP-38 0.02 μ M	ns	0.9434
No PACAP vs. CgPACAP-38 0.1 μ M	ns	0.8494
hPACAP-38 vs. HSP70	ns	0.1734
hPACAP-38 vs. CgPACAP-38 0.02 μ M	ns	0.8423
hPACAP-38 vs. CgPACAP-38 0.1 μ M	ns	0.7612
HSP70 vs. CgPACAP-38 0.02 μ M	ns	0.2347
HSP70 vs. CgPACAP-38 0.1 μ M	ns	0.3393
CgPACAP-38 0.02 μ M vs. CgPACAP-38 0.1 μ M	ns	0.8993
TGF-β		
No PACAP vs. hPACAP-38	ns	0.9370
No PACAP vs. HSP70	ns	0.8007
No PACAP vs. CgPACAP-38 0.02 μ M	*	0.0487
No PACAP vs. CgPACAP-38 0.1 μ M	ns	0.6920
hPACAP-38 vs. HSP70	ns	0.8621
hPACAP-38 vs. CgPACAP-38 0.02 μ M	ns	0.0558
hPACAP-38 vs. CgPACAP-38 0.1 μ M	ns	0.7506
HSP70 vs. CgPACAP-38 0.02 μ M	ns	0.0753
HSP70 vs. CgPACAP-38 0.1 μ M	ns	0.8849
CgPACAP-38 0.02 μ M vs. CgPACAP-38 0.1 μ M	ns	0.0962
MYD88		
No PACAP vs. hPACAP-38	ns	0.4056
No PACAP vs. HSP70	ns	0.0664
No PACAP vs. CgPACAP-38 0.02 μ M	**	0.0044
No PACAP vs. CgPACAP-38 0.1 μ M	*	0.0420
hPACAP-38 vs. HSP70	ns	0.2552
hPACAP-38 vs. CgPACAP-38 0.02 μ M	*	0.0177
hPACAP-38 vs. CgPACAP-38 0.1 μ M	ns	0.1466
HSP70 vs. CgPACAP-38 0.02 μ M	ns	0.1267
HSP70 vs. CgPACAP-38 0.1 μ M	ns	0.6279
CgPACAP-38 0.02 μ M vs. CgPACAP-38 0.1 μ M	ns	0.3419



Supplementary Figure S1. Relative expression levels of antimicrobial peptides in RTS11 cells during live infection with *Yersinia ruckeri* (MOI of 0.001) after PACAP pre-treatment. RTS11 cells were pre-treated with CgPACAP-38 (0.02 and 0.1 μM), 0.1 μM of hPACAP-38 or 0.1 μM of HSP70 peptide 24 h before the exposure to live *Y. ruckeri* and the relative expression levels of Cathelicidin-1 (**A**) and Hecpudin (**B**) were analyzed on day 3 post-infection (day 4 after PACAP treatment). Relative expression was determined following the $2^{-\Delta\Delta CT}$ method and EF-1 α was used as the reference gene. Data was expressed as fold change relative to the expression level in the No Bacteria group, and was represented as the mean \pm SD of 3 experimental replicates. Differences among treatments were considered to be significantly different when compared to the No PACAP control (RTS11 exposed to *Y. ruckeri*, dashed line) by a One-way ANOVA followed by a Fisher's least significant difference (LSD) post-hoc test. Similar lowercase letters represents no statistically significant differences at $p < 0.05$.

Supplementary Table S7. Statistical analyzed performed to the relative expression levels of antimicrobial peptides in RTS11 cells during live infection with *Y. ruckeri* (MOI of 0.001) after PACAP pre-treatment

Uncorrected Fisher's LSD	Summary	Individual P Value
Cathelicidin-1		
No PACAP vs. hPACAP-38	ns	0.3471
No PACAP vs. HSP70	ns	0.6916
No PACAP vs. CgPACAP-38 0.02 μM	ns	0.8840
No PACAP vs. CgPACAP-38 0.1 μM	ns	0.1658
hPACAP-38 vs. HSP70	ns	0.5758
hPACAP-38 vs. CgPACAP-38 0.02 μM	ns	0.4221
hPACAP-38 vs. CgPACAP-38 0.1 μM	ns	0.6224
HSP70 vs. CgPACAP-38 0.02 μM	ns	0.8011
HSP70 vs. CgPACAP-38 0.1 μM	ns	0.3028
CgPACAP-38 0.02 μM vs. CgPACAP-38 0.1 μM	ns	0.2083
Hecpudin		
No PACAP vs. hPACAP-38	ns	0.2717
No PACAP vs. HSP70	ns	0.7830
No PACAP vs. CgPACAP-38 0.02 μM	ns	0.2892

No PACAP vs. CgPACAP-38 0.1 μ M	ns	0.3741
hPACAP-38 vs. HSP70	ns	0.3980
hPACAP-38 vs. CgPACAP-38 0.02 μ M	ns	0.9652
hPACAP-38 vs. CgPACAP-38 0.1 μ M	ns	0.9131
HSP70 vs. CgPACAP-38 0.02 μ M	ns	0.4214
HSP70 vs. CgPACAP-38 0.1 μ M	ns	0.5127
CgPACAP-38 0.02 μ M vs. CgPACAP-38 0.1 μ M	ns	0.9442