PACAP system evolution and its role in melanophore function in teleost fish skin

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Running Title: *PACAP system and fish skin pigmentation*

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Highlights

- PACAP receptor genes duplicated and acquired distinct functions during the teleost radiation
- PACAP receptor expression in tilapia skin is modified by a light/dark challenge
- PACAP stimulates pigment aggregation in tilapia skin melanophores
- PACAP activation of Pac_1a increases cAMP but RAMP1 attenuates the increase

Abstract

Pituitary adenylate cyclase-activating polypeptide (PACAP) administered to tilapia melanophores *ex-vivo* causes significant pigment aggregation and this is a newly identified function for this peptide in fish. The G-protein coupled receptors (GPCRs), *adcyap1r1a* (encoding Pac_1a) and *vipr2a* (encoding $Vpac_2a$) are the only receptors in melanophores with appreciable levels of expression and are significantly ($p < 0.05$) down-regulated in the absence of light. $Vpac_2a$ is activated exclusively by peptide histidine isoleucine (PHI), which suggests that Pac_1a mediates the melanin aggregating effect of PACAP on melanophores. Paradoxically activation of Pac_1a with PACAP caused a rise in cAMP, which in fish melanophores is associated with melanin dispersion. We hypothesise that the duplicate *adcyap1ra* and *vipr2a* genes in teleosts have acquired a specific role in skin and that the melanin aggregating effect of PACAP results from the interaction of Pac_1a with Ramp that attenuates cAMP-dependent PKA activity and favours the Ca^{2+}/Calmodulin dependent pathway.

Keywords: gene duplication; family B GPCRs; melanin aggregation; functional divergence; PACAP; RAMPs; skin melanophores; teleost

1. Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) is an important neuropeptide with well-conserved functions in vertebrates. PACAP and its receptors have a widespread tissue distribution and act on the central nervous system (CNS), cardiovascular, gastrointestinal, respiratory, reproductive and immune systems and skin (Halvorson, 2013,Reglodi et al., 2012,Sherwood et al., 2007,Sherwood et al., 2000,Tamas et al., 2012,Vaudry et al., 2009,Vaudry et al., 2000). PACAP is a member of the secretin-like peptide family and shares sequence, structure and functional similarities with vasoactive intestinal peptide (VIP) (Sherwood et al., 2000,Cardoso et al., 2007,Cardoso et al., 2010). In mammals, two mature PACAP peptide isoforms have been described: the predominant form is PACAP-38, and a second form PACAP-27, results from post-translational processing of the longer peptide (Vaudry et al., 2009,Arimura et al., 1991,Cox, 1992). The biological action of PACAP is triggered when it binds to class 2 subfamily B1 (a.k.a secretin-like receptors or class II) G-protein coupled receptors (GPCRs), PAC_1, $VPAC_1$ and $VPAC_2$. PAC_1 is the specific receptor for PACAP but the peptide also activates $VPAC_1$ and $VPAC_2$ and this explains why its functions overlap with VIP. PACAP receptors are typical GPCRs with seven transmembrane domains but, in contrast to other GPCR families in which extracellular loops (ECs) are involved in ligand recognition, its large N-terminal domain (N-ted) is the most important region for peptide binding (Couvineau and Laburthe, 2012).

Splice variants of both PAC_1 and VPAC exist in mammals, although the functions of VPAC variants are still unknown (Dickson and Finlayson, 2009,Poyner and Hay, 2012). PAC_1 splice variants have modified mechanisms of signal transduction and their divergent patterns of expression in the nervous system are indicative of different functions (Dickson and Finlayson, 2009,Blechman and Levkowitz, 2013,Journot et al., 1995,Spengler et al., 1993). PAC_1 and VPAC signalling cascades are triggered by intracellular trimeric G-protein complexes that, when coupled to the receptor C-terminal domain, activate either the adenylate-cyclase (AC) pathway increasing cAMP production or the intracellular calcium (iCa^{2+}) mobilization pathway involving phospholipase C and inositol 1,4,5-triphosphate (IP3) activity (Harmar et al., 2012,Laburthe and Couvineau, 2002,Langer, 2012). Activation of subfamily B1 GPCRs may also involve the assembly of homo- or hetero-receptor dimers and interaction with accessory proteins that regulate receptor function and cellular response (Couvineau and Laburthe, 2012,Ng et al., 2012,Yu et al., 2012). Receptor activity-modifying proteins (RAMPs) are a class of accessory membrane proteins that interact with and modulate the activity of class 2 B1 GPCRs by changing receptor pharmacology (Archbold et al., 2011). RAMPs are single transmembrane proteins with a large extracellular amino terminal domain and a short cytoplasmic domain. In mammals, three related proteins RAMP1, RAMP2 and RAMP3 have been characterized (Sexton et al., 2001) and their association with VPACs has been

demonstrated in mammals. Interaction of RAMP2 with $VPAC_1$ enhances phosphoinositol (PI) but not cAMP and co-transfection of RAMPs with $VPAC_2$ suggests that they do not interact (Archbold et al., 2011,Christopoulos et al., 2003,Couvineau and Laburthe, 2012,Wootten et al., 2013).

Teleost fish possess duplicate PACAP genes, *adcyap1a* (protein; Pacapa) and *adcyap1b* (protein; Pacapb) and six PACAP receptor genes *adcyap1r1a* (protein; Pac_1a) and *b* (protein; Pac_1b), *vipr1a* (protein; $Vpac_1a$) and *b* (protein; $Vpac_1b$) and *vipr2a* (protein; $Vpac_2a$) and *b* (protein; $Vpac_2b$) that are homologues of mammalian *ADCYAP* (protein; PACAP) and PACAP receptor genes *ADCYAP1R* (protein; PAC_1), *VIPR1* (protein; $VPAC_1$) and *VIPR2* (protein; $VPAC_2$). Recent studies of fish Pac_1a and Pac_1b paralogues indicate that their function resembles human PAC_1 but that the duplicate gene copies acquired specialized functions early in the teleost radiation (Cardoso et al., 2007,Roch et al., 2009). Activation of teleost Pac_1, $Vpac_1$ and $Vpac_2$ triggers the same signalling pathways as in mammals, birds and amphibians. It remains to be established if the conservation of the PACAP system in teleosts and mammals extends to their interaction with RAMPs. Although Ramps have been identified in teleosts (Nag et al., 2007,Nag et al., 2012) their association with Pac_1, $Vpac_1$ or $Vpac_2$ signalling has not been explored.

PACAP regulates skin colour in frogs (Tang et al., 2014). PACAP in the CNS modifies amphibian, *Xenopus laevis*, skin melanophore cell activity and stimulates pituitary proopiomelanocortin (POMC) biosynthesis and α-MSH (melanocyte-stimulating hormone) release that activates the melanocortin receptors (MCs), which stimulate a cAMP dependent mechanism (Kidane et al., 2007,Koch and Lutz-Bucher, 1992,Rene et al., 1996). PACAP also has a direct action on amphibian skin and provokes melanin pigment granule dispersal via activation of a specific endogenous $VPAC_2$ receptor (Marotti et al., 1999,Pereira et al., 2002). Human skin, expresses RAMPs, although their association with the ligand preference of class 2 B1 GPCRs is restricted to studies of the calcitonin peptide receptor subfamily, which are involved in keratinocyte cell growth, cutaneous immunity and skin vasodilation (Hasbak et al., 2006,Mikami et al., 2011,Roggenkamp et al., 2013).

In teleosts, body colour change is regulated by a rapid response of the sympathetic system and also by the endocrine system. The α-Msh released from the pituitary and melanocyte-concentrating hormone (Mch) from the hypothalamus have opposing effects on pigment migration in melanophores (reviewed by (Mizusawa et al., 2013). Fish skin also responds to PACAP and juvenile catfish (*Clarias gariepinus*) exposed to water borne PACAP over several weeks became redder (Lugo et al., 2008). In sea bream skin only the gene for *adcyap1r1a* is expressed (Cardoso et al., 2007) although in rainbow trout both *adcyap1r1* and

vipr2 are expressed (Lugo et al., 2011) but their role in fish skin physiology remains elusive. The presence of Ramps in fish skin is also largely unstudied.

The function of PACAP in pigmentation in fish was evaluated by studying its role in melanophore regulation. Furthermore, the hypothesis that the divergent expression of the two *adcyap1r1* transcripts in fish skin is an example of their functional specialization after duplication was tested. In analogy with the situation in mammalian skin, the presence of Ramps in fish skin and their involvement in Pac_1 receptor signalling and physiology was assessed in the melanophores.

2. Material and methods

2.1 Peptides

All peptides were purchased from Sigma-Aldrich (Spain) and were of human (h) origin with the exception of peptide histidine isoleucine (PHI) that was from rat (r). Human secretin (SCT) peptide was purchased from Bachem (Germany). The mammalian PACAP, VIP and PHI peptides are identical, or share high sequence identity with the predicted fish homologues (Supplementary Figure 1 A, B and C) and interact with, and stimulate the fish PACAP receptors (Cardoso et al., 2007,Kwok et al., 2006,Wong et al., 1998)).

2.2 Animal maintenance

Animal maintenance and manipulation was in accordance with Portuguese legislation and covered by a "Group-1" license obtained from the Direção-Geral de Veterinária, Ministério da Agricultura, do Desenvolvimento Rural e das Pescas, Portugal. Juvenile immature tilapia (*Oreochromis mossambicus*) of 9 ± 1 g body weight/ 7 ± 2 cm body length (a stock bred and reared in the PRODEP Marine Experimental Station, University of Algarve) were kept in a fresh water closed circuit system (approximately 60 litres) at 26 ºC $\pm$ 1ºC under a constant photoperiod (12 light (L): 12 dark (D)) and fed *ad libitum* once a day with commercial cichlid food (Nutrafin basix®; Rolf C. Hagen, Inc, Canada). Food was withheld for 24 hours before sampling.

2.3 Skin *ex-vivo* pigment motility assays

For *ex-vivo* skin melanophore assays, fish (n = 6) under normal photoperiod were netted, wrapped in a damp cloth and scales (harbouring melanocytes) were plucked from below the dorsal fin using fine tweezers and washed with tilapia saline solution (TSS) (140 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM $NaHCO_3$, 2 mM NaH_2PO_4, 5.5 mM glucose, pH = 7.8) (McCormick et al., 1992) and maintained on ice prior to peptide incubations. *Ex-vivo* peptide assays were performed in *eppendorf* tubes and tilapia scales with skin were incubated with PACAP-27 (1 μM), hPACAP-38 (1 μM), hVIP (1 μM), rPHI (1 μM) and hSCT (1 μM)

158 diluted in 1 x TSS, for 60 min at RT. After peptide stimulation, scales were mounted on clean
159 glass slides (75 x 25 mm; Industrial Quality, Germany) using a press-seal hybridization
160 chamber (19 x 32 x 0.15 mm; Sigma-Aldrich, Spain) filled with the peptide solution and
161 changes in melanin pigment translocation were observed by phase-contrast microscopy
162 (Olympus BH-2, UK). Control experiments were carried out in parallel with 1 x TSS without
163 peptides.
164 Skin melanophore assays were also performed in the presence of drugs known to
165 interfere with signalling pathways that regulate pigment motility in melanophores (Thaler and
166 Haimo, 1992). These included IBMX (2.5 mM, 3-Isobutyl-1-methylxanthine, Sigma-Aldrich,
167 Spain) a potent non-specific inhibitor of cAMP phosphodiesterases that convert cAMP to 5'-
168 AMP, (Beavo et al., 1970) or W7 (100 μM N-(6-aminohexyl)-5-chloro-1-
169 naphthalenesulfonamidean, Sigma-Aldrich, Spain) an inhibitor of Ca^{2+}/Calmodulin (CaM)-
170 activated phosphodiesterase (Hidaka et al., 1981). Tilapia skin was incubated for 10 min with
171 the drugs diluted in 1 x TSS and subsequently challenged with 1 μM PACAP-27 in the presence
172 of the drugs for 60 min at RT. As a control of the effect of the drug treatment on pigment
173 transport, epinephrine, a potent melanin aggregator (1μM for 5 minutes, Sigma-Aldrich, Spain),
174 was used (Clark et al., 1987,Rozdzial and Haimo, 1986) in the presence or absence of IBMX
175 (2.5 mM) or W7 (Thaler and Haimo, 1992,Thaler and Haimo, 1990). To confirm the
176 responsiveness of skin melanophores (pigment dispersion) to an increase in intracellular cAMP
177 levels, tilapia skin was incubated for 10 min at RT with forskolin (10 μM), an activator of
178 adenylyl cyclase (Seamon et al., 1981).
179 Skin melanophore assays were performed with scale obtained from 6 different fish in 4
180 independent assays each using 2-4 scales/treatment. Bias in the analysis of melanin aggregation
181 state was removed by carrying out blinded trials. The total number of scales assayed and the
182 total melanophores analysed for each experiment is described in Table 1. Digital images of
183 scales were captured using a Leica DFC 480 camera (Germany) and Leica IM50 Image Master
184 Software and assembled using Adobe Photoshop. Image J software was used to quantify
185 individual melanophore response to the peptide treatments in the presence and absence of the
186 drugs. Melanin translocation (aggregation or dispersion) response was scored using the
187 melanophore index (MI) (Hogben and Slome, 1931). All the assays were performed using fish
188 skin with > 95% MI5 melanophores and in the dark to eliminate the effect of the light that
189 favours melanin pigment aggregation.
190
191 **2.4 Fish skin assays**
192 To induce morphological changes in skin pigmentation (designated hereafter as
193 darkening skin), tilapia maintained in the conditions indicated above (section 2.2) were exposed
194 for 8 days to total darkness (n = 5, 0:24 LD) or maintained under a standard photoperiod (n = 5,

12:12 LD) and the skin from experimental and control fish was collected for RNA isolation and cDNA synthesis. Sampling of dark challenged fish skin was performed under red light. Approximately 1 cm^2 of skin was removed from below the dorsal fin and snap frozen in liquid nitrogen.

2.5 Tilapia melanophore enriched skin cell cultures

Scales from the dorsal fin region were collected and melanophore enriched cultures were derived from the skin attached to the scale (12-15 scales) from different fishes. The skin was isolated and finely sliced and the scale-skin fragments were digested with 400 µl of a 0.25 % trypsin/EDTA solution for 90 min at 37 ºC with agitation. The released cells were added to 1600 ml of L-15 medium (Sigma Aldrich, Spain) supplemented with 10 % sterile foetal bovine serum and 0.1 % penicillin: streptomycin antibiotic mixture (10.000 U:10 mg/ml, Sigma Aldrich, Spain) and 250 µg/ml (1:100) sterile filtered amphotericin B solution (Sigma-Aldrich, Spain) in a 6 well plate. Melanophores were identified by their dark colour in phase-contrast microscopy and were transferred with the aid of a micropipette to a new well of a 6 well-plate to generate melanophore-enriched cultures. Other cell types that were transferred with melanophores were subsequently removed by medium aspiration. Melanophore-enriched cultures containing approximately 40 to 50 melanophores were snap frozen and stored at -80 ºC for total RNA extraction and cDNA synthesis.

2.6 RNA isolation and cDNA synthesis

Tilapia cDNA was used to amplify receptors, peptide precursors and *ramps*. The cDNA was synthesized from total RNA (tRNA) extracts of several tissues to confirm *in silico* gene predictions and characterize the tissue distribution. For tissue collection, fish were anaesthetized with 2-phenoxyethanol (1 $ml.l^{-1}$ water) and killed by decapitation. Skin and other tissues were collected and immediately frozen in liquid nitrogen and stored at -80 ºC. Total RNA from tilapia tissues was isolated using Tri-Reagent (Sigma Aldrich, Spain) and treated with 1 U DNase (DNA-free Kit, Ambion, UK) for 30 min at 37 °C in accordance with the manufacturer's instructions. DNase treated total RNA (500 ng) was denatured at 65 °C for 5 min, quenched on ice for 5 min and used for cDNA synthesis in a 20 µl reaction volume containing 10 ng of pd(N)6 random hexamers (GE Healthcare, UK), 2 mM dNTPs, 100 U of MMLVRT and 20 U RNasin® Plus RNase inhibitor (Promega, Spain). cDNA was synthesized for 10 min at 20 °C followed by 60 min at 42 °C and 72 °C for 5 min. The quality and quantity of the cDNA was assessed by PCR amplification of ribosomal subunit *18s* rRNA (*18s*) using the following cycle: 94 ºC for 3 min; 25 cycles of 94 ºC for 35 sec, 57 ºC for 30 sec, 72 ºC for 45 sec, followed by 72 ºC for 5 min.

2.7 Database mining

Adcyap1, *vip* and *adcyap1r, vipr1* and *vipr2* genes were identified and retrieved from the cichlid, Nile tilapia (*Oreochromis niloticus*) genome ENSEMBL annotation (http://www.ensembl.org/Oreochromis_niloticus/Info/Index) (July 2014 release) using the tblastn algorithm. Sequence queries were performed with the deduced mature peptides of *Takifugu rubripes* Pacapa (ABG73207), Pacapb (ABG73208), Vip (DQ659330) and the zebrafish peptides (Vipa, ABV83048 and Vipb, NP_001108027). Tilapia receptors were retrieved using the *Takifugu* PACAP receptor sequence homologues: Pac_1a (Q5WML1); Pac_1b (Q5WML0); $Vpac_1a$ (CAC82588); $Vpac_1b$ (CAC82587); $Vpac_2a$ (CAC83861); $Vpac_2b$ (CAC82587). Searches for PACAP and PACAP receptors genes and transcripts were extended to other fish genomes (12 genome assemblies, November 2014) available in ENSEMBL (http://www.ensembl.org), the recently published European sea bass (*Dicentrarchus labrax*) genome (Tine et al., 2014), the cartilaginous fish, the elephant shark (*Callorhinchus milii*) genome (http://esharkgenome.imcb.a-star.edu.sg/blast/), the Japanese lamprey (*Lethenteron japonicum*) genome (http://jlampreygenome.imcb.a-star.edu.sg) and an "in house" sea bream (*Sparus auratus*) gene transcript nucleotide database. The available genome of the Mexican tetra (*Astyanax mexicanus*) in ENSEMBL corresponds to its caveform and is designated hereafter as cavefish. The tilapia genome and other fish databases were also used to search for homologues of human RAMP1 (NP_005846.1), RAMP2 (NP_005845.2) and RAMP3 (NP_005847.1). The sequences of *Takifugu obscurus ramp1a* (BAE45310.1), *ramp1b* (BAE45306.1), *ramp2tv1* (BAE45308.1), *ramp2tv2* (BAE45311.1), *ramp3* (BAE45305.1) and *ramp5* (BAE45307.1) gene transcripts were used to interrogate the databases. The Nile tilapia (*O. niloticus*) sequences were aligned with the other fish and tetrapod homologues and used to design primer pairs for isolation of *adcyap1r*, *vipr1*, *vipr2*, *adcyap1*, *vip* and *ramp* transcripts from Mozambique tilapia (*O. mossambicus*).

2.8 *In silico* comparisons, phylogeny and gene synteny analysis

Multiple sequence alignments were generated using the Clustal X (version 2) software programme (Larkin et al., 2007,Thompson et al., 1997) and the GeneDoc software (http://iubio.bio.indiana.edu/) was used to annotate the alignment and to calculate percentage of sequence identity/similarity of the deduced amino acid sequences. The PAC/VPAC receptor sequence alignment was submitted to ProtTest (2.4) to select the best model that characterizes receptor protein evolution according to the Akaike Information Criterion (AIC) statistical model (Abascal et al., 2005). The Jones-Taylor-Thornton (JTT) (Jones et al., 1992) matrix based model was chosen and phylogenetic analysis was performed with 88 vertebrate receptor sequences that included the tilapia receptors, the orthologues from other fish, *Xenopus* and human. Phylogenetic trees were built using the Maximum Likelihood (ML) method in PhyML

3.0 implemented in ATGC (http://www.atgc-montpellier.fr/phyml/) (Guindon et al., 2010) and using the Neighbor-Joining (NJ) (Saitou and Nei, 1987) method in Mega 5.2 (Tamura et al., 2011) with 100 and 1000 bootstrap replicates, respectively (Felsenstein, 1985). ML trees were searched using the Nearest-Neighbor-Interchange (NNI) heuristic method and constructed with 4-substitution rate categories gamma shape (0.941). The vertebrate SCT receptor sequences (Cardoso et al., 2014) were also included in the analysis and both trees were rooted with the STCR clade. The NJ and ML methods generated trees with similar topologies.

A similar strategy to that outlined above was used with the teleost Ramps. Phylogenetic tree was performed with the Jones-Taylor-Thornton (JTT) matrix selected after ProtTest (2.4). Analysis included 69 sequences and the ML tree was constructed with 4-substitution rate categories gamma shape (1.116) and proportion of invariable sites (0.019) with 100 bootstrap replicates. The ML and NJ trees generated similar tree topologies and a hypothetical root was added between the RAMP1/3 and RAMP2/5 clades based upon the model proposed for RAMP family evolution (Benítez-Páez and Cárdenas-Brito, 2008).

The gene environment of the teleost *adcyap1r1*, *vipr2*, *adcyap1* and *vip* was characterized and the neighbouring gene homologues were identified based upon the ENSEMBL database gene annotation and complemented with sequence homology searches.

2.9 Quantitative PCR (q-PCR)

Receptor and peptide transcript abundance in tilapia skin (with no scales) and melanophore enriched cultures was quantified using quantitative real-time PCR (q-PCR). Primer sequences and annealing temperatures are described in Supplementary Table 1. Four reference genes *β-actin*, glyceraldehyde-3-phosphate dehydrogenase (*gapdh1*), *18s*, and melanocortin 1 receptor (*mc1r,* a.k.a. melanocyte-stimulating hormone receptor) were used to calculate relative expression units of the tilapia transcripts. Transcript absolute values were normalized for differences in reverse transcription efficiencies against the geometric mean of the reference genes. The geometric mean of *β-actin* and *18s* was used to normalize the results for tissue distribution of PACAP receptor transcripts. For normalization of PACAP receptor transcripts in skin collected from fish under a light/dark challenge the geometric mean of tilapia *β-actin* and *gapdh1* was used. Gene expression analysis on the melanophore cell cultures was normalized using the *mc1r* (a marker of melanophores) (Higdon et al., 2013,Kobayashi et al., 2012,Selz et al., 2007). Reference genes were selected taking into consideration their stable expression level in the tissues analysed. Q-PCR analysis was performed in duplicate (< 5% variation between replicates) using an Icycler iQ™Real-Time PCR Detection System (Bio-Rad, Portugal) and SsoFast EvaGreen supermix (Bio-Rad, Portugal) and 300 nM of the forward and reverse primer in 96-well micro plates (Bio-Rad, Portugal). Optimized cycling conditions

consisted of 95 ºC for 30 s, followed by 45 cycles of 95 ºC for 5 s and 58 - 62 ºC for 10 s. Melting curves were performed to detect nonspecific products and primer dimers. Standard curves were prepared from M13 amplified PCR products of each transcript cloned in pGEMT-easy (Promega, Spain). Control reactions were included in all runs to confirm the absence of genomic DNA.

2.10 Cloning and recombinant vector construction

A sea bream (*Sparus auratus*) Pac_1a construct was available in house (Cardoso et al., 2007) and was used to infer the activity of the tilapia receptor homologue as they shared over 90% aa sequence identity (Supplementary Figure 2). The complete coding sequence of tilapia *vipr2a* was amplified from brain cDNA using specific primers and proofreading DNA polymerase (iProof, BioRad, Portugal, Supplementary Table 1). The PCR thermocycle consisted of: 98 ºC for 30 sec, followed by 45 cycles (98 ºC 10 sec, 64 ºC for 30 sec, 72 ºC for 2 min) with a final extension of 72 ºC for 10 min. The PCR product was cloned into the pcDNA3.1 vector (Directional TOPO Expression Kit, Invitrogen, USA) and used to transform TOP10 competent bacteria. Positive bacterial colonies were screened by PCR using receptor specific primers. Plasmid DNA was isolated from those giving a PCR product of the expected size using the standard alkaline lysis method and was sequenced to confirm identity. Human RAMP1 (conceded by Dr Vanessa Schein, UFRGS, Brazil) was amplified with proofreading DNA polymerase (iProof, BioRad, Portugal) using specific primers (Supplementary Table 1) and cloned into pcDNA3.1.

2.11 Mammalian cell cultures and transfections

HEK293 cells (ECACC collection, UK) were maintained in complete Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, Spain) with 4.5 g/L glucose, 110 mg/L sodium pyruvate and L-glutamine supplemented with 10 % sterile foetal bovine serum and 0.1 % penicillin: streptomycin antibiotic mix (10.000 U:10 mg/ml, Sigma) and 250 µg/ml sterile filtered 1:100 amphotericin B solution (Sigma, Spain) in a humid 5 % CO_2 incubator (Heraeus, Portugal) at 37 °C. One day prior to transfection, 2–3 x 10^5 cells were seeded into 6 well plates (Sarstedt, Portugal) and cells were transiently transfected using Fugene 6 transfection reagent (1 : 6 DNA : Fugene, Roche, Germany) following the manufacturer's protocol. Simultaneous transient transfections of a vector expressing green fluorescent protein were performed to estimate the efficiency and success of cell transfections. The capacity of the PACAP peptides PACAP-27 and hPACAP-38 to activate the teleost receptor cAMP-signalling pathway was assayed 72 hours after cell transfections. HEK293 stable cell lines expressing human RAMP1 were generated to assess the effect of transmembrane accessory proteins on the pharmacology of PACAP receptor function.

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343 **2.12 cAMP and Ca^{2+} intracellular signalling**

344 The capacity of PACAP-27 and hPACAP-38 peptides to stimulate intracellular cAMP
345 production by binding Pac_1a and $Vpac_2a$ and RAMP1-Pac_1a complex in the transfected cells was
346 measured using a cAMP dynamic 2 kit (Cisbio, France) following the manufacturer's protocol.
347 Approximately 15,000 cells were assayed per well and incubations were performed in white 384
348 well small Volume™ HiBase Polystyrene microplates (Greiner, Germany). Prior to the assay,
349 cells were ressuspended in 1 x PBS with 1 mM of 3-isobutyl-1-methylxantine (IBMX, Sigma)
350 and incubated for 5 min at 37 ºC. Peptides (1 µM - 0.01 nM) diluted in 1 x PBS / 1 mM IBMX
351 were added to the cells for 30 min at 37 ºC. cAMP production was monitored using a Biotek
352 Synergy 4 plate reader (Biotek, USA). Data was normalized following the manufacturer's
353 recommendations.

354 Intracellular Ca^{2+} (iCa^{2+}) release (RFU) was also measured for Pac_1a in the presence of
355 PACAP peptides using the Ca^{2+} sensitive fluorescent dye Fluo-4 NW (Molecular Probes,
356 Invitrogen). Prior to the assay, plates (96 well black/plates, µClear bottom, Greiner, Germany)
357 were coated with sterile poly-L-lysine (0.1 mg/ml, Sigma, Spain). Assays were performed with
358 approximately 50,000 cells according to manufacture's instructions. Background RFU of
359 transfected cells was measured prior to stimulation and dose-response curves were determined
360 for PACAP-27 and hPACAP-38 (1 µM to 0.01 nM). Calcium mobilization stimulated by the
361 peptide was measured every 10 sec over a total period of 2 min using a Biotek Synergy 4 plate
362 reader (Biotek, USA).

363

364 **2.13 Statistical analysis**

365 Significant changes in melanin response to the peptides in *ex-vivo* assays were assessed
366 using a two-tailed Student t-test. Q-PCR expression data are presented as mean ± SEM.
367 Significant differences in the abundance of receptor distribution in tissue was assessed with a
368 One-way Anova and Tukey's multiple comparison test. Significant difference in receptor
369 expression in the skin of light/dark challenged tilapia was determined using a two-tailed Student
370 t-test. Receptor activation assays are presented as the mean ± SEM of three independent
371 experiments carried out in triplicate and significant differences in cAMP or iCa^{2+} was assessed
372 using a two-tailed Student t-test. All the analyses were performed using Prism GraphPad
373 version 5. Statistical significance was considered at $p < 0.05$, $p < 0.01$ and $p < 0.001$.

374

375 **3. Results**

376 **3.1 Effect of PACAP and related peptides on melanin mobilization in the skin**

377 Incubations of tilapia skin with PACAP and VIP and also with peptide histidine
378 isoleucine (PHI) and secretin (SCT), which are all members of the secretin-like peptide family,

indicated that PACAP was the most potent inducer of melanin aggregation in melanophores (Figure 1A, Table 1). The aa sequence of hPACAP-27 is 100% identical with most of the fish homologues (PACAP-27a, Supplementary Figure 1 A) and hereafter the peptide is designated PACAP-27.

PACAP-27 or hPACAP-38 peptides added to fish skin had a similar effect on pigment aggregation and 45.1 ± 11.2 % ($p < 0.05$) and 40.3 ± 7.1 % ($p < 0.05$), respectively, of the total melanophores were fully aggregated (MI 1-2) compared to 20.0 ± 4.2 % MI 1-2 in the control. The melanin aggregating effect of hVIP (1 μM) and rPHI (1 μM) were not significantly different from the control (30.5 ± 6.3 % and 26.4 ± 7.6 %, respectively) (Figure 1A). Secretin (SCT), which is absent from teleost genomes (Cardoso et al., 2014), did not modify the state of melanin aggregation (Table 1). The results suggest that PACAP regulated pigment translocation in fish skin melanophores and stimulated melanin aggregation. Forskolin (10 μM, a stimulator of cAMP production) promoted melanin pigment dispersion in tilapia skin melanophores (data not shown, Logan et al., 2006,Sheets et al., 2007). The results, suggest that the action of PACAP-27 and hPACAP-38 on tilapia skin pigment aggregation was unlikely to be triggered by a rise in the intracellular levels of cAMP.

The signalling pathways involved in the melanin aggregating effect of PACAP were assessed using IBMX (2.5 mM) and W7 (100 μM) (Table 1, Figure 1B). IBMX (a non-specific inhibitor of cAMP phosphodiesterases) caused dispersion of melanin in 78.5 ± 2.7% of skin melanophores (MI5) compared to 51.5 ± 5.3 % in the control (Figure 1B, Table 1). Incubation of skin with W7 (an inhibitor of Ca^{2+}/CaM-activated phosphodiesterase) did not change the pigment translocation pattern in relation to the control (Table 1, Figure 1B). Incubation of skin melanophores with PACAP-27 (1 μM) and IBMX significantly ($p < 0.05$) decreased the melanin aggregating effect of the peptide (42.4 ± 11.2% to 15.0 ± 2.3). Incubation of skin with PACAP-27 and W7 also caused a significant ($p < 0.05$) reduction in the number of aggregated melanophores (42.4 ± 11.2% to 15.0 ± 1.9). Epinephrine (1μM) had a significant ($p < 0.01$) melanin aggregating effect (89.9 ± 2.5% of melanophores) compared to the control. IBMX (2.5 mM) blocked the effect of epinephrine (1μM) and melanin aggregation occurred in only 8.8 ± 2.0 % of the melanophores. W7 did not affect the melanin aggregating effect of epinephrine (1μM) (Table 1).

3.2 PACAP system and RAMPs

Two *adcyap1* (*a* and *b*), two *vip* (*a* and *b*), six Pacap receptors (*adcyap1r1a* and *b*, *vipr1a* and *b* and *vipr2a* and b) genes and five *ramp* genes were identified in the Nile tilapia (*O. niloticus*) genome and amplified in the Mozambique tilapia (*O. mossambicus*). The deduced mature peptide sequence of the genes and transcripts in both species of tilapia were 100%

identical. A similar gene number was also retrieved from other teleost genomes (Supplementary Table 2, 3 and 4).

3.2.1 PACAP and VIP in fish

In tilapia and other teleosts, duplicates of the human *ADCYAP1* and *VIP* genes were identified (Supplementary Table 2, Supplementary Figure 3, (Cardoso et al., 2007, Ng et al., 2012). The fish *adcyap1* genes (*adcyap1a* and *b*) produced paralogue Pacap-27 and Pacap-38 peptides (a and b) and in most fishes (including tilapia and other teleosts, elephant shark and coelacanth) Pacap-27a was 100% identical to human and *Xenopus* PACAP-27 and was the most conserved peptide of this family in vertebrates (Supplementary Figure 1A). The exceptions were cod, cavefish and zebrafish in which the predicted paralogue Pacap-27b was most similar to human PACAP-27a. Teleost Pacap-27b only differed from the tetrapod homologues (96% aa identical) at a single aa residue but in the cavefish it was 100% identical (Supplementary Figure 1A). The teleost duplicate Pacap-38 was also highly conserved and Pacap-38a and Pacap-38b differed at 3 and 4 aa residues and shared 92% and 89% identity, respectively with the human peptides. The cod, cavefish and zebrafish Pacap-38 was less conserved.

In tilapia, cavefish and zebrafish duplicate *vip* genes (*vipa* and *b*) were identified but in other fishes only a single gene copy existed (Supplementary Table 2). In teleosts the predicted mature peptides of Vip shared less conservation than Pacap with the human homologue (Supplementary Figure 1B). The predicted tilapia Vipa and Vipb peptides shared 81% aa identity with human VIP and the other teleosts shared between 77% to 85% identity with the exception of the Amazon molly that shared only 59% identity. In the early ray-finned fish the spotted gar, the cartilaginous elephant shark and the coelacanth (a lobe-finned fish) a single *adcyap1* and *vip1* gene was retrieved. The genome regions of the spotted gar and duplicate *adcyap1* and vip1 genes in teleost shared high synteny with the homologue genome region in human (Supplementary Figure 3) indicating that duplication of the peptide genes was teleost specific (Supplementary Figure 3). *Adcyap1* and *vip* genes were retrieved from the Japanese lamprey genome and the deduced mature peptides shared 85% and 66% aa identity with the human homologue (Supplementary Figure 1A and B).

3.2.2 PACAP receptor genes in fish

The deduced tilapia Pac_1a and b mature protein sequence shared 92 % and 89 % aa sequence similarity with the *Takifugu* homologues (Table 2). The alternatively spliced-exon in the fish Pac_1a hop isoform (*adcyap1a-hop*) was also amplified from tilapia and caused an 82 bp insertion in the C-terminal intracellular domain (Cardoso et al., 2007,Kwok et al., 2006,Fradinger et al., 2005).

The teleost analysed also contained duplicate *adcyap1r1*, *vipr1* and *vipr2* genes and phylogenetic analysis suggested that receptor gene duplication was a consequence of the teleost genome tetraploidization event (Figure 2). The exception was the cavefish that lacked the gene homologue for *adcyap1r1a* and the zebrafish that lacked the *vipr2b* gene (Supplementary Table 3, Figure 2). The absence of *adcyap1r1b* and *vipr2b* genes in the cod was presumably a consequence of the incomplete genome assembly. The genome region flanking *adcyap1r1a* was conserved in several fishes (Supplementary Figure 4) and mapped to short contigs in the cavefish genome. The genes, *c2cd4c*, *chico*, *ccdc94* and *shda* in linkage with zebrafish *adcyap1r1a* mapped to KB882255.1 in cavefish but *adcyap1r1a* was not identified and further studies will be required to confirm if specific gene loss occurred in the cavefish lineage.

The conserved gene environment that flanked the teleost paralogue *vipr2b* gene (*esyt2a*, *pflkpb* and *ptpnr2*) was distributed on chr 2 and chr 7 in the zebrafish genome suggesting that this gene may have been lost (Supplementary Figure 4). Single copies of *adcyap1r1*, *vipr1* and *vipr2* genes were retrieved from the spotted gar and with the exception of *vipr2* were also identified in the coelacanth. In the Japanese lamprey genome two putative receptor genes for PACAP and VIP have been described (Cardoso et al., 2014,Ng et al., 2012) and in the cartilaginous elephant shark genome, putative *vipr1* and *vipr2* genes were identified but the *adcyap1r1* gene was missing (Supplementary Table 3).

3.2.3 RAMP genes in fish

The tilapia and other teleost genomes contained five predicted gene homologues of human and *T. obscurus* RAMPs (Supplementary Figure 5, Supplementary Table 4). The deduced mature protein sequence of tilapia Ramp1 and Ramp4 were 52% identical and shared 42% aa identity with human RAMP1. Phylogenetic analysis of piscine *ramp1* and *ramp4* indicated that they were duplicate genes in the teleost lineage and we have designated them *ramp1a* and *ramp1b*, respectively. *Ramp* genes were also identified in other fish genomes such as the cartilaginous elephant shark, the spotted gar and the coelacanth but were not found in the Japanese lamprey genome assembly (Supplementary Table 4). In the elephant shark orthologues of vertebrate RAMP1, RAMP3 and RAMP2/RAMP5 were identified and in the spotted gar a single gene of RAMP2 and RAMP5 was identified. Homologues of the fish *ramp5* genes were not found in human or *Xenopus* genomes and the fish members tended to group with the RAMP2 clade. Overall the results support the notion that RAMP2/RAMP5 shared a common origin and that both genes emerged during an early gene duplication event in the vertebrate lineage (Benítez-Páez and Cárdenas-Brito, 2008) and *ramp5* gene was subsequently deleted in tetrapods. In the coelacanth, five putative *ramp* genes with sequences divergent from vertebrate

homologues were identified; it was unclear if this was a species-specific evolutionary event or a problem with the genome assembly.

3.3 Expression of the PACAP system and RAMPs in tilapia

Q-PCR analysis revealed that the six predicted PACAP receptors and *adcyap1r1a-hop* had a widespread tissue distribution in tilapia (Figure 3). PACAP receptor transcripts were low abundance with the exception of *adcyap1r1a*, which was highly abundant in brain and *vipr1a* that had a moderate expression in all the tissues analysed (Figure 3). In tilapia *adcyap1r1a* and *adcyap1r1a-hop* ($p < 0.001$) and the paralogue *adcyap1r1b* ($p < 0.05$) transcripts were significantly more abundant in brain relative to other tissues. The duplicate *vipr1* and *vipr2* transcripts were present in brain, kidney, duodenum, gills, liver and white muscle. *Vipr1b* transcripts were significantly ($p < 0.001$) higher in the kidney compared to the gills and brain. *Vipr2b* transcripts were significantly ($p < 0.05$) higher in brain relative to all other tissues (Figure 3).

In the skin PACAP receptor transcript abundance was low and changed in normal and darkening tilapia skins (Figure 4). In normal tilapia skin, transcripts for the paralogues *vipr1a*, *vipr1b* and *vipr2a* were more abundant relative to other receptors and *vipr2b* was low abundance/undetectable and was excluded from further analysis. *Adyap1r1a* and *vipr2a* were significantly down-regulated ($p < 0.05$) in darkening skin compared to normal skin. In contrast, *vipr1b* transcripts were significantly up-regulated ($p < 0.05$) in darkening skin suggesting that its function in skin diverged from that of *adcyap1r1a* and *vipr2a* (Figure 4). *Adcyap1r1a-hop*, *adyap1r1b* and *vipr1a* transcript abundance did not change in response to a light/dark challenge and were also excluded from further analysis. Of the duplicate PACAP and VIP peptide encoding transcripts, *adcyap1a* and *vip* (*a* and *b*) were low abundance/undetectable in skin and only *adcyap1b* was detected at quantifiable levels (Figure 4). Most of the *ramps*, with the exception of *ramp1b*, showed low abundance in skin and *ramp5* was undetectable. No significant differences in *ramp* expression between normal and darkening skins were observed (Figure 5). In melanophore enriched tilapia cell cultures (Figure 6), *adcyap1r1a* was relatively more abundant than *vipr1b* and *vipr2a* (Figure 6A). *Ramp1b* was the most abundant transcript in melanophores (Figure 6B).

3.4 Intracellular signalling

Activation by PACAP of sbPac1a and tiVipr$_2$a expressed in HEK293 cells was characterised (Figure 7). PACAP peptides were tested because; i) they have the most potent effects on pigment aggregation in tilapia melanophores *in vitro* and ii) *adcyap1b* transcripts were detected in fish skin. Both PACAP-27 and hPACAP-38 promoted a dose-dependent increase in intracellular levels of cAMP when they were added to human HEK293 cells

transiently transfected with fish sbPac$_1$a (Figure 7A). PACAP-27 and hPACAP-38 were equipotent when they activate sbPac$_1$a and had EC_{50} values of 1.422 e^{-8} M and 1.457 e^{-8} M, respectively. PACAP-27 and hPACAP-38 binding to sbPac$_1$a also stimulated an increase in intracellular Ca^{2+} and had an EC_{50} respectively of 1.243 e^{-8} M and 4.175 e^{-8} M (Figure 7B). TiVipr2a transiently transfected in HEK293 cells was not stimulated by PACAP (Figure 7A) and was only activated by PHI (data not shown and (Wu et al., 2008).

The association between a rise in cAMP and melanin dispersion in tilapia melanophores and the melanin aggregating effect of PACAP suggested that fish Pac$_1$a, may use an alternative signalling mechanism that leads to a decrease in intracellular cAMP levels. To test this hypothesis the effect of PACAP on HEK293 cells co-transfected with sbPac$_1$a and RAMP1 from human was evaluated by measuring the change in cAMP. PACAP-27 and hPACAP-38 stimulated cAMP production in a HEK293 cell line co-expressing sbPac$_1$a and hRAMP1 but at 10-fold lower levels than in a HEK293 cell line expressing sbPac$_1$a alone (Figure 7A). The results indicated that PACAP mediated activation of sbPac$_1$a in the presence of hRAMP1 modified cAMP pathway signalling.

4. Discussion

Hormones and neurotransmitters regulate the function of pigment containing cells in skin and eye and GPCRs are major signal transducers in this process. PACAP is an important vertebrate neuroendocrine factor that activates GPCRs and regulates pigment dispersion in amphibians via an increase in cAMP. In teleost fish, gene duplicates are suggested to have contributed to their exuberant skin colour and complex pigment patterns (Braasch et al., 2008,Braasch et al., 2009). The present study investigated if genes duplicates of the PACAP system in fish are involved in skin colour change and revealed PACAP as a new player in the endocrine regulation of fish pigment mobilization. PACAP receptors are differentially expressed in skin, receptor abundance is responsive to light exposure and PACAP-27 significantly stimulates melanin pigment aggregation *ex-vivo*. However, the activation of PACAP receptors by PACAP-27 causes a rise in intracellular cAMP, which is normally associated with skin melanin pigment dispersal. We hypothesize that the physiological pigment translocation response caused by PACAP-27 most likely involves fish Pac$_1$a with RAMP in skin, which attenuates or inhibits the rise in intracellular cAMP associated with activation of Pac$_1$a alone. Furthermore, the reduction of cAMP-dependent PKA activity favours a $Ca^{2+/}$CaM dependent phosphatase that promotes melanin aggregation.

4.1 Evolution of PACAP and its receptors in fish

Teleosts are the most diverse and successful group of vertebrates and more than 23,000 species have been identified (Venkatesh, 2003). Their genomes duplicated early in evolution

and the presence of gene duplicates in fish has been associated with the physiological plasticity of this group of vertebrates (Braasch et al., 2009,Glasauer and Neuhauss, 2014). In teleost genomes, duplicates of class 2 B1 GPCRs and ligands have been extensively studied (Sherwood et al., 2000,Cardoso et al., 2007,Cardoso et al., 2010,Roch et al., 2009,Nag et al., 2012,Cardoso et al., 2014,Wu et al., 2008,Cardoso et al., 2005,Cardoso et al., 2004,Martins et al., 2014,Cardoso et al., 2014,Hwang et al., 2013) but their functional role in fish physiology remains unclear. PACAP and PACAP receptors emerged early in the evolution of vertebrates and members of the gene family are present in representatives of early vertebrate genomes, such as the lamprey, and both peptide and receptor genes duplicated in the ancestral teleost and persisted during the fish radiation (Sherwood et al., 2000,Cardoso et al., 2007,Cardoso et al., 2014,Ng et al., 2012,Wu et al., 2008,Cardoso et al., 2004). *Adcyap1r1* genes were only identified in bony vertebrates and two PACAP peptides and two receptors genes were present in most of the teleost genomes analysed. In fish with genomes that did not undergo tetraploidization, such as the spotted gar, a representative of the ray-finned fish that preceded the teleost radiation, and the coelacanth, basal lobe-finned fish, a single receptor gene copy was found. Within the teleosts, the exceptions were cod and the cavefish that, in common with other teleosts, underwent genome tetraploidization (Glasauer and Neuhauss, 2014) and retained duplicate *adcyap1* but only a single *adcyap1r1* was identified and in the cavefish no *adcyap1r1*a was found (Figure 8). The cavefish lacks melanin pigmented cells and eyes (McCauley et al., 2004,Protas et al., 2006,McGaugh et al., 2014) and this intriguing observation seems to support the link between PACAP receptors and pigmentation. However, the incomplete nature of the cavefish genome assembly makes the loss of *adcyap1r1a* during evolution uncertain and further studies are required to confirm that they lack duplicate PACAP receptors.

4.2 PACAP regulates melanophore pigment motility in fish skin

In tetrapods, PACAP is a central endocrine factor that regulates the secretion of pituitary hormones involved in skin melanophore cell activity. Studies in amphibians have demonstrated that PACAP is a key factor regulating vertebrate skin colour and that this action has been conserved in tetrapods (Marotti et al., 1999,Kidane et al., 2008). In tilapia, the two PACAP peptides and six receptor genes identified in this study are the duplicates of the human and amphibian homologues and the present study indicates that in common with tetrapods, elements of the piscine PACAP system are expressed in fish skin and involved in melanin pigment translocation. Duplicates that are homologues of human *ADCYAP1R1* and *VIPR* are expressed in fish skin but only *adcyap1r1a*, *vipr1b* and *vipr2a* have modified expression in darkening skin and *adcyap1r1a* is the most abundant receptor transcript in piscine melanophore enriched skin cultures. The results suggest that *adcyap1r1a*, *vipr1b* and *vipr2a* acquired a specific function in fish skin and that the former receptors may regulate the status of melanin

pigment in tilapia. It remains to be established if their functions are conserved across teleosts as homologue receptors were identified in all jawless, cartilaginous, ray-finned and lobe-finned fish genomes explored and further experiments directed at confirming this hypothesis are required.

PACAP has a direct action on frog skin and provokes pigment granule dispersal in *X. laevis* melanophores via activation of an endogenous $VPAC_2$ (Marotti et al., 1999,Pereira et al., 2002,Yamaguchi et al., 2007). The effect of PACAP on melanophore granule status in frog (melanin dispersion) is opposite to that observed in tilapia (melanin aggregation) and highlights that gene sequence homology and apparent similarity of physiological systems across evolution does not guarantee conserved function. The divergent functions of vertebrate PAC_1 may result from differences in the mechanisms that govern pigment translocation in fish and frog melanophores. Fish skin possesses larger and faster-responding melanophores than frog skin and the cellular microtubule motors that translocate pigment work differently (Zaliapin et al., 2005). For example, in fish pigment movements are assisted predominantly by kinesin 1, while in frog kinesin 2 has this role. Further work will be required to establish at the cellular level how Pac_1a activation in fish melanophores is coupled to pigment translocation.

The functional divergence observed between PAC_1 in frog and fish may be related to the remarkable percentage of duplicate genes that have been retained in the genome of fish most likely as a consequence of acquisition of new functions (neofunctionalization) or partitioning of the ancestral molecule function between the duplicated gene copies (subfunctionalization) (Sato and Nishida, 2010,Taylor et al., 2003,Postlethwait et al., 2004). The PACAP endocrine system in fish is duplicated and receptor transcript expression in sea bream was taken to suggest that the persistence of duplicate *adcyap1r1a* (widespread) and *adcyap1r1b* (brain specific) genes is a consequence of their distinct function in fish (Cardoso et al., 2007). In tilapia, *adcyap1r1a* and *adcyap1r1b* are both brain-specific but the former receptor is approximately 40 times more abundant than *adcyap1r1b*, supporting the notion first observed in sea bream that the paralogues may have acquired distinct functions. The different expression pattern of the duplicate PACAP receptors in tilapia skin after a light/dark challenge, the abundance of *adcyap1r1a* in enriched melanophore cultures, and the role of PACAP, the ligand, on melanin aggregation *ex-vivo* suggest that Pac_1a has acquired a specific role in fish skin pigmentation.

4.3 PACAP signalling in fish skin

Rapid changes in pigment motility can occur in amphibian and teleost cells. In amphibians and fish an increase in intracellular cAMP induces pigment dispersion and a decrease promotes pigment aggregation (Logan et al., 2006,Busca and Ballotti, 2000,Nery and Castrucci, 1997,Tuma and Gelfand, 1999). In contrast, the role of the iCa^{2+}-dependent pathway

633 in melanophores is controversial, this pathway is not directly involved in melanin translocation
634 and a species-dependent skin pigmentation response occurs in fish (Tuma and Gelfand,
635 1999,Sammak et al., 1992,Svensson et al., 1997). Perturbations in cAMP levels interfere with
636 microtubule (MT) (dynein and kinesin) and actin filament (AF) (myosin V) proteins that govern
637 the movement of melanin pigment. A decline in cAMP increases the frequency of minus-end
638 motility episodes that are associated with aggregation (Tuma and Gelfand, 1999,Rodionov et
639 al., 2003). In tilapia melanophores melanin pigment aggregation by epinephrine, nor-
640 epinephrine and Mch depends on the activity of PKA and of a protein phosphatase (eg. PP2B or
641 calcineurin) (Thaler and Haimo, 1992,Thaler and Haimo, 1990,Oshima et al., 2001,Oshima and
642 Wannitikul, 1996).
643 Functional characterisation of fish Pac_1a revealed that both PACAP-27 and hPACAP-38
644 peptides are equipotent in stimulating cAMP production (Cardoso et al., 2007,Wong et al.,
645 1998) and that the presence of RAMP1 attenuates the production of intracellular cAMP. Since
646 high cAMP is associated with pigment dispersion this suggests that the melanophore response
647 observed in tilapia skin in the present study (melanin aggregation) is a result of the change in
648 receptor pharmacology (Figure 9 A). We hypothesise that the combination of attenuated levels
649 of cAMP and the probable increase in iCa^{2+} leads to stimulation of CaM and protein
650 phosphatases (PP) that promote melanin aggregation (Figure 9 B). The similar functional
651 characteristics of the PACAP receptor from goldfish, zebrafish and sea bream despite the
652 differences in their amino acid sequence suggests this may be a common mechanism in teleosts
653 (Cardoso et al., 2007,Kwok et al., 2006,Wong et al., 1998,Fradinger et al., 2005,Wu et al.,
654 2008). It also supports the notion that the characteristics of the sea bream Pac_1a tested *in vitro*
655 will be similar for the tilapia orthologue and so can be related to the *in vivo* response in tilapia.
656 In vertebrates, MSH and MCH peptides are classical endocrine factors that regulate skin
657 pigmentation. In tilapia (and other fish), background adaptation does not modify plasma or
658 pituitary α-Msh levels or Mc1r expression and Msh/Mc1r seem to play a minor role in the
659 regulation of skin pigmentation (van der Salm et al., 2005). In contrast, an increase in plasma
660 Mch levels and up-regulation of the peptide in the hypothalamus is observed when fish are
661 adapted to a light (white) background (Mizusawa et al., 2013,Kishida et al., 1989,Suzuki et al.,
662 1995). In fish skin, rapid changes in colour also involve the neurotransmitter norepinephrine
663 (NE) released by the sympathetic system and this stimulates pigment aggregation (Mizusawa et
664 al., 2013). Our study suggests that PACAP is a regulatory factor of fish skin and the presence of
665 PACAP transcripts in skin and the action of PACAP-27 on melanin status in melanophores
666 suggests that the PACAP system may also participate in the local and rapid response of skin
667 colour change (Figure 9 A). If the PACAP system interacts with Msh/Mch to modulate skin
668 pigmentation in fish still remains to be established. In tetrapods, PACAP modulates central

669 MSH secretion but in fish this remains to be demonstrated, as does the potential effect of
670 PACAP on Mch release (Tanaka et al., 2009).
671 PACAP, MSH and MCH peptides and also NE activate GPCRs and regulate
672 intracellular levels of cAMP coupled to distinct G-protein complexes. In fish skin, Msh
673 produced in the adenohypophysis or locally, binds to Mch-r1 that is associated with the
674 intracellular Gs-protein complex and increases melanophore cAMP production (Oshima et al.,
675 2001). Fish Mch is mainly secreted by the neurohypophysis and activates Mch-r2 that is
676 coupled to the Gi-protein complex that inactivates adenylate-cyclase and signals via the
677 inhibition of cellular cAMP levels (Hamamoto et al., 2011,Kawauchi, 2006). However, Mch
678 actions in fish are not conserved and tilapia and catfish skin contain another receptor type, Mch-
679 r1, that signals via cAMP and promotes and stimulates melanin dispersion suggesting that
680 regulation of skin colour pattern in fish may be species dependent (Oshima et al., 2001). In the
681 studies of duplicate PACAP receptors in fish they mainly signal via Gs (cAMP release) and Gq
682 (calcium release) (Cardoso et al., 2007,Kwok et al., 2006,Wong et al., 1998,Fradinger et al.,
683 2005,Wu et al., 2008), although it is possible that other intracellular signalling pathways are
684 activated in parallel particularly if accessory proteins are involved. For example, the association
685 of human $VPAC_1$ with S-SCAM (synaptic scaffolding molecule) inhibits cAMP production via
686 Gi-coupling and favours receptor internalization (Gee et al., 2009).
687 The interaction of RAMPs with class 2 B1 GPCRs was first demonstrated for the
688 calcitonin receptors and was associated with promiscuity of ligand-binding and increased
689 signalling diversity and physiological responses (Couvineau and Laburthe, 2012). RAMPs have
690 also been described to interact with the mammalian glucagon, parathyroid and VPAC receptors,
691 although no changes in the cAMP intracellular signalling response have been reported
692 (Archbold et al., 2011). To date the effect of RAMP on fish Pac_1a pharmacology has not been
693 explored and to our knowledge the present study represents the first evidence that they may
694 modify the receptors intracellular signalling response as previously reported for the calcitonin
695 receptors. It was not possible to establish if in the present study the attenuated cAMP response
696 associated with PACAP stimulation of cells co-transfected with Pac_1a and RAMP *in vitro* also
697 occurs *in vivo* particularly since an heterologous expression system was used (fish receptor,
698 mammalian RAMP and mammalian cell line). Despite differences between fish and human
699 RAMPs (only 43% identical) the results from the cell transfection studies suggest that RAMP
700 interacts with the fish Pac1a and reduces the potency of PACAP to increase cAMP. We
701 hypothesise that functional conservation between the vertebrate RAMP systems exists and it is
702 possible that the homologue system may be more effective at modifying receptor signalling
703 potentially via the recruitment of other intracellular signalling molecules. Future studies with a
704 homologous system should contribute to resolve how the interaction of Pac_1a and RAMP
705 modifies the response to PACAP and will contribute to more precisely establish how this affects

intracellular signalling and causes melanin aggregation in melanophores. RAMPs and PACAP receptors emerged early in the vertebrate radiation (Figure 8) and their involvement in skin pigmentation may be ancient and their interaction may contribute to skin colour diversity. A future challenge will be to establish if the interaction of RAMPs and PACAP receptors also contribute to the diversity of PACAPs physiological roles in vertebrates.

In conclusion, we provide evidence that PACAP is involved in the regulation of melanophore function in a teleost fish. In teleosts, duplicate PACAP receptors exist and we suggest that Pac_1a but not Pac_1b mediate this effect in tilapia skin melanophores. The results of the study indicate that during the teleost radiation Pac_1a acquired a specific role in the local control of fish melanin translocation causing pigment aggregation in skin melanophores. This contrasts to amphibians, where PACAP by activating its receptor stimulates an increase in cAMP and induces pigment dispersion. The reason for the different action of PACAP in fish may be the result of the interaction of Pac_1a with an accessory protein of the RAMP family that attenuates/blocks the rise in intracellular cAMP that normally occurs when PACAP activates its receptor. Overall it appears that functional divergence occurred after PACAP receptor duplication in the teleost lineage and this probably contributed to their persistence in the genome. The interaction between the fish PACAP system and the classical Mch and NE pathways involved in pigmentation was not established and will be the target of future studies.

5. Acknowledgments

The authors thank Dr Vanessa Schein (UFRGS, Brazil) for providing the human RAMP1 vector clone. JCRC and JF are supported by an auxiliary research contract FCT Pluriannual funds attributed to CCMAR. RCF (SFRH/BPD/89811/2012), RSM (SFRH/BPD/66742/2009) and VF (SFRH/BPD/80447/2011) are funded by FCT. This work was supported by the European Regional Development Fund (ERDF) COMPETE - Operational Competitiveness Programme and Portuguese funds through FCT – Foundation for Science and Technology, under the project "PEst-C/MAR/LA0015/2013".

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Tables:

Table 1: Melanophore index (MI, %) in tilapia skin after incubation with the PACAP (PACAP-27 and 38), VIP, PHI and SCT peptides and of PACAP-27 with IBMX or W7. The number of scales and individual melanophores counted are indicated and the melanophore index (MI) was classified as aggregated (MI 1-2), partially dispersed (MI 3-4) and dispersed (MI 5) according to (Hogben and Slome, 1931). Skin was incubated with 1 μM of PACAP-27, hPACAP-38, hVIP and rPHI and also with IBMX (2.5 mM) or W7 (100 μM) in the presence or absence of PACAP-27 (1 μM). The results represent the average of four independent experiments (scored blind) by two independent observers and are expressed as the proportion of each melanophore stage with variability given as SEM. Control assays were carried out in the presence of 1 x TSS alone. Epinephrine (1 μM) was used to test the efficacy of melanophore response with IBMX and W7. The hSCT (1 μM) skin assays were performed independent of the other assays and has its own control. The hPACAP-27 is 100% identical in aa sequence with most of the fish orthologues (Pacap-27a, Supplementary Figure 1 A). h-human; r-rat.

Table 2: Percentages of amino acid sequence identity / similarity of the tilapia PACAP receptors with the human, zebrafish, *Takifugu* and sea bream orthologues. Fish accession numbers are provided in Supplementary Table 3. Human receptors are PAC_1 (NP_001186566), $VPAC_1$ (NP_004615) and $VPAC_2$ (NP_003373). * incomplete EST (lacks TM7).

Figures

Figure 1: Response of tilapia skin melanophores to PACAP-27, hPACAP-38, hVIP and rPHI peptides (A) and to PACAP-27 in the presence of IBMX or W7 (B). Results represent the average percentage scores of aggregated (MI 1 and 2) and dispersed (MI 5) pigment in skin melanophores after 60 min of peptide treatment. Tilapia skin melanophores and TSS alone were used as the control. In A) tilapia skin was incubated with 1 μM of PACAP-27, hPACAP-38, hVIP and rPHI peptides diluted in 1 x TSS. In B) the effect of IBMX (2.5 mM) and W7 (100 μM) on PACAP-27 (1 μM) aggregation was tested. Values represent mean ± SEM of 4 experiments each using 2-4 scales. Significant differences were detected using an unpaired two-tailed Student t-test; significant differences in aggregation in relation to PACAP-27 are denoted by a ($p < 0.05$) and b ($p < 0.001$). To facilitate interpretation MI3 and MI4 melanophores (available in Table 1) were not included. h-human, r-rat.

Figure 2: Phylogenetic tree of the tilapia and other fish Pacap receptors. Consensus tree was constructed using the Maximum Likelihood method and a JTT matrix with 100 bootstrap replicates. The tree was rooted with the vertebrate (human, *Xenopus* and fish) SCTR branch (Cardoso et al., 2014) and bootstrap support nodes for the main vertebrate clades are indicated. The tree was constructed using the deduced mature protein sequences (from TM1 to TM7) of the receptors listed in Supplementary Table 3. The two Japanese lamprey receptors were obtained from (Cardoso et al., 2014,Ng et al., 2012). The deduced coelacanth Pac_1 and $Vpac_1$, sea bream $Vpac_2$ and cavefish $Vpac_2b$ were not used, as their sequences were very incomplete. Accession numbers were: human PAC_1 (NP_001186566), $VPAC_1$ (NP_004615), $VPAC_2$ (NP_003373) and *Xenopus* PAC_1 (NP_001072799), $VPAC_1$ (XP_002941910.2) and $VPAC_2$ (NP_001120616).

Figure 3: Tissue distribution of the *adcyap1r1*, *vipr1* and *vipr2* transcripts in tilapia. Receptor expression levels are normalized using the geometric mean of two tilapia reference genes (*18s* and *β-actin*). Expression of *adcyap1r1a* and *adcyap1r1b* was high abundance in the brain but low abundance/undetectable in the other tissues analysed and the results are not presented. Data corresponds to the mean ± SEM of tissues (n = 4). One-way Anova and Tukey's multiple comparison test was used to assess differences in receptor transcript expression between tissues. Bars with different letters are significantly different ($p < 0.001$ and $p < 0.05$).

Figure 4: Expression of the receptors and peptides of the tilapia Pacap system in normal and darkening skin. Transcript expression was obtained by q-PCR and normalized using the geometric mean of the tilapia reference genes *β-actin* and *gapdh1*. Data is presented as the mean

± SEM (n = 5 biological replicates) and statistical significance is considered at $p < 0.05$ (*, two-tailed unpaired Student's t-test). The relative expression levels of *adcyap1a*, *vipa*, *vipb* and *vipr2b* are not presented, as they were low abundance/undetectable in the skin.

Figure 5: Expression of *ramps* in normal and darkening tilapia skin by q-PCR. Transcript expression was obtained by q-PCR and normalized using the geometric mean of the reference genes (tilapia *β-actin* and tilapia *gapdh1*). Data is presented as the mean ± SEM (n = 5 biological replicates). The results for *ramp 5* are not presented, as it was low abundance/undetectable in skin.

Figure 6: Detection of Pacap receptors (A) and Ramp (B) transcripts in tilapia melanophores. Transcript expression determined by q-PCR in enriched melanophore skin cell cultures is normalized using the mean of *mc1r* expression (melanophore pigment gene). Data is presented as the mean ± SEM for the fish Pacap receptors (n = 9) and *ramps* (n = 5). Each cDNA was synthesised from cultures containing approximately 40-50 melanophores. *Adcyap1r1a-hop*, *adcyap1r1b* and *vipr1a* are not represented, as they were undetectable in tilapia melanophore enriched cultures.

Figure 7: Profile of PACAP peptides on the activation of Pac_1a in the production of cAMP (A) and mobilization of iCa^{2+} release (B). (A) PACAP stimulated cAMP production in cells transfected with teleost $sbPac_1a$ and cells co-transfected with $sbPac_1a$ and hRAMP1, but not $tiVpac_2a$. Receptors were stimulated with 1 μM to 0.01 nM of the PACAP-27 and hPACAP-38 peptides: $sbPac_1a$ (◆), $sbPac_1a$ + hRAMP1 (◇), $tiVpac_2a$ (▲). To facilitate visualization $sbPac_1a$ assays (alone or with hRAMP1) are represented by solid lines and $tiVpac_2a$ with dashed lines. Values represent the mean ± SEM of at least three independent experiments performed in triplicate. (B) PACAP promoted iCa^{2+} release. Receptors were stimulated with 1 μM to 0.01 nM of the PACAP-27 (●) and hPACAP-38 (▲) peptides. RFU (relative florescent units) values represent mean ± SEM of at least three independent experiments performed in triplicate.

Figure 8: Gene duplications and losses of the *adcyap1r1 and viprs*, *adcyap1and vip* and *ramp* during the chordate radiation. *Adcyap1r1, vipr1, vipr2* genes are represented by circles, *adcyap1* and *vip* genes by pentagons and *ramps* genes by squares. Crossed symbols represent putative gene loss. The major events associated with gene family evolution are indicated. In lamprey, duplicate PACAP/VIP receptors (*i* and *ii*) and single *vip* and *adcyap1* genes exist and they are the ancestral of the gnathostome PACAP/VIP system (Ng et al., 2012). In *Ciona intestinalis* putative *gcgr/vipr* genes have been described (represented by an open circle,

Cardoso et al., 2006) but the gene orthologue of the related specie the tunicate *Chelyosoma productum adcyap1* transcripts (represented by a dashed pentagon, McRory and Sherwood, 1997) remains to be found (Ng et al., 2012,Cardoso et al., 2010). In the elephant shark a putative *ramp2*/*ramp5* gene exists. In coelacanth (*Latimeria chalumnae*) *ramp* genes are represented by open squares and their identity remains to be clarified. The genome duplication events that occurred in the vertebrate radiation (1R, 2R) and the teleost specific (3R) are indicated. Peptide, receptors and ramp that resulted from the teleost 3R are represented by *a* and *b*. "?" represent uncertain events and unknown identity.

Figure 9: Scheme outlining the proposed endocrine regulation by Pacap of tilapia skin melanophores (A) and cross-talk between signalling pathways (B). The working hypothesis to explain the effect of PACAP on tilapia melanin aggregation is proposed to involve the Pac_1-Ramp protein complex which attenuates/inhibits the increase in cAMP caused by Pac_1 alone. The reduction in cAMP will decrease the cAMP-dependent PKA activity that is responsible for protein phosphorylation. The inhibition of PKA activity favours Ca^{2+}/CaM protein phosphatase (PP) that is stimulated by the increase of cytosolic Ca^{2+} levels occurring via the PLC/IP3 signalling. The overall outcome of the proposed change in intracellular signalling is cytoskeleton protein dephosphorylation and pigment aggregation (Thaler and Haimo, 1990,Oshima and Wannitikul, 1996). Dashed and blocked lines indicate inhibitory effects and arrows indicate stimulatory effects. *Adcyap1* transcripts are expressed in tilapia skin and melanophores and this suggests it may act as a paracrine factor in skin pigmentation. Although $Vpac_2a$ is expressed in skin, PHI (the ligand) does not have an effect on melanin aggregation or dispersion and its role in skin remains to be established. Dark circles represent the melanin pigment.

Supplementary Tables:

Supplementary Table 1: Primer pairs used in q-PCR analysis and cloning.

Supplementary Table 2: Accession numbers of the *adcyap1* (Prp/Pacap) and *vip* (Phi/Vip) genes in fish. The fish genes were retrieved from ENSEMBL database and from the elephant shark genome database. Sea bass genes were retrieved from the sea bass genome assembly and sea bream ESTs from an "in house" database generated by assembly of all sequence data for this species in NCBI. ni- not identified. * incomplete sequence; # deduced from the genome.

Supplementary Table 3: Accession numbers of the fish *adcyap1r1*, *vipr1* and *vipr2* genes. The fish genes were retrieved from ENSEMBL, sea bass genome assembly and sea bream ESTs from an "in house" database generated by assembly of all sequence data for this species in NCBI. For each specie, accession number of paralogue *a* is at the top and *b* follows below. ni- not identified. * incomplete sequence; + EST data.

Supplementary Table 4: Accession numbers of the fish *ramp* genes. The fish genes were retrieved from ENSEMBL and also retrieved from the sea bass genome assembly or from an "in house" database generated by assembly of all sequence data for sea bass and sea bream in NCBI. The tilapia *ramp2* transcript was obtained from the NCBI database and is not predicted in the genome assembly. The sea bream putative *ramp1* and *ramp5* transcripts were identified but were very incomplete. The existence of the coelacanth *ramp* genes was unconfirmed as their deduced mature protein sequences are very divergent from other vertebrate homologues. No putative *ramp* genes were found in lamprey genomes. (+) not annotated in the genome and identified using similarity with the homologue vertebrate sequence; (*) not included in phylogenetic analysis. ni- not identified.

Supplementary Figures:

Supplementary Figure 1: Amino acid sequence conservation of the fish Pacap (A), Vip (B) and Phm/Phi (C) deduced peptides with the human and *Xenopus* homologues. Percent amino acid sequence identity of the fish and *Xenopus* mature peptides with the human homologues are indicated. Note - of all the peptides analysed Pacap is the most conserved. *a* and *b* discriminate the paralogues identified in teleost genomes (Cardoso et al., 2007,Roch et al., 2009,Ng et al., 2012). The amino acids that differ from the human peptides are highlighted in bold. (A) Pacap-27 and Pacap-38 isoforms are represented and the amino acids that are underlined are only present in Pacap-38. The deduced teleost Pacap-27a (+, Pacapb in zebrafish, cavefish and cod) is 100% identical with the human homologue and is conserved across the vertebrates. In the spotted gar genome and in the Japanese lampreys no Pacap-38 peptides were predicted. (B) and (C) two Vip and Phi peptides were predicted in tilapia, cavefish and zebrafish. The Phi/Vip gene precursor in stickleback was incomplete and so Phi is not represented. (*) - conserved aa, (:) - conserved aa with similar properties and (.) - conserved aa with weakly similar properties. Accession numbers: Human (*Homo sapiens*) PACAP (CAA42962) and PHM/VIP (AAA61287.1); Rat (*Rattus norvegicus*) PHI/VIP (NP_446443.1); *Xenopus* PACAP (NP_001096425.1) and PHI/VIP (XP_002936422.2). The Japanese lamprey sequences were obtained from (Ng et al., 2012).

Supplementary Figure 2: Sequence alignment of the sea bream and tilapia Pac_1a. Receptor TM domains are boxed and the signal peptide in italics and underlined. The deduced mature protein sequence of both fish receptors are highly similar and only vary at a few amino acids (aa's) in the N-terminus region. (*) - fully conserved aa's; (:) - conserved aa's with similar properties and (.) - conserved aa's with weakly similar properties.

Supplementary Figure 3: Comparison of the homologous genome regions harbouring the *adcyap1* (A) and *vip* (B) genes in fish and human. The duplicate *adcyap1* and *vip* genes (*a* and *b*) emerged from the teleost genome duplication event (Cardoso et al., 2007,Ng et al., 2012). Gene environment revealed that the teleost paralogue *adcyap1* and *vip* genome regions share synteny with the human and spotted gar homologue regions. The teleost *adcyap1a* and *vipa* genome regions retained genes that are only shared with human and spotted gar. The *mettl4*, *enosf1*, *smchd1*, *tyms* genes that are part of the teleost *adcyap1a* gene environment (A) and *fbxo5* and *mtrf1l* gene within the teleost *vipa* genome region (B) are not present in the *adcyap1b* and *vipb* paralogue regions. (B) Only a single *vip* gene was found in tetraodon and the genes that are in linkage with the zebrafish, cavefish and tilapia *vipb* gene map to different tetraodon chromosomes. Horizontal lines represent chromosome fragments and the length of the genome regions analysed (Mb) are indicated within brackets. Block arrows represent genes and their

orientation in the genome. Orthologue genes are represented in the same colour and gene symbols are indicated and the numbers within the arrows represent the number of predicted gene members in tandem.

Supplementary Figure 4: Comparison of the homologous genome regions harbouring the *adcyap1r1* (A) and *vipr2* (B) genes in fish. The paralogue *adcyap1r1* (*a* and *b*) and *vipr2* (*a* and *b*) genome regions are represented. The gene environments of the cavefish (*A. mexicanus*) *adcyapr1* and zebrafish (*D. rerio*) *vipr2* are boxed. (A) The gene environment of the fish orthologous *adcyapr1a* in the cavefish genome assembly is on several scaffolds and *adcyapr1a* and *ghrhra* were not identified. *C2cd4c*, *chico*, *ccdc94* and *shda* genes in close linkage with *adcyap1r1a* in zebrafish map to scaffold KB882255.1 in the cavefish genome. (B) The genes *esyt2a*, *pfkpb* and *gtpbp4* in linkage with teleost *vipr2b* map to zebrafish chr2 and *ptprn2* maps to chr7 suggesting that chromosomal gene rearrangement may be responsible for loss of *vipr2b* in zebrafish. Horizontal lines represent chromosome fragments and the lengths of the genome regions analysed are indicated within brackets. Block arrows represent genes and their orientation in the genome. Orthologue genes are represented in the same colour and gene symbols are indicated.

Supplementary Figure 5: Phylogenetic tree of the fish Ramps. A consensus tree was constructed with the deduced mature protein sequence of the fish, *Xenopus* and human genes (Supplementary Table 4) using the Maximum Likelihood method with the JTT matrix and 100 bootstrap replicates. Sixty-nine vertebrate sequences were used and bootstrap support values are only indicated for the main vertebrate clades. A hypothetical root was added to the tree between the vertebrate RAMP1/3 and RAMP2/5 clades to comply with the existing model for the RAMP family evolution that proposes RAMP5 is a divergent branch of an early RAMP2 duplication (Benítez-Páez and Cárdenas-Brito, 2008). In the teleost radiation the *ramp1* gene duplicated and originated Ramp1a and Ramp1b (previously named Ramp4). In coelacanth 5 putative *ramp* genes were identified but the deduced amino acid sequences are very divergent from the other fish and tetrapod sequences and thus were not included in the phylogenetic analysis. Human RAMP1 (NP_005846.1), RAMP2 (NP_005845.2), RAMP3 (NP_005847.1); *Xenopus* RAMP1 (NP_001072813), RAMP2 (XP_002932549.1) and RAMP3 (NP_001107376); *Takifugu obscurus* Ramp1a (BAE45310.1), Ramp1b (BAE45306.1), Ramp2tv1 (BAE45308.1), Ramp2tv2 (BAE45307.1), Ramp3 (BAE45305.1) and Ramp5 (BAE45311.1).

1295 **Table 1**

Table 1

	Scales	Melanophores	MI 1-2	MI 3-4	MI 5
Peptide assays					
PACAP-27	11	4027	45.1±11.2	22.1±1.4	32.8±10.1
hPACAP-38	13	4714	40.3±7.1	27.3±2.9	32.4±8.2
hVIP	10	3142	30.5±6.3	27.1±4.0	42.4±8.6
rPHI	11	6708	26.4±7.6	27.2±4.0	46.4±10.5
Control (TSS)	16	6687	20.0±4.2	27.3±4.3	52.7±7.7
hSCT	9	4709	7.8±0.9	49.1±1.4	43.1±1.5
hSCT-Control (TSS)	9	4601	22.2±10.2	26.3±6.0	51.5±5.3
Assays with drugs					
PACAP-27	12	5434	42.4±11.2	37.5±6.9	20.1±5.4
PACAP-27 + IBMX	12	4517	15.0±2.3	20.7±2.5	64.3±3.5
IBMX	9	3739	6.2±1.2	15.3±1.5	78.5±2.7
PACAP-27 + W7	12	6134	15.0±1.9	27.3±2.7	57.7±3.1
W7	9	4534	15.4±5.5	21.1±5.4	63.5±5.3
Epinephrine	9	6568	89.9±2.5	13.4±2.6	1.1±0.9
Epinephrine + IBMX	9	4594	8.8±2.0	29.3±15.2	61.9±17.1
Epinephrine + W7	9	5863	82.4±2.8	9.4±0.9	8.2±3.6
Control (TSS)	9	4601	22.2±10.2	26.3±6.0	51.5±5.3

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1298

1299 **Table 2**

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Table 2

	ENSEMBL	Human	Zebrafish	Takifugu	Sea bream
Pac1a	ENSONIG00000009271	70/79	82/88	89/92	90/93
Pac1b	ENSONIG00000019468	70/79	79/87	82/89	87/91
Vpac1a	ENSONIG00000005778	54/69	67/81	83/90	67/73*
Vpac1b	ENSONIG00000015597	52/69	66/78	74/85	ni
Vpac2b	ENSONIG00000008080	49/65	ni	64/76	ni
Vpac2a	ENSONIG00000014886	57/71	62/75	78/85	ni

1301

Figure 1

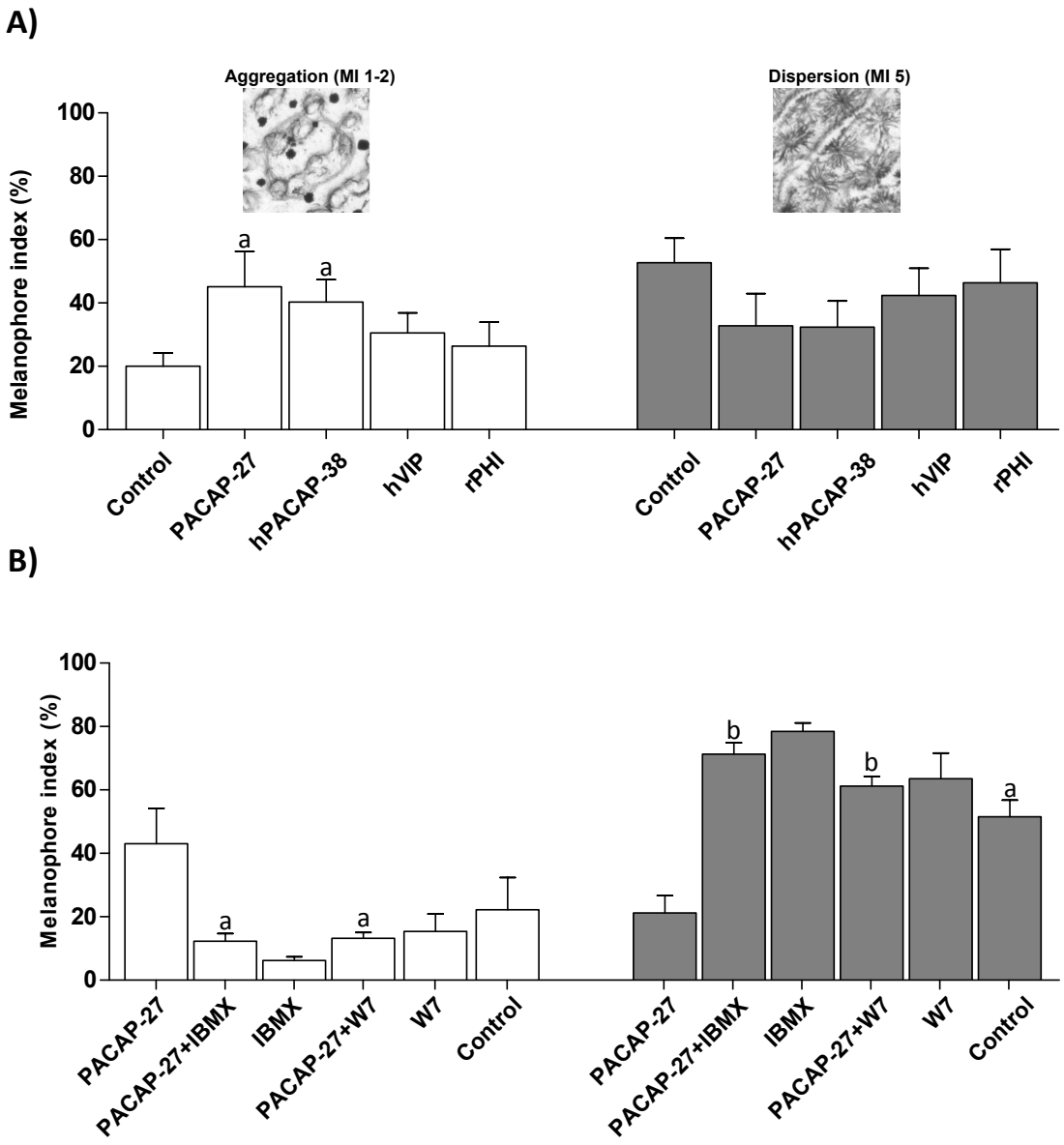

A)
Aggregation (MI 1-2)
Dispersion (MI 5)
Melanophore index (%)
100
80
60
40
20
0
a
a
Control
PACAP-27
hPACAP-38
hVIP
rPHI
Control
PACAP-27
hPACAP-38
hVIP
rPHI
B)
Melanophore index (%)
100
80
60
40
20
0
a
a
b
b
a
PACAP-27
PACAP-27+IBMX
IBMX
PACAP-27+W7
W7
Control
PACAP-27
PACAP-27+IBMX
IBMX
PACAP-27+W7
W7
Control

Figure 2

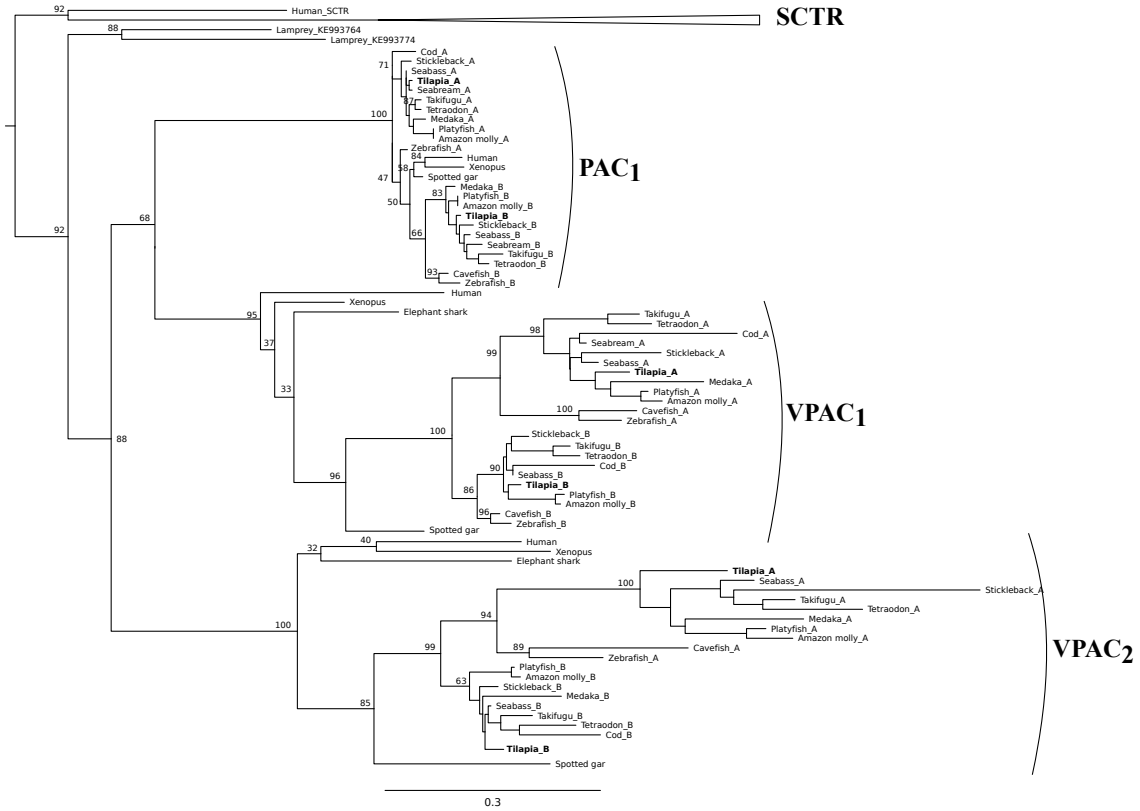
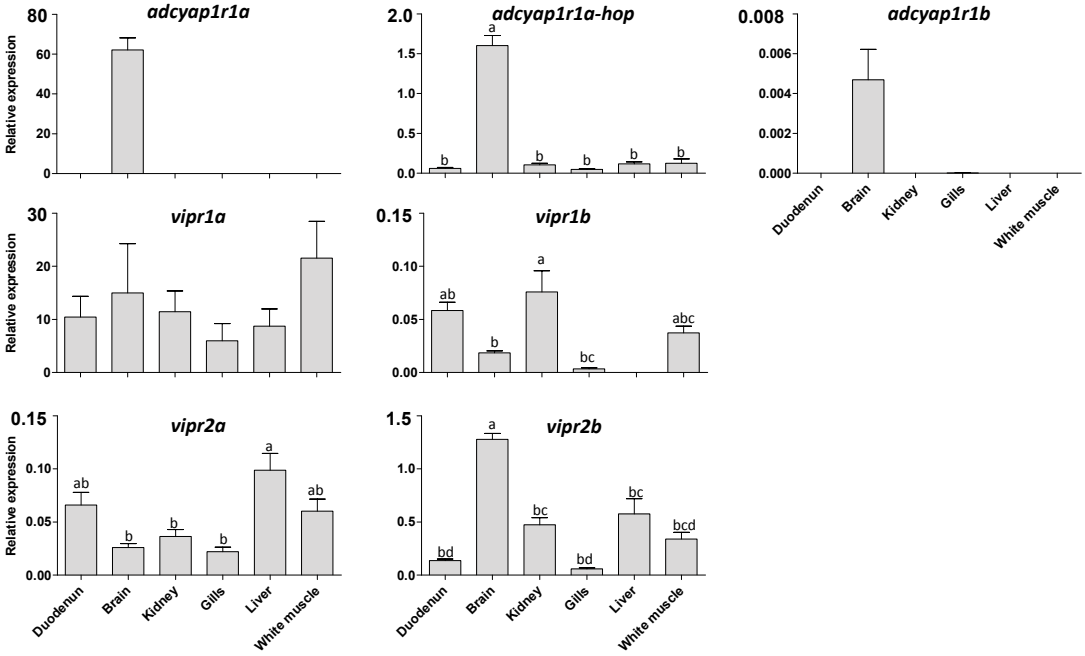
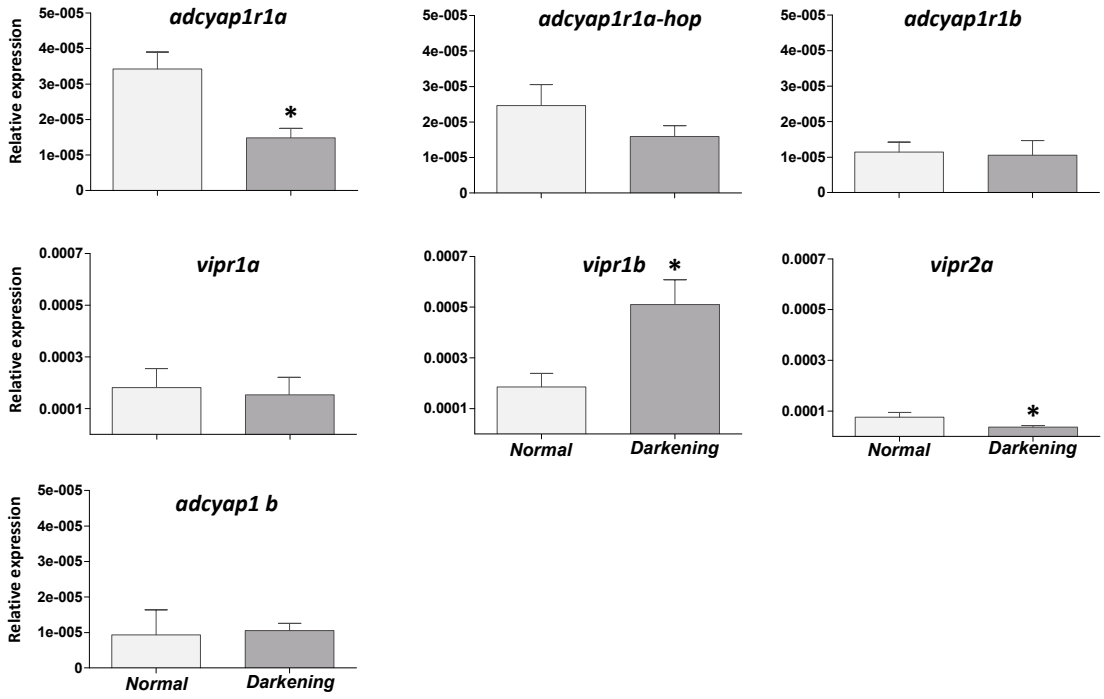

SCTR
PAC1
VPAC1
VPAC2
Human_SCTR
Lamprey_KE993764
Lamprey_KE993774
Cod_A
Stickleback_A
Seabass_A
Tilapia_A
Seabream_A
Takifugu_A
Tetraodon_A
Medaka_A
Platyfish_A
Amazon molly_A
Zebrafish_A
Human
Xenopus
Spotted gar
Medaka_B
Platyfish_B
Amazon molly_B
Tilapia_B
Stickleback_B
Seabass_B
Seabream_B
Takifugu_B
Tetraodon_B
Cavefish_B
Zebrafish_B
Elephant shark
Cavefish_A
Cod_B
0.3

Figure 3


adcyap1r1a
adcyap1r1a-hop
adcyap1r1b
vipr1a
vipr1b
vipr2a
vipr2b
Relative expression
Duodenum
Brain
Kidney
Gills
Liver
White muscle

1320 **Figure 4**



1321

1322

Figure 5

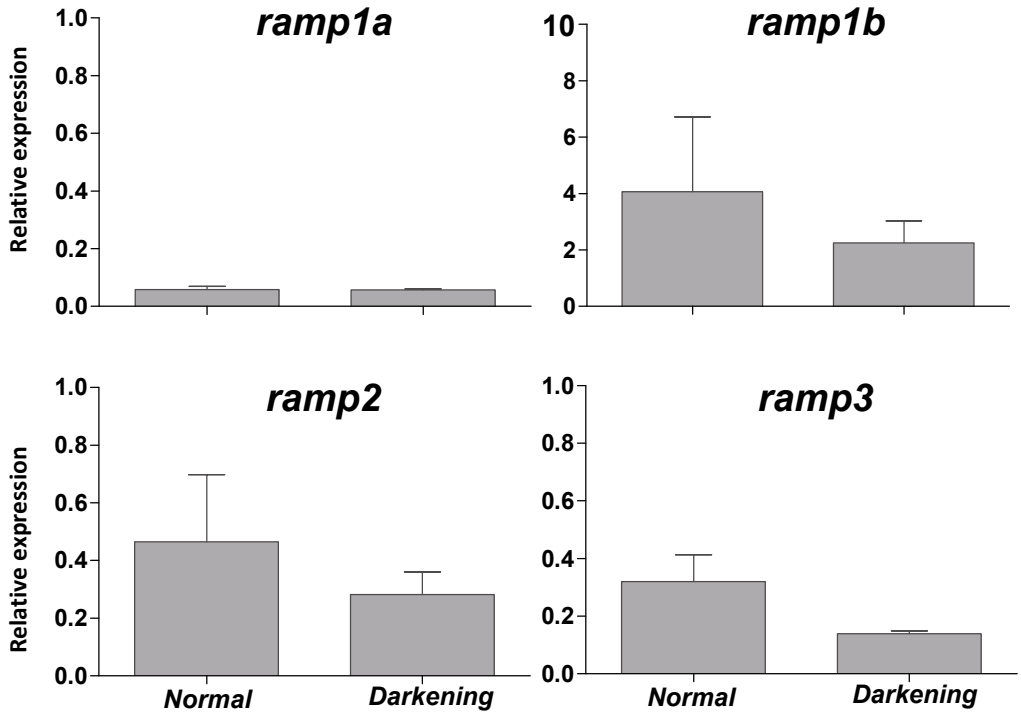

ramp1a
ramp1b
ramp2
ramp3
Relative expression
Relative expression
1.0
0.8
0.6
0.4
0.2
0.0
10
8
6
4
2
0
Normal
Darkening
Normal
Darkening

Figure 6

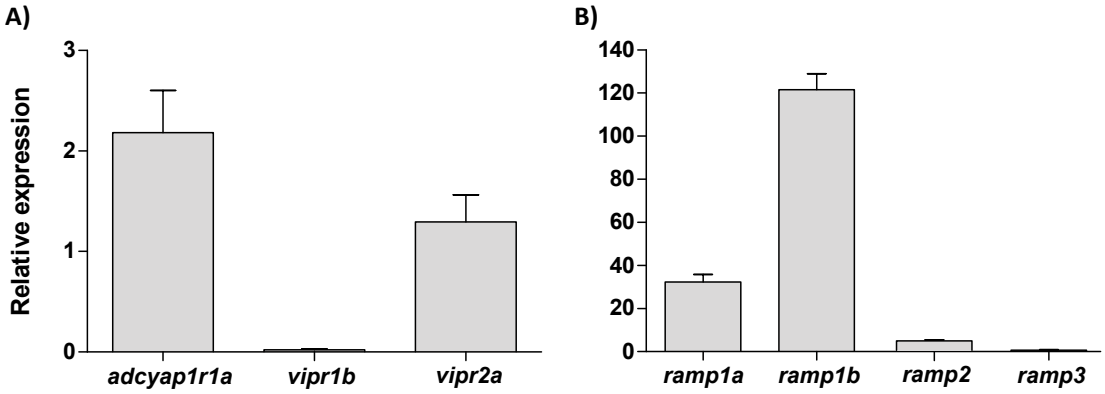

A)
Relative expression
3
2
1
0
adcyap1r1a
vipr1b
vipr2a
B)
140
120
100
80
60
40
20
0
ramp1a
ramp1b
ramp2
ramp3

Figure 7

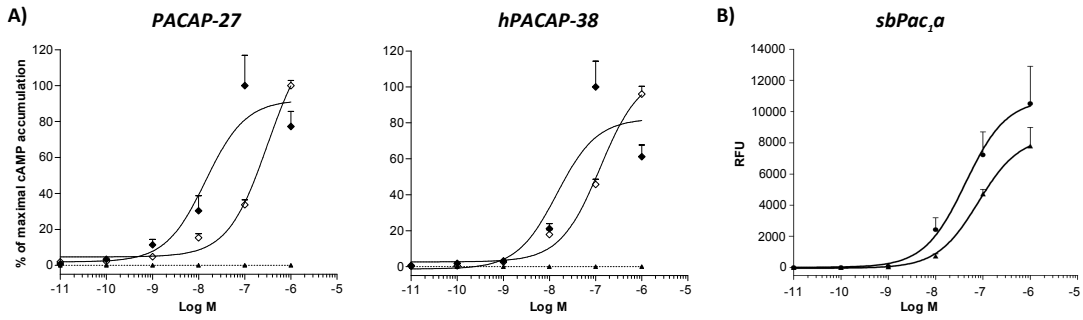

A)
PACAP-27
hPACAP-38
B)
sbPac1a
% of maximal cAMP accumulation
RFU
Log M
Log M
Log M

Figure 8

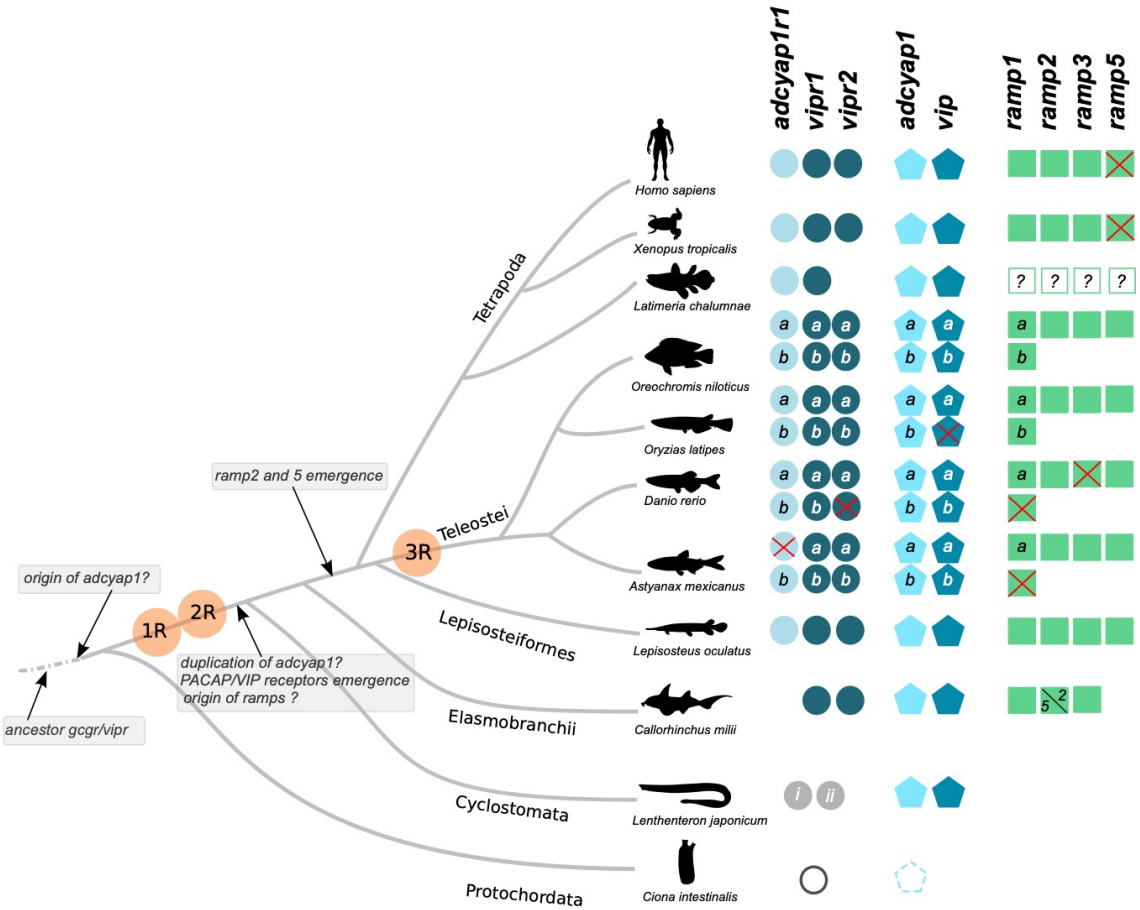
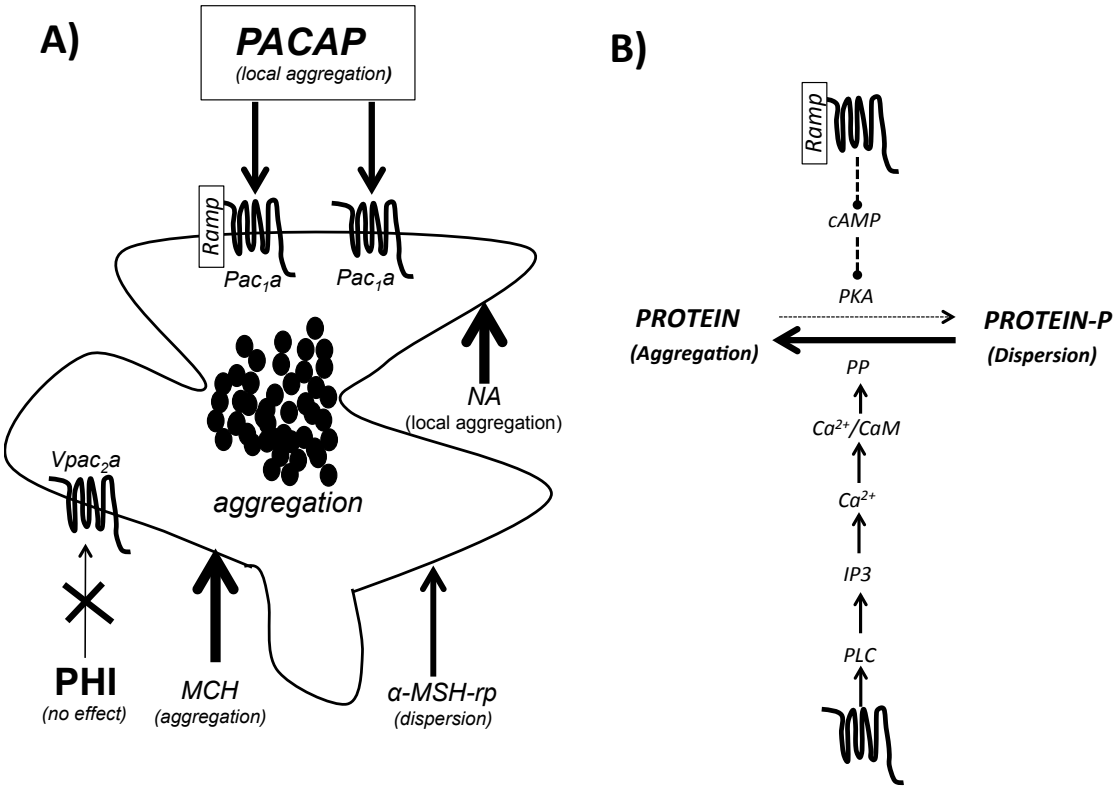

adcyap1r1
vipr1
vipr2
adcyap1
vip
ramp1
ramp2
ramp3
ramp5
Homo sapiens
Xenopus tropicalis
Latimeria chalumnae
Oreochromis niloticus
Oryzias latipes
Danio rerio
Astyanax mexicanus
Lepisosteus oculatus
Callorhinchus milii
Lenthenteron japonicum
Ciona intestinalis
Tetrapoda
Teleostei
Lepisosteiformes
Elasmobranchii
Cyclostomata
Protochordata
ramp2 and 5 emergence
origin of adcyap1?
3R
2R
1R
duplication of adcyap1?
PACAP/VIP receptors emergence
origin of ramps ?
ancestor gcgr/vipr

Figure 9


A)
PACAP
(local aggregation)
Ramp
Pac1a
Pac1a
NA
(local aggregation)
aggregation
Vpac2a
PHI
(no effect)
MCH
(aggregation)
α-MSH-rp
(dispersion)
B)
Ramp
cAMP
PKA
PROTEIN
(Aggregation)
PROTEIN-P
(Dispersion)
PP
Ca2+/CaM
Ca2+
IP3
PLC

Supplementary Table 1

	Primer sequence (5' – 3')	Ta (ºC)	Efficiency (%)
adcyap1a	Fwd- acggcaattccttaccgtccc	62	94
(PRP/PACAP*a*)	Rev- caacattttccccccgcttttt		
adcyap1b	Fwd- agattagacttgaaaacgacgca	62	92
(PRP/PACAP*b*)	Rev- catggtgttgtcgtcactcttc		
vip1	Fwd- tgatgagctgattgatgagcc	59	92
(PHI/VIP1)	Rev- gcttctctttcctgataagac		
vip2	Fwd- ggtgacctgacagaaaacctg	58	96
(PHI/VIP2)	Rev- acttctctttccctgcagca		
adcyap1r1a	Fwd- aaaggacctgtggtggcatct	60	95
(PAC1a)	Rev- gagcgtgccagacgcaggt		
adcyap1r1ahop	Fwd- aaaggacctgtggtggcatct	60	91
(PAC_1Ahop)	Rev- tgtgatggtggataactctga		
adcyap1r1b	Fwd- gctggaaatgaatccagtatt	62	94
(PAC_1B)	Rev- taccagacgttccctcttgct		
vipr1a	Fwd- ccttatttcagctacttcagtg	60	91
(VPAC1a)	Rev- atggtgtagccgatcttcact		
vipr1b	Fwd- actacggggtcatggccagt	62	96
($VPAC_1$B)	Rev- ttcgttatcgcccacgctgag		
vipr2a	Fwd- gtatggcgaaaccacagactg	62	93
($VPAC_2$A)	Rev- gagaggcggaaggagtaggt		
vipr2b	Fwd- tcaaaccagccatcactggtg	60	99
($VPAC_2$B)	Rev- ataaatcctcgttatcaccca		
ramp1	Fwd- gctcctgtggttcgttatag	60	98
	Rev- tgtccagtctgaacttgccc		
ramp2	Fwd- atgacactcaccagattctta	58	96
	Rev- ggaggcagctcattctgtctga		
ramp3	Fwd- gggatgatgagaggactgtca	58	93
	Rev- gaggttacaccagttctgtg		
ramp4	Fwd- tctctgcttggcgaatttcaca	60	93
	Rev- atcctcaaggccacctggtatg		
ramp5	Fwd- gatatgcgtggaatctgtggct	60	99
	Rev- tcctgaggggcatcttcagtc		
mc1r	Fwd- atacttcacgcggacttcgga	64	96
	Rev- ggcgagtggaggtttcggttt		
β-actin1	Fwd- tgacctcacagactacct	58	90
	Rev- gctcgtaactcttctcca		
gadph1	Fwd- gctggtcatcggtaaca	58	97
	Rev- gccttctcaatggtggta		
18S	Fwd- tgacggaagggcaccaccag	60	103
	Rev- aatcgctccaccaactaagaacgc		

Supplementary Table 2

	adcyap1r1	***vipr1***	***vipr2***
Coelacanth	ENSLACG00000013363*	ENSLACG00000004349*	*ni*
Latimeria chalumnae			
Tilapia	ENSONIG00000009271	ENSONIG00000005778	ENSONIG00000008080
Oreochromis niloticus	ENSONIG00000019468	ENSONIG00000015597	ENSONIG00000014886
Sea bream+	AJ514930.1	SRR278741_isotig34555	SRR278741_isotig30899*
Sparus auratus	AJ514931.1	*ni*	*ni*
Stickleback	ENSGACG00000017184	ENSGACG00000012836	ENSGACG00000001845
Gasterosteus aculeatus	ENSGACG00000005402	ENSGACG00000003341	ENSGACG00000017549
Sea bass	DLAgn_00007350	DLAgn_00001640	DLAgn_00009610
Dicentrarchus labrax	DLAgn_00145280	DLAgn_00076520	DLAgn_00078890
Takifugu	ENSTRUG00000012410	ENSTRUG00000008126	ENSTRUG00000017701
Takifugu rubripes	ENSTRUG00000004289	ENSTRUG00000017596	ENSTRUG00000005848
Tetraodon	ENSTNIG00000006236	ENSTNIG00000007927	ENSTNIG00000007985
Tetraodon nigroviridis	ENSTNIG00000018630	ENSTNIG00000012733	ENSTNIG00000008704
Amazon molly	ENSPFOG00000006236	ENSPFOG00000015954	ENSPFOG00000009610
Poecilia formosa	ENSPFOG00000009610	ENSPFOG00000003737	ENSPFOG00000002160
Platyfish	ENSXMAG00000006344	ENSXMAG00000000298	ENSXMAG00000004624
Xiphophorus maculatus	ENSXMAG00000016932	ENSXMAG00000009513	ENSXMAG00000006533
Medaka	ENSORLG00000013021	ENSORLG00000011942	ENSORLG00000005878
Oryzias latipes	ENSORLG00000017774	ENSORLG00000009354	ENSORLG00000019029
Cod	ENSGMOG00000001536	ENSGMOG00000000393	ENSGMOG00000011750
Gadus morhua	*ni*	ENSGMOG00000002209	*ni*
Cavefish	*ni*	ENSAMXG00000015142	ENSAMXG00000005208
Astvanax mexicanus	ENSAMXG00000010490	ENSAMXG00000014845	ENSAMXG00000009437*
Zebrafish	ENSDARG00000029989	ENSDARG00000028878	ENSDARG00000012353
Danio rerio	ENSDARG00000053724	ENSDARG00000059058	*ni*
Spotted gar	ENSLOCG00000009545	ENSLOCG00000009610	ENSLOCG00000012346
Lepisosteus oculatus			
Elephant shark	*ni*	SINCAMG00000014757	SINCAMG00000007313
Callorhinchus milii			

+ EST data

* incomplete sequence

Supplementary Table 3

	***adcyap1* (Prp/Pacap)**	***vip* (Phi/Vip)**
Coelacanth	ENSLACG00000016776	ENSLACG00000004320
Latimeria chalumnae		
Tilapia	ENSONIG00000006092	ENSONIG00000001111
Oreochromis niloticus	ENSONIG00000009205	ENSONIG00000019842
Sea bream	DQ659328	Contig7512
Sparus auratus		
Stickleback	ENSGACG00000004163	ENSGACG00000001298*
Gasterosteus aculeatus	ENSGACG00000017084	
Sea bass+	DLAgn_00077420	DLAgn_00015910
Dicentrarchus labrax	DLAgn_00007540	
Takifugu	ENSTRUG00000003782	ENSTRUG00000001139
Takifugu rubripes	ENSTRUG00000010059	
Tetraodon	ENSTNIG00000017117	ENSTNIG00000007449
Tetraodon nigroviridis	ENSTNIG00000018649	
Amazon molly	ENSPFOG00000017025	ENSPFOG00000008366
Poecilia formosa	ENSPFOG00000003779	
Platyfish	ENSXMAG00000002216	ENSXMAG00000017180
Xiphophorus maculatus	ENSXMAG00000016709	
Medaka	ENSORLG00000011205	ENSORLG00000003905
Oryzias latipes	ENSORLG00000017872	
Cod	ENSGMOG00000005491	GeneScaffold_4161#
Gadus morhua	ENSGMOG00000007155	
Cavefish	ENSAMXG00000007261	ENSAMXG00000003550
Astvanax mexicanus	ENSAMXG00000013310	ENSAMXG00000006697
Zebrafish	ENSDARG00000004015	ENSDARG00000078247
Danio rerio	ENSDARG00000027740	ENSDARG00000079443
Spotted gar	ENSLOCG00000011028	ENSLOCG00000015508
Lepisosteus oculatus		
Elephant shark	SINCAMG00000008747	SINCAMG00000003053
Callorhinchus milii		

+ EST data

* incomplete sequence

deduced from the genome

Supplementary Table 4

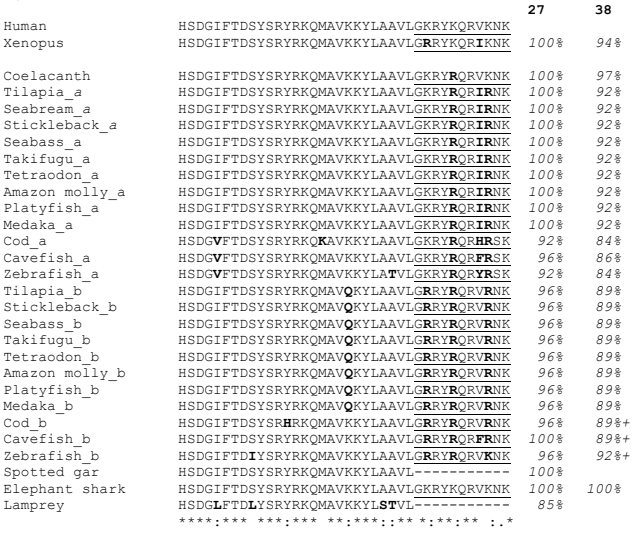
	ramp1a/ramp1b	ramp2	ramp3	ramp5
Coelacant*	ENSLACG00000001045	ENSLACG00000005969	ENSLACG00000002219	ENSLACG00000005454
Latimeria chalumnae	ENSLACG00000006022			
Tilapia	ENSONIG00000004458	XP_003442092.1	ENSONIG00000008085	ENSONIG00000018101
Oreochromis niloticus	ENSONIG00000013384			
Sea bream	SRR2787417isotig49497*	SRR278741/ Contig4235	ni	SRR278741/isotig51923*
Sparus auratus	Contig14134/ Contig11408			
Stickleback	ENSGACG00000002191	ENSGACG00000008501	ENSGACG00000002155	groupV_5.19Mb+
Gasterosteus aculeatus	ENSGACG00000014723			
Sea bass	DLAgn_00048510	DLAgn_00192410	DLAgn_00074550	ni
Dicentrarchus labrax	DLAgn_00137260			
Takifugu	ENSTRUG00000014618	ENSTRUG00000015089	ENSTRUG00000004598	ENSTRUG00000005984
Takifugu rubripes	ENSTRUG00000007700			
Tetraodon	ENSTNIG00000007561	Ch3_12.88Mb+	ENSTNIG00000000529	Ch2_2.14Mb+
Tetraodon nigroviridis	ENSTNIG00000008322			
Amazon molly	ENSPFOG00000002861	ENSPFOG00000013682	ENSPFOG00000011986*	ENSPFOG00000012809
Poecilia formosa	ENSPFOG00000013837			
Platyfish	ENSXMAG00000002037	ENSXMAG00000009128	ENSXMAG00000006290	ENSXMAG00000016882
Xiphophorus maculatus	ENSXMAG00000007864			
Medaka	ENSORLG00000018110	ENSORLG00000004263	ENSORLG00000006002	Ch19_ 20.86Mb+
Oryzias latipes	ENSORLG00000002375			
Cod	ENSGMOG00000002122	ENSGMOG00000015667	ENSGMOG00000004845	ENSGMOG00000003857
Gadus morhua	ENSGMOG00000016155			
Cavefish	ENSAMXG00000010837	ENSAMXG00000012707	ENSAMXG00000015099	ENSAMXG00000011334
Astyanax mexicanus	ni			
Zebrafish	ENSDARG00000056704	ENSDARG00000037895	ni	ENSDARG00000087020
Danio rerio	ni			
Spotted gar	ENSLOCG00000004383	ENSLOCG00000012382	ENSLOCG00000013297	ENSLOCG00000012389
Lepisosteus oculatus				
Elephant shark	SINCAMG00000017078	SINCAMG00000006267	SINCAMG00000000642	ni
Callorhinchus milii				

* sequences removed from the analysis

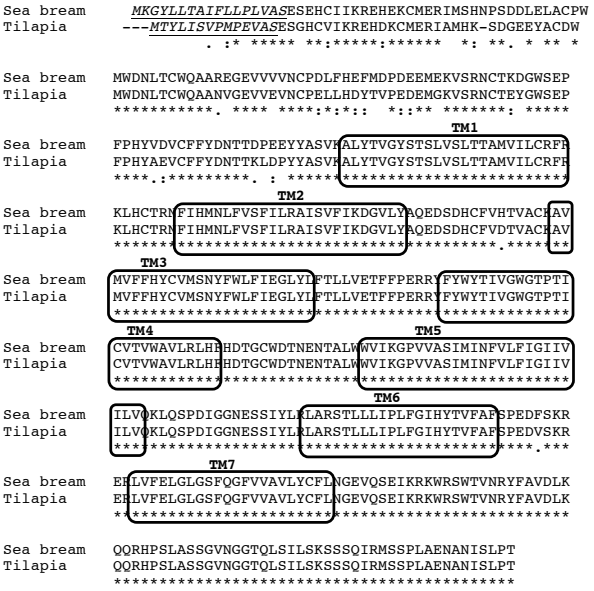
+ deduced from the genome

Supplementary Figure 1

A) PACAP

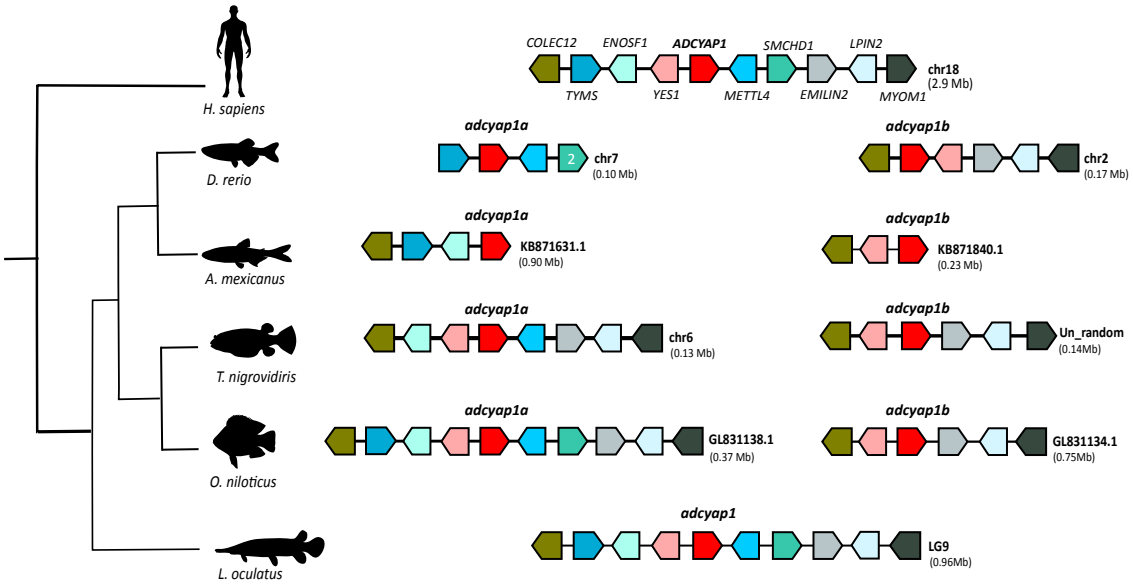


Supplementary Figure 2

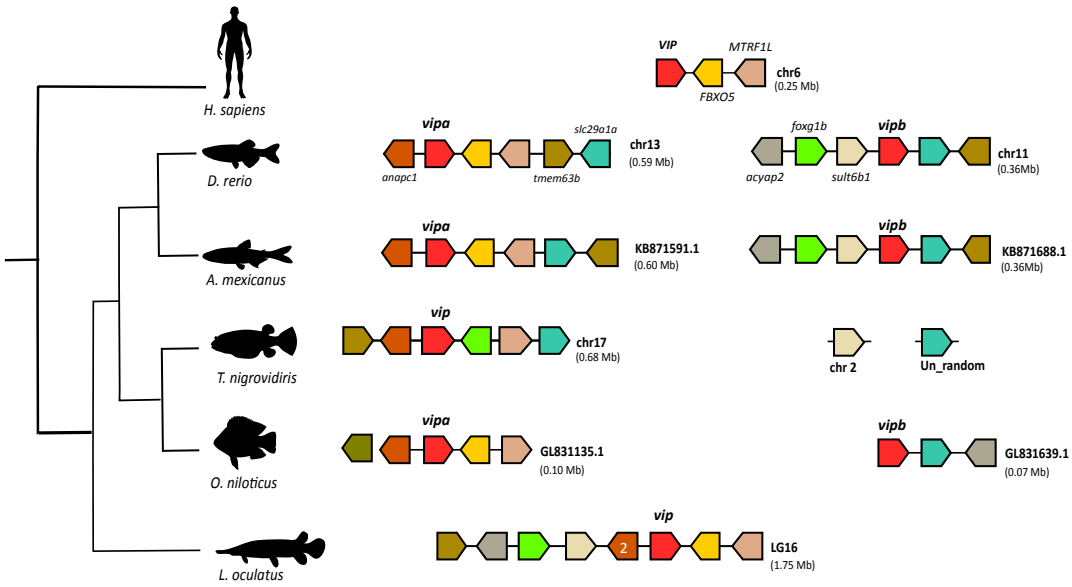


Supplementary Figure 3

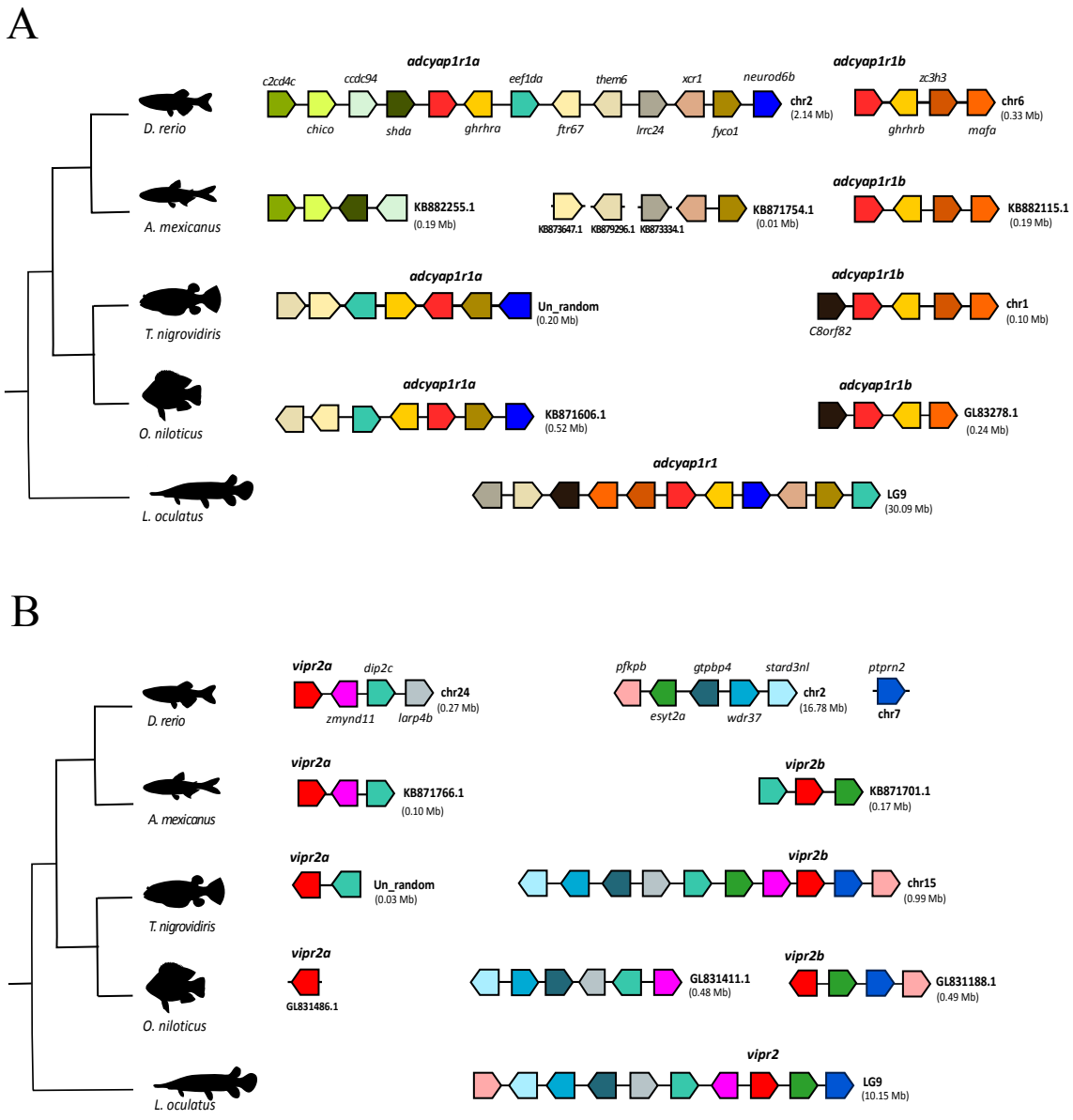
A



B



Supplementary Figure 4



Supplementary Figure 5