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Supplemental information

Long-lasting redundant *gnrh1/3* expression in GnRH neurons enabled apparent switching of paralog usage during evolution

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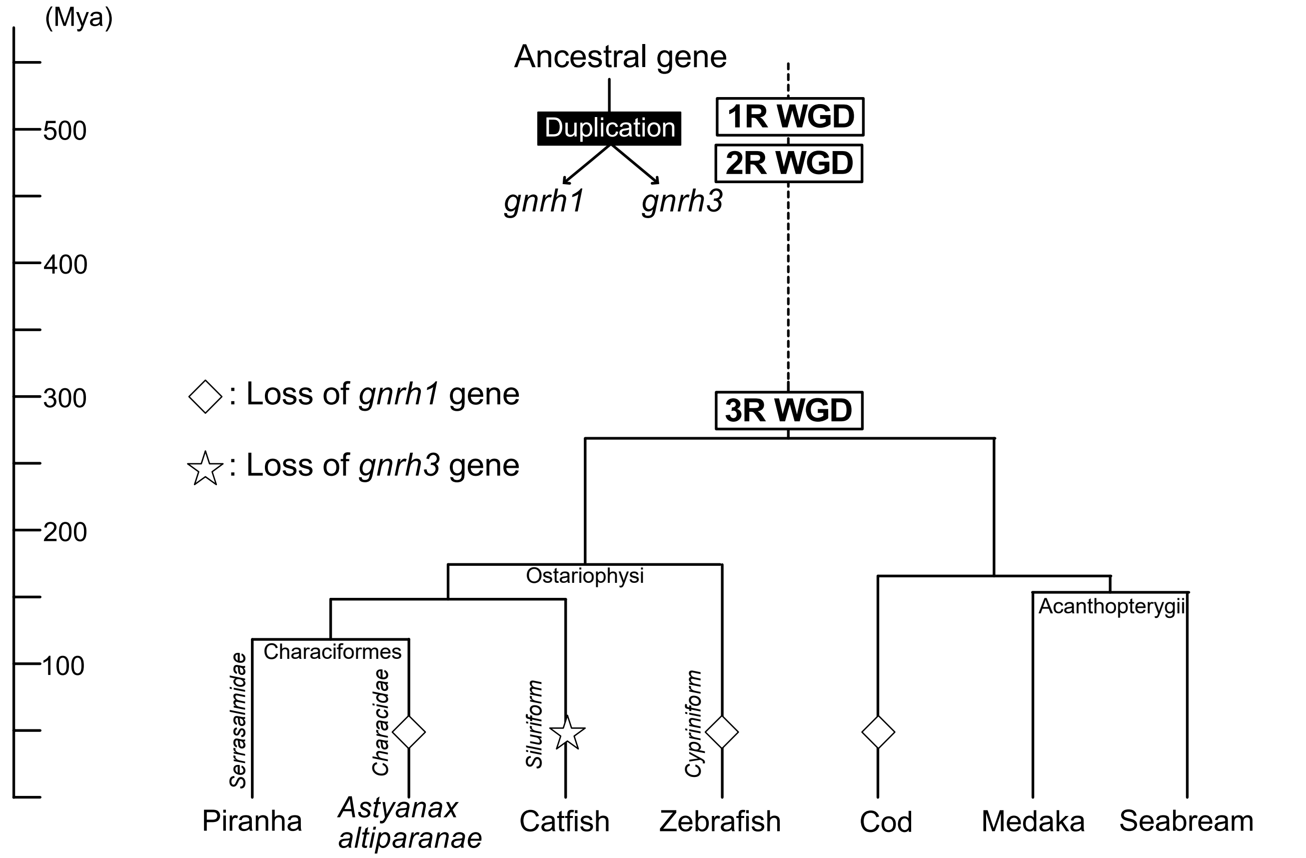


	Piranha	*Astyanax altiparanae*	Catfish	Zebrafish	Cod	Medaka	Seabream
gnrh paralog	*gnrh1* *gnrh3*	*gnrh3*	*gnrh1*	*gnrh3*	*gnrh3*	*gnrh1* *gnrh3*	*gnrh1* *gnrh3*
(Reference)	(This study)	[S1]	[S2]	[S4]	[S6]	[S7]	[S9]
Hypophysiotropic *gnrh*	?	*gnrh3?*	*gnrh1*	*gnrh3*	*gnrh3?*	*gnrh1*	*gnrh1*
(Reference)			[S3]	[S5]		[S8]	[S10]

Figure S1. Phylogenetic tree of teleost species and their hypophysiotropic GnRH paralogs, related to introduction and Figure 5.
The *gnrh1* and *gnrh3* genes were arisen from an ancestral gene during the first/second-round (1R/2R) whole genome duplication (WGD), which occurred ~550 million years ago (Mya). Many teleost species have hypophysiotropic GnRH1 neurons as the main regulator of gonadotropin release in Acanthopterygii. However, in Cypriniform and Characidae species as well as Atlantic cod, *gnrh1* has been lost and *gnrh3* is expressed in hypophysiotropic GnRH neurons instead. Since three forms of GnRH peptide are expressed in a Serrasalmidae species, pacu, we expected species in this group, including piranha, may have both *gnrh1* and *gnrh3*.

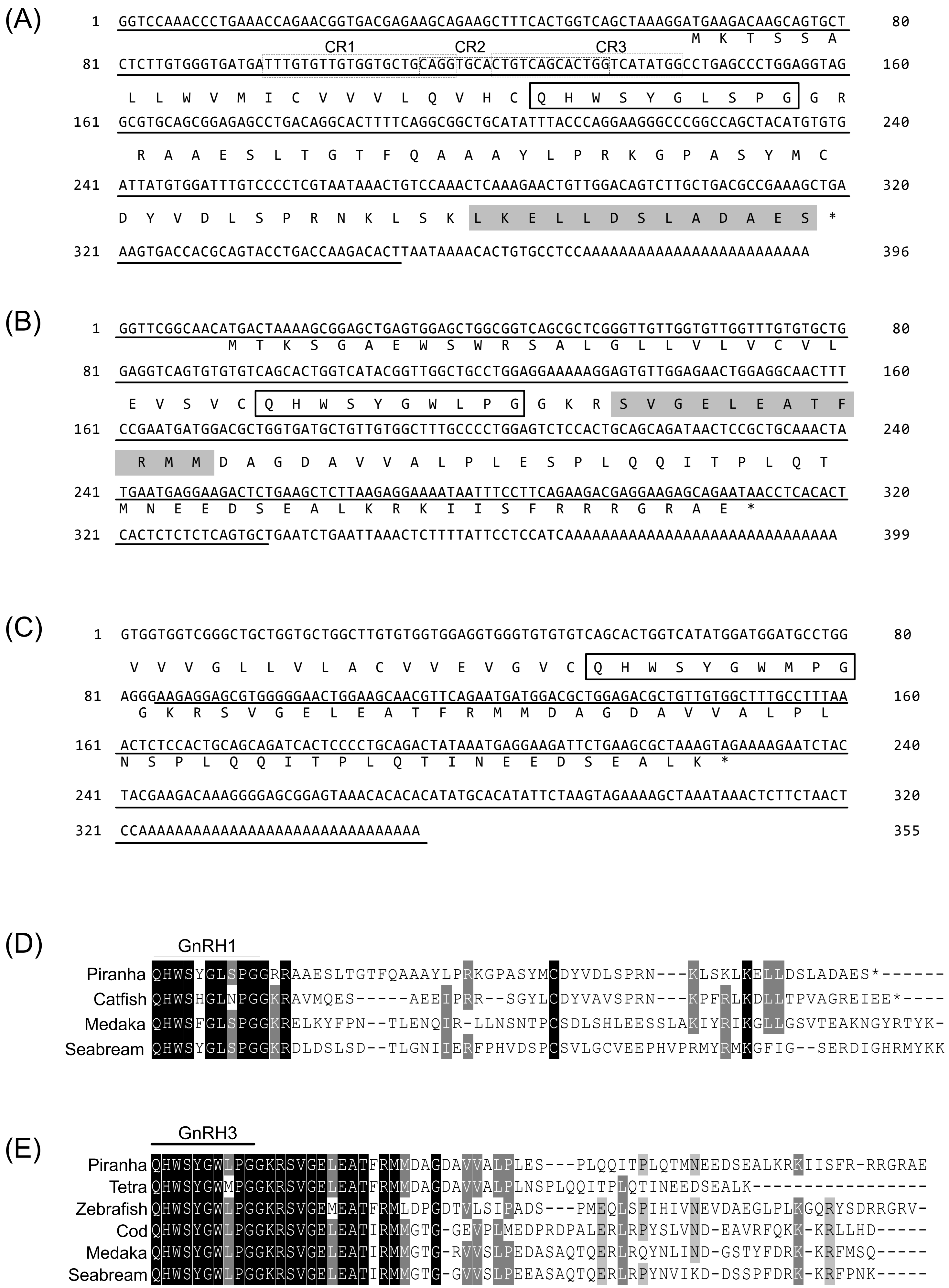
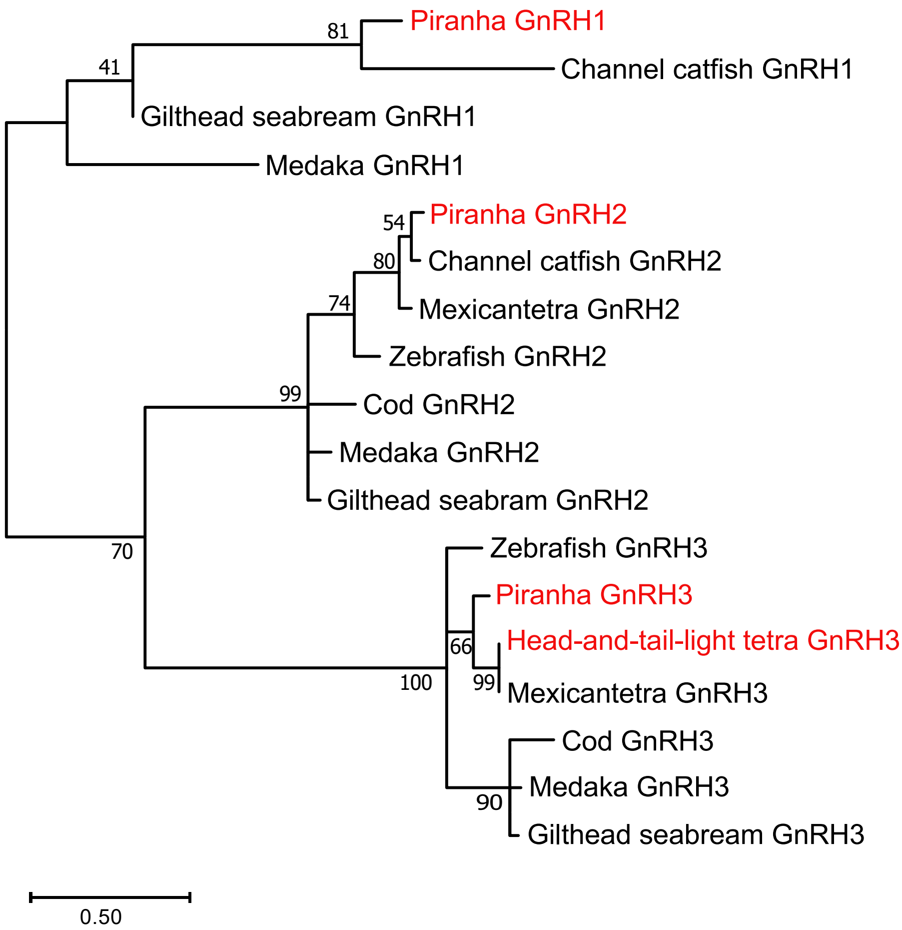

(A)
1 GGTCCAAACCCTGAAACCAGAACGGTGACGAGAAGCAGAAGCTTTCACTGGTCAGCTAAAGGATGAAGACAAGCAGTGCT 80
M K T S S A
CR1 CR2 CR3
81 CTCTTGTGGGTGATGATTTGTGTTGTGGTGCTGCAGGTGCACTGTCAGCACTGGTCATATGGCCTGAGCCCTGGAGGTAG 160
L L W V M I C V V V L Q V H C Q H W S Y G L S P G G R
161 GCGTGCAGCGGAGAGCCTGACAGGCACTTTTCAGGCGGCTGCATATTTACCCAGGAAGGGCCCGGCCAGCTACATGTGTG 240
R A A E S L T G T F Q A A A Y L P R K G P A S Y M C
241 ATTATGTGGATTTGTCCCCTCGTAATAAACTGTCCAAACTCAAAGAACTGTTGGACAGTCTTGCTGACGCCGAAAGCTGA 320
D Y V D L S P R N K L S K L K E L L D S L A D A E S *
321 AAGTGACCACGCAGTACCTGACCAAGACACTTAATAAAACACTGTGCCTCCAAAAAAAAAAAAAAAAAAAAAAAAA 396
(B)
1 GGTTCGGCAACATGACTAAAAGCGGAGCTGAGTGGAGCTGGCGGTCAGCGCTCGGGTTGTTGGTGTTGGTTTGTGTGCTG 80
M T K S G A E W S W R S A L G L L V L V C V L
81 GAGGTCAGTGTGTGTCAGCACTGGTCATACGGTTGGCTGCCTGGAGGAAAAAGGAGTGTTGGAGAACTGGAGGCAACTTT 160
E V S V C Q H W S Y G W L P G G K R S V G E L E A T F
161 CCGAATGATGGACGCTGGTGATGCTGTTGTGGCTTTGCCCCTGGAGTCTCCACTGCAGCAGATAACTCCGCTGCAAACTA 240
R M M D A G D A V V A L P L E S P L Q Q I T P L Q T
241 TGAATGAGGAAGACTCTGAAGCTCTTAAGAGGAAAATAATTTCCTTCAGAAGACGAGGAAGAGCAGAATAACCTCACACT 320
M N E E D S E A L K R K I I S F R R R G R A E *
321 CACTCTCTCTCAGTGCTGAATCTGAATTAAACTCTTTTATTCCTCCATCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 399
(C)
1 GTGGTGGTCGGGCTGCTGGTGCTGGCTTGTGTGGTGGAGGTGGGTGTGTGTCAGCACTGGTCATATGGATGGATGCCTGG 80
V V V G L L V L A C V V E V G V C Q H W S Y G W M P G
81 AGGGAAGAGGAGCGTGGGGGAACTGGAAGCAACGTTCAGAATGATGGACGCTGGAGACGCTGTTGTGGCTTTGCCTTTAA 160
G K R S V G E L E A T F R M M D A G D A V V A L P L
161 ACTCTCCACTGCAGCAGATCACTCCCCTGCAGACTATAAATGAGGAAGATTCTGAAGCGCTAAAGTAGAAAAGAATCTAC 240
N S P L Q Q I T P L Q T I N E E D S E A L K *
241 TACGAAGACAAAGGGGAGCGGAGTAAACACACACATATGCACATATTCTAAGTAGAAAAGCTAAATAAACTCTTCTAACT 320
321 CCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 355
(D)
GnRH1
Piranha QHWSYGLSPGGRRAAESLTGTFQAAAYLPRKGPASYMCDYVDLSPRN---KLSKLKELLDSLADAES*------
Catfish QHWSHGLNPGGKRAVMQES-----AEEIPRR--SGYLCDYVAVSPRN---KPFRLKDLLTPVAGREIEE*----
Medaka QHWSFGLSPGGKRELKYFPN--TLENQIR-LLNSNTPCSDLSHLEESSLAKIYRIKGLLGSVTEAKNGYRTYK-
Seabream QHWSYGLSPGGKRDLDSLSD--TLGNIIERFPHVDSPCSVLGCVEEPHVPRMYRMKGFIG--SERDIGHRMYKK
(E)
GnRH3
Piranha QHWSYGWLPGGKRSVGELEATFRMMDAGDAVVALPLES---PLQQITPLQTMNEEDSEALKRKIISFR-RRGRAE
Tetra QHWSYGWMPGGKRSVGELEATFRMMDAGDAVVALPLNSPLQQITPLQTINEEDSEALK----------------
Zebrafish QHWSYGWLPGGKRSVGEMEATFRMLDPGDTVLSIPADS---PMEQLSPIHIVNEVDAEGLPLKGQRYSDRRGRV-
Cod QHWSYGWLPGGKRSVGELEATIRMMGTG-GEVPLMEDPRDPALERLRPYSLVND-EAVRFQKK-KRLLHD-----
Medaka QHWSYGWLPGGKRSVGELEATIRMMGTG-RVVSLPEDASAQTQERLRQYNLIND-GSTYFDRK-KRFMSQ-----
Seabream QHWSYGWLPGGKRSVGELEATIRMMGTG-GVVSLPEEASAQTQERLRPYNVIKD-DSSPFDRK-KRFPNK-----

Figure S2. Alignment of piranha *gnrh1*, *gnrh3* and head-and-tail-light tetra *gnrh3*, related to Figure 1 and Figure 2.

(**A-C**) Nucleotide sequence and deduced amino acid sequence of piranha *gnrh1* (**A**), *gnrh3* (**B**) and head-and-tail-light tetra *gnrh3* gene (**C**). Each box shows mature GnRH peptides. Three dotted boxes (CR1, CR2, CR3) in (**A**) represent gRNA sequences used for generation of *gnrh1* knockout (KO) piranha. Antigen sequence for antibody production is shaded in gray. Underlines show probe sequences for *in situ* hybridization. (**D**) Alignment of the deduced amino acid sequences of mature GnRH1 and GnRH-associated peptide (GAP1) in piranha with those in channel catfish (catfish), medaka and gilthead seabream (seabream). Mature GnRH1 sequence of piranha is identical to that of gilthead seabream. Conserved amino acids in all 4 species and 3 species are shaded black and gray, respectively. (**E**) Alignment of the deduced amino acid sequences of mature GnRH3 and GnRH-associated peptide (GAP3) in piranha and head-and-tail-light tetra with those in zebrafish, cod, medaka and gilthead seabream (seabream). Mature GnRH3 sequence of piranha is identical to those of all species aligned except for head-and-tail-light tetra (Tetra). Conserved amino acids in all 6 species and 4 species are shaded black and gray, respectively.

(A)



(B)

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Wild type      TGTCAGCACTGGTCATATGGCCTGAGCCCTGGA
                    Q  H  W  S  Y  G  L  S  P  G

1bp deletion   TGTCAGCACTGGT-ATATGGCCTGAGCCCTGGA
                    Q  H  W   Y  M  A  *

10bp deletion  TGTCA----------TATGGCCTGAGCCCTGGA
                              H  M  A  *

9bp deletion   TG---------GTCATATGGCCTGAGCCCTGGA
                          W  S  Y  G  L  S  P  G
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(C)

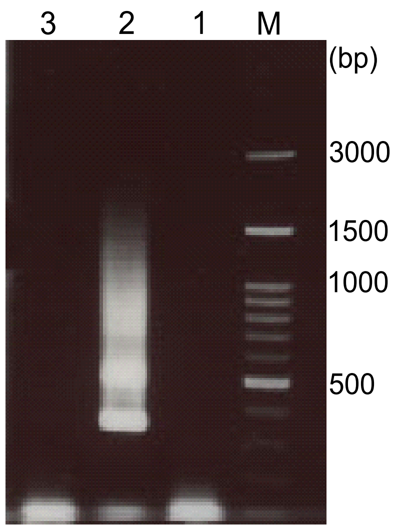

Figure S3. Phylogenetic tree of *gnrh1* and *gnrh3*, information of the knockout, and a PCR result, related to Figure 1 and Figure 2.

(**A**) Phylogenetic tree of GnRH paralogs in piranha, head-and-tail-light tetra, and other teleosts (Maximum likelihood). The number at each node shows the percentage of the bootstrap value for 1000 replicates. Scale bar represents 0.50 substitutions per site. (**B**) Genomic and deduced mature peptide sequences of wild type and KO *gnrh1* gene. Both 1-bp and 10-bp deletion mutation resulted in a frameshift and a premature stop codon (asterisk). In all mutations, it is expected that functional GnRH peptides are not produced. **(C)** Detection of *gnrh1* mRNA was failed in various Characidae fish cDNA. Degenerate primers that amplified piranha *gnrh1* failed to amplify *gnrh1* of neontetra (lane1), head-and-tail-light tetra (lane2) and glowlight tetra (lane3). Note that the bands observed in lane 2 are nonspecific amplicons. M, DNA markers (bp).

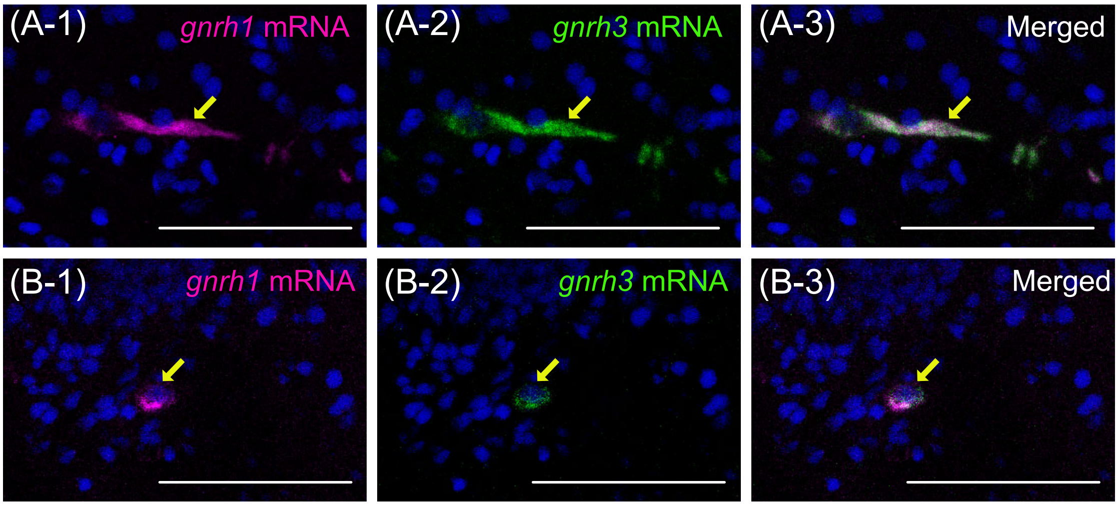

Figure S4. Other representative sections of double-labeled *in situ* hybridization of *gnrh1* and *gnrh3* in piranha POA, related to Figure 1.

(A, B) Double *in situ* hybridization indicates that co-expression of *gnrh1* and *gnrh3* is observed in many *gnrh*-expressing cells in POA. Magenta signals in (A-1), (A-3), (B-1) and (B-3) show *gnrh1* mRNA, and green signals in (A-2), (A-3), (B-2) and (B-3) indicate *gnrh3* mRNA. Nuclear counterstaining using methyl green is shown as blue signals.

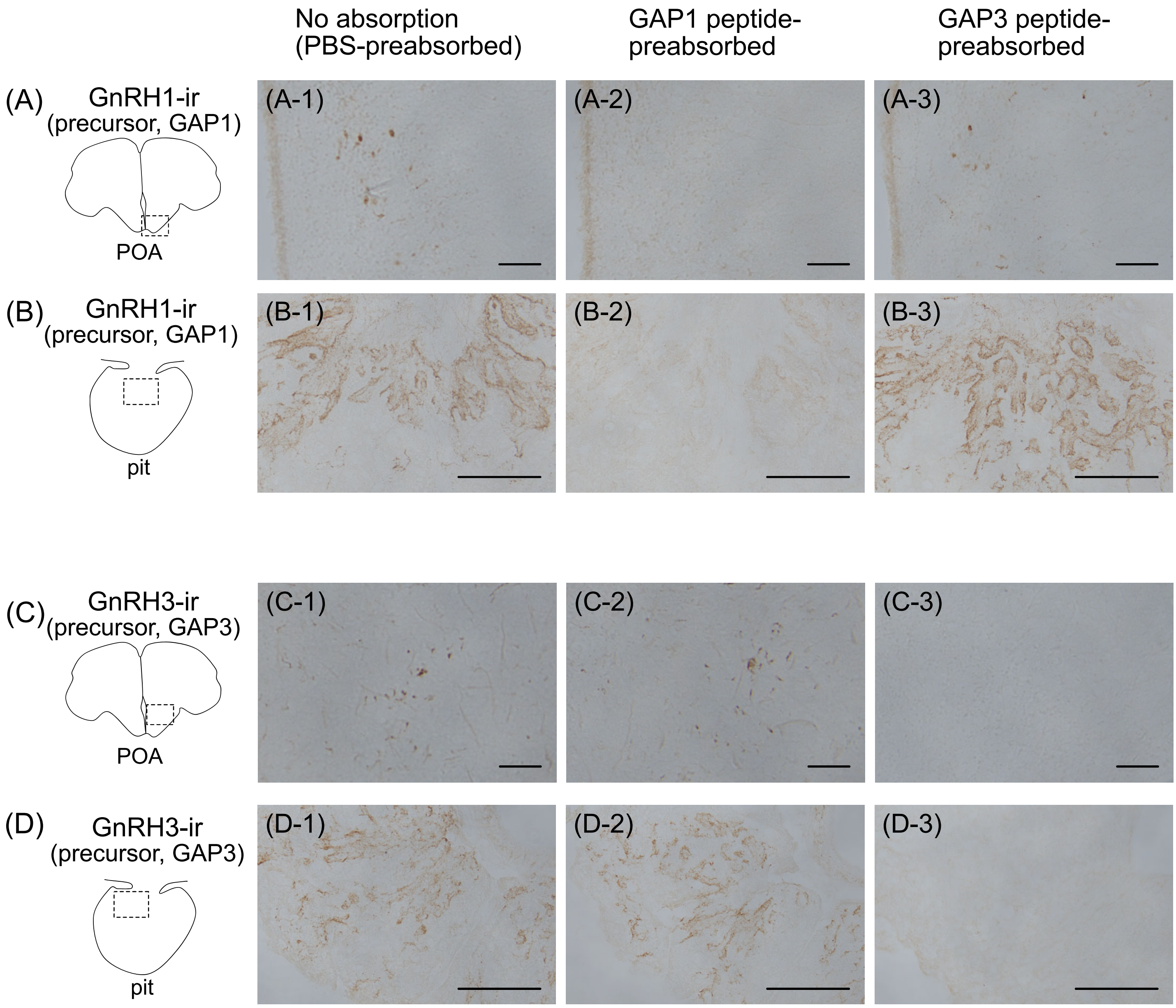

Figure S5. Specificities of produced GnRH precursor antibodies, related to Figure 1 and Figure 2.

(**A, B**) Immunohistochemistry for GnRH1 precursor of transverse section of piranha POA (**A**) and pituitary (**B**). Immunoreactive cells and fibers are labeled using a GnRH1 precursor antibody (**A-1, B-1**) and are not labeled with a GnRH1 precursor antibody preabsorbed with GnRH1 precursor peptides (**A-2, B-2**). (**A-3, B-3**) In immunohistochemistry using a GnRH1 precursor antibody preabsorbed with GnRH3 precursor peptide, immunoreactive cells and fibers are labeled. (**C, D**) Immunohistochemistry for GnRH3 precursors shows that immunoreactive cells and fibers are labeled in the POA (**C**) and the pituitary (**D**) when preabsorbed with PBS (**C-1, D-1**) or GnRH1 precursor peptide (**C-2, D-2**), but are not labeled when preabsorbed with GnRH3 precursor peptide (**C-3, D-3**). Scale bars, 100 μm.

1	AATTCTTTCTAGTCATCGTAGTTTTAAGACTGTAAAGTTTGGTCAGTAGGAGGTGGCCAAATCTGCTCTGTTTTTTTCTC	80
81	ACGGATTTGAATAAGCGTCAGTGGGTTTTTTGCCATTGATACTGCTACACAAAACACAAATCTGTGTCACATATGAAGAA	160
161	AAAGATTGGACATGGGCCACTTTTACCTGCAGTGTGAATGTAACTTATGAATCTTTAAAAAGCTCTCTTGCGTAGTAACA	240
241	GTAGCCTTGCTGTACCAGTACATATAGATCGGAGCATTGAGTTTGTGGTTTGCCAATGACTGGCAGTTAATGCATTCGTG	320
321	TTTCAGAGTTCTGGAATATTTTAGGAGTTTAAGACTCTGAAATTGGTGAAATGTTGAAGTTAGGCAGAGCAGCCTTTTCT	400
401	GAGGCTTTTCTTGATTTTCAAGGGCACGTCAGGTTCAGTTTGAAGCCATATTATATCGTGCATGCTTAAGGAGGCAAAAT	480
481	CTTGATATCTGTGTAGCATATGAAAGAAAAGGGTGATGGATTCCATAACCTCATATTTCTATATCAATTTTCTAGGTTAG	560
561	GCATGCAGTTAAATATGCAATATAAATGTATTAGTGCTGTCTGTTTTTGACGTTTCTGTTGTGCAAATGCAAATGACAGC	640
641	AGTTTGTATCACTTTCGTTTGTTTGCTGGTTTGTTCCAAATCTACCTCTCACTTAGGATACTGACCGTTTTTTTTTTTTT	720
721	TTTTTTTTTTTTTTTTTTGCTGTTTTTGTGAGTTTGGGTTTTTTTTGGCCATTGTCAATGCTGTTCTCTTGGATTCTCAT	800
801	GCGAAGGCTTGTGCTGACTGTGAGTGGATAGACGCTAGGATGTTAAATGAAAGATAAATTACCAAATTGCTGTTGTATTT	880
881	TTGAGTTATGCACACCCTGTGGCTATCAACTCGAGGTATAATGATACTAGCCTATTAATTAATATGCAGAACAGTTCACC	960
961	AGTAGCAAGACAACACTGCTTTGAATGATGATTTGTGTATTGTGTGACTGTTTCTCAACAGATATTTCTTAGTTTTTTTT	1040
1041	CTTGACATTATTAGTAGTGTAACTACTAATAAAAATGTCTTTGTTCAGGCAAAACCAAAAAGCTTTGAATGGGGTGCTTA	1120
1121	CCTGTCAGATAGAGTGCTTCCCTCAGCGTAATCGCATAAAACCGTTCCTTGCTGATTTGACAGGCCTGAAATCATTGTCA	1200
1201	CTGACCGCATTAGCATCAAGTCGTGGTCATCCGTATGCCCACTTAGCACTTATTTATATTGTCCACGCTTTGTCTGTAAG	1280
1281	CCTATTACAATAATATGTTTGTGTAGTGTTTGCTGATTCCTTACCTACTGCCCTGCAGTTCATGTAATGCTTTCAATGTT	1360
1361	AATATTATTCCCATGGGGTCAGACCTGCTCTTGATATAAGAATGCATGTCTGGAGACACTTGTTAAGCTTTTGCACACCC	1440
1441	AATCACCTGCAGACAAGGGTACGCTCCCTCTAAAACCCACCCAGATGTCTCTTGTCCAGGTCTAGAACACGAGCAAAACA	1520
1521	AGTTAGCTAGCAAAACGCTATCGTTAGCTAATTAAACTTGTTAACGCTTGCCGGGGGCTCATCTTCTTTTTTACTGTGTG	1600
1601	TTCTCCACACTTGCTGCTCACATCTACATGCTTATTTGGAGTTAATATGGGGGACTTTTATGTTACATCCATGTGCTAAC	1680
1681	AACAGGCTTCTTATACTTTGGACAGCAAACGCACAAAATTAGCAAGTCACTTTTTTTATTTTTTATTTTTTTGAGCTTGT	1760
1761	TTCCACCCAAATATATGCATGTTCCCAAAATTTCTTGTAGTTGTCCCCTAATGTTCTCGTTACGAGATCATGTCTGGCCC	1840
1841	CCATGCGAGTGTTAATATCAGCTGGATTATTGCTGTGTGGTTAAAAACTAAGTCAGACCTGTTTTCTGGCAATAGCTTAA	1920
1921	AGTTAATGGGATTCAATATGCTGTTACTTCTCTGATGTATGTAATTGTAGGTACTCCTGTTGAATGAAGGCAATCAAAGC	2000
2001	AGACTTCTGCACTTTAAGTGTGAGTCTAAATTTACTGGACCCTTGACGAAAGGGCCCAGGCTATTTATGTATGTATGTTA	2080
2081	TGTATGTAATCAGGCTATATTTATGGCAGGTGAAAAAAGAGATGTCCTCTACTTTCACCTCACTATCTTGCACTCACTAT	2160
2161	TACGGAAGTGTATTTCCATAAACAGACTGTTATAATCAAACTCGTCTTGTGTCTTCATTTTTCCCGTTTGTGTTTTTGGT	2240
2241	CTTTAATCTTGTTGTTTGAGTGAACACTCCAACTCTGTACGAGTGACGCTCAGAACGAATCATTCAAACTGCTCCATTAA	2320
2321	AATGCTTCAGTTATTCTAATCAGGTATTTTGCACGTGATAAACGCTGCAAGCGTTACAAAGAAAGGGGAACTTCAGTCGG	2400
2401	AATTTTGCCCCTGACTGGTTAGAAAGTGTCTTTGACTGGCCCAAGAGCTGTTTATGATCCCAAATTCCCAACTTAAAGGG	2480
2481	GAATAATTGAGCCTTTGAGATGTAAGCAAAGTCATTCTGAGCAGTTTGATGCGAAACGGTTCATTGTAGAGAAACTGCCT	2560
2561	CAGATTTCTTTATGGTGGTGATTGAAACCAGGAGTCACAGTGTCTACAGCATAAATCCACTTTTTTACTATCCAAAATGA	2640
2641	ACTGTACACAAGTTTTCAGACATTTTATGCCTAATTTGCATAATCACAAATTTTAAAAAGGCATCAGCCGTGTCTTCAGA	2720
2721	ACCACTGTGAGCAAGCTTTAAGATTTGATACATTTTTCTGGATTGAATCATTTCACACCAAACCACTCTGAAGGACTATT	2800
2801	TACAGCTTAACCATTTCATTATGTAGAAAAATAGACAAATCAGTGGAAGTCCAATCCAAAAAAAGGTTCAGCGTGGCTGG	2880
2881	GGACTTTCATTCATTCAACAAAGCAGGAGCTTGTGCTCAATTGTTTGGAGACTGAGCTCCACTGATTAAAGGTGCCGTGT	2960
2961	GTAAGATGTAGTGACATCTGGTGGTGAGGTTGCAGATTGCAACCAGCTAAATATTACTCTTAAATATCAATGTAACTTTT	3040
3041	GTGTCTTCATGTTTTCGCTTCTTGGATGATGATCCACCCTCCTTTGCTTTTTCTTGTGCCGCAAAATACGATCCTCCATT	3120
3121	TAATAAAGTTTGGGTCCTCAAACTTCTCTCAACACAATGATTAGCCAGCACTGGCAGCAGCCACTGATTCTTCTCTCCAC	3200
3201	GTCTGTACCACTAAATCAGCAACATAAACACGGCAGACGCTCTGGCCTACTGTAGAAACATGGCAGCGCAGCATGGCTGT	3280
3281	TGGAAGAGGACCCACTCCCTGTAGATTTTAAGGGCTCATTCTAAGTGAACGAAAACACAACAAGCTATAGTTACAGGTGA	3360
3361	TAACGCACTAATGAAAACAGGGTTTTGTATACTATATTTTGCTTCTGCCAATAGACTTCTTACACACTGCACCTTTAAAC	3440
3441	GCTCCAGCTTATTTGGAATGAAAATTTGTAGCCACACCAGCTCTGCGGATAAAATTGTACACTCCTGTTTTAGATGGTGA	3520
3521	AACAAACTATAGACCAAAGTGCACAGAAAGTAAGACATATTTACTTACAATGTATCCATCCATCCATCCATCCATTTTCT	3600
3601	AAGCCGCATCTCCGTCAGGGTCGCGGGGGGAAGCTGGAGCCTATCCCAGCAGTCTTCGGGCGGAAGGCAGGATACACCCT	3680
3681	GGACAGGTCGCCAGTCCATCACAGGGCATACACACAGACACAGACAGTCACTCACACCTAGGGACAATTTAGCACACCCA	3760
3761	ATTGGCCTGACTGCATGTCTTTGGACTGTGGGAGGAAAGAACCCGGAGGAAACCCACGCAGACACGGGGAGAACATGCAA	3840
3841	ACTCCACACAGAGAGGACCCCGGTCACCCGGCCGGGGAATCGAACCCAGGCCCTCCTTGCTGTGAGGCGACAGCGCCACC	3920
3921	GTGCCGCCCTTACTTACAATGTTTTTTTTTTTTTCTACTTACAATGTAGAAATAAAAAAACCAACATAGGCCATAGAATT	4000
4001	GTTTGAACTCACGTCCTGTTGTCTTGAAATCAACCAGAATAAAGCAAAAGAAATTTCAAACTCTTTGCAAAGACTTAGC	4080
4081	AGAAAGTATAGTGTTATATTAGAAAGCCTGGCACAACTGCACTCCTGGTCAATCACTTGGAGTTCAGTCAGTACCGTTGA	4160
4161	CTCTTCTCATTCACACTTGGGAGCACTTTAAAAACTTCAGCATTAGCCTTCTCTGAGTAATCTCTTAAAAAGGCTCAGTG	4240
4241	GGAAGTGTCTGAAAGGTGCTTACTTCCGTCATCCTTTCATTCTGCTCTTAGCCCCCTGCAGTGAGGGGCTGGAGAAGCGA	4320
4321	TTGCGTGAGACGAGCAAGAGCTCTATAAGAACACTAAAGCTGAGAGCAGGAGTGTCCAAACCCTGAAACCAGAACGGTGA	4400
4401	CGAGAAGCAGAAGCTTTCACTGGTCAGCTAAAGGTAAGTCAGATCCAAGTACGTGTCCTTCTGTTGGCACACTTAGAGCA	4480
4481	CTGGTTCACAAACCAGTCCTCAGGGCCTCTGGATGTTCCAGGTTACTGATCAGAATACCTGATGAAAGGTTTGAAAACTA	4560
4561	ATGCCTTTGAGCAATATTTAGCAGATCAGTGTCCCACAGTTCAAAGAGCTTAGACTGCTTTTGCTGGACAGTTCTGATCC	4640
4641	TAAATTTTAAAGACTTTTATTTTAAAAGGGGGTATAGGTTTAATTTGAAAAGCTAACATCCAAATTTATCTTGGAAAGCC	4720
4721	ACAGTTTCCCTTGATATCCCCTTTGGTATCAAGGCTTTTATGTTGAAAAGCCTTATTTATCCCCAATACCTGAGTATTGA	4800
4801	GACTTTGGAAAGCTACAGTTTCTATGATATTTGGGCATCTTATATTTTTAAAAAAATGCATATTTAACTCAATTGAGTGG	4880
4881	TTTTCAATGTCTGAAGGTGGCTGTTTTCTGTTGCTCTTTTCAGGATGAAGACAAGCAGTGCTCTCTTGTGGGTGATGATT	4960
4961	TGTGTTGTGGT	

Figure S6. Upstream sequences of piranha *gnrh1,* related to Figure 3 and Figure 4.

The upstream sequence of piranha *gnrh1,* which was used for generation of transgenic medaka and zebrafish, Tg(*pngnrh1*:RFP).

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1     TTTAAAACAATCATTCCTACGCACAGTGAACTAAGTGCTGTTTCTTAGAGATGAAGTGTGTCTCAGTTTAACTGTTTAGT   80
81    TATCTTGTTTGTTGTTTTCAGTGAACTGACCGCTGAGTGGAGCGCATTCAGACAAATTCAGTTTAACCGATTCAGTTTTT   160
161   CAGTATTTAATTTGTCAAACGACGTCACATTAATTTTATAACACTTAATTTTTTTTATTTGAGTTATTTGTTTACCTGTG   240
241   CACCAGTCATTAAACTCTTGGAAAAATAAATCAAAATCATTTCTTCCTGGTCCTGAGTCTTTCAACCACCACTCAGCTAT   320
321   CCTGCAACACATTATTGTACAGTAAGAAGAAATCACAGAAATTACACATTTCATTTTAGATCTTTATTTGGTAATACAAT   400
401   GTGCATATGTGTGTTTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTATGTGTGTGTGTGTGTGTGTGTGTGTGT   480
481   GTGTGTGTGTGCGCATGCATGTTGAGTCCGAAAGAGGCAAAGCGGTCCACCTTCACATACTGTTTACATAGTATAATAGT   560
561   ATACATTTAAAATAACCCTAGGATATTGCTATATTGCTTAAAAACTGTACTTACATCAGAGAGTGAGGAAACCATGCTAC   640
641   TCTGTGATGCTCCGTGCAGAGGGTCTTTGACCTAGTAGACAACCCATCTCCATATGAATGGACTGTAAAAAGGAAAAAAG   720
721   TAAATAAATCCCACATTCTTGTTGATACGGCGCCATGAGGTATAGAGATGCTTGAGGTAAATTTGAAAATTAATTTCATT   800
801   GAGCTGTATCCTAAGAAGCATGACAGAAGGATTATGTTCAATAAGAATAAAAGATGAGCGCAGACACATAAAGAAGTACA   880
881   AAGATGACGCAGCACTCTCCTGAAATGTGCTACCACTCATTCCAACATTCAAAACAGAACAGCACCCCCTGCTGTGAGTA   960
961   TCAATGCAAACCCAAATCCTCATTTCATTCACAAAAGTAGTTCACCTCAAATGTCACAATAGTCAGTTACTCAAAATCTG   1040
1041  CACAAGAAGATTGGCAGGAATTTGTGAATTCAGGCTAAGCGTGAAGTTACTTTTCATGCACTGTTAGTGATAAAGTTTCT   1120
1121  GTGCAGGTATATTTTCCCTCAAAGGTACAACAGTGGTTTTAAGGTCCAATTGTGAACCTTAAATAAGTTTTTTCCGGCTG   1200
1201  AAAAATACATATTTTCACGTTTTCATAAGCTAGTGCTTTAAAACAGAACAATAAAATAAAAGCCTGGCGATGAGACGGCG   1280
1281  TGTGCAGAGTCAATACAAATATCATTTCAGTGTATTATAGTACAGTTATGAACCCTGACTAAAGGCACAGAGATGTACCC   1360
1361  TTGAGGGTTCCATCCTAGTGACAAGAGAATAGTGCCCCAGTGGCAGTTTAGTACCTTTATTTATTAAGCGTACAATCACA   1440
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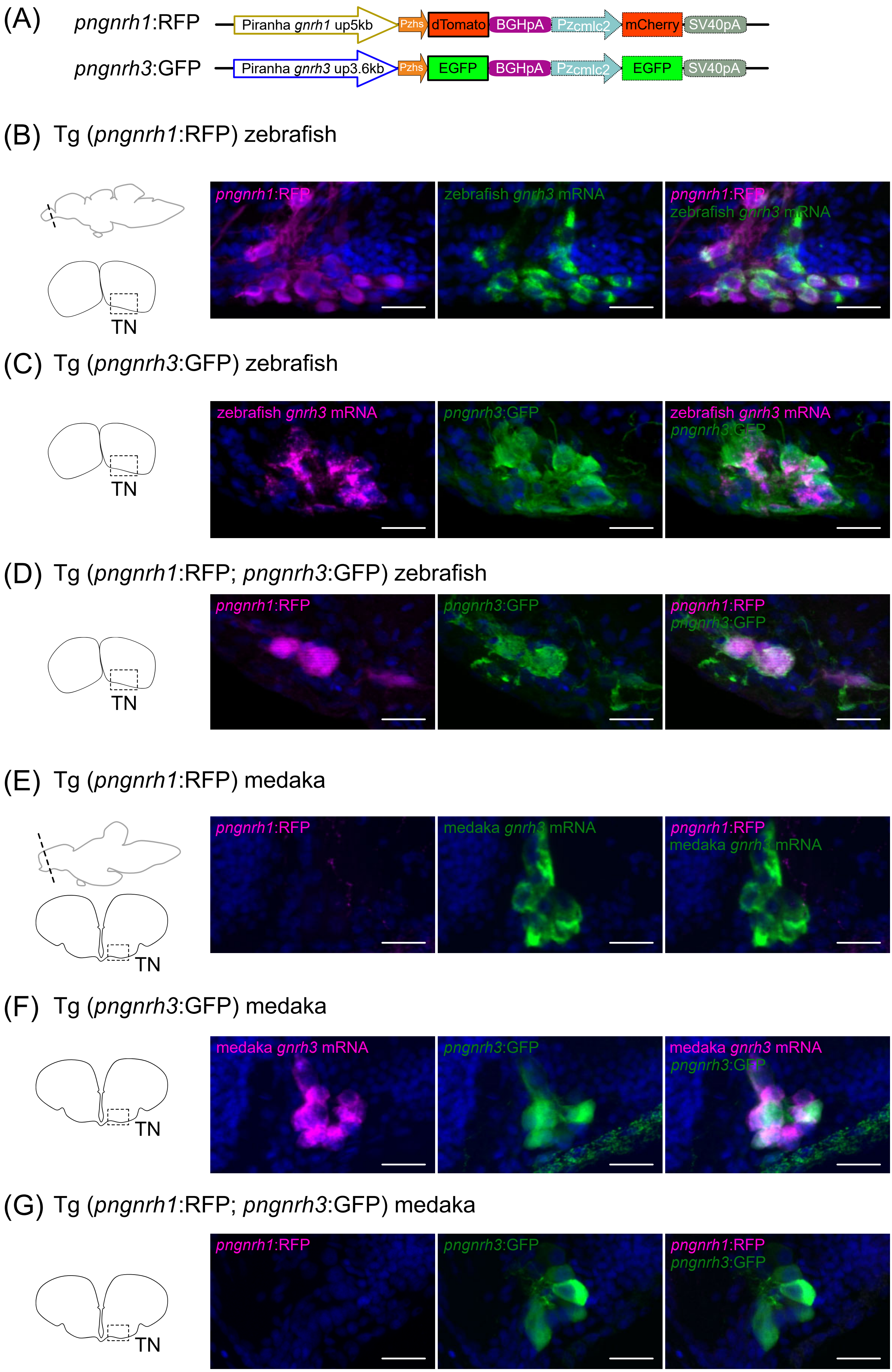
2401  CTGATGTCGTGTTTAGCTTTTTGCCATTTAGTATATTGTTAGCGAGCTGTTTCAATATATCCAGTTTACATGGCTAATCC   2480
2481  AGTGTTTTGTTTACAGTTATTTTCACCTCCATTGATTTATCGCTCTGTAGACGTTAGCTGACATTACTCTAATAGTTGTG   2560
2561  AAGGAACTTTGTCAACAAATCATTTACATTGTTTATTTAGTTTGGACTCCCACACGGTTGTGTTTGATTCATCCTATGAC   2640
2641  AAAGATAAAGTGACTCACCTTTCTGTGGTGAGCTGACGTCTATTTTCCTGAGGTTAGTAACTGTCCTGAAGGCAATAGCA   2720
2721  TATCTTGCTTTACACATCTTTTTCTTGTGAAAAGGGTCTATGGAAGTCCCTGCATGGTCTAGCTGGCTTGGATGGCCCAA   2800
2801  AAGACAAATCAGAATACCAACAAGAACATCTCCTACTACTCCTTATGGACAGTCTGAGCTTGGAGTATCCCAGTACAAAA   2880
2881  TACTGGGGTGTGGCCAGACACCACAGGCCCCCCTCTACCAATGTGACTCTCTGCCCCCCAATAGTAAAGTCACTGATCTC   2960
2961  ACTCATCTTCAGATCACCATTTTTTCTTTCAAAAAATTACTGATGACGAGCGAGGACATTTCAGCAAGTCTTGATGTTAG   3040
3041  CTAACAAAGACAAAACAGCTAGAAAAATACAAATATGTAACAATGTTACAAATAACATTAAAGTATTCTTGAGGTTTGTC   3120
3121  TTTATATGTGGTTATGGGTGAAATTCCACTATAGATCAAACACTCTAACGTTTAATGATAACAAAGCCATAAATGCTAAA   3200
3201  TGCTTCTTTTAGCATGTGCTAATCTACACTCCAGGCACTATAGTAAAGATAAGCTTTATTAAAGCTCTAGCAGCTGTGTT   3280
3281  TTCTTTGTCACTGTGAGCTGTTAAAGCATTAAAAATAAAGCTTTTGCTACATTAACCATCAGAGATAATCTCTTGTGTGA   3360
3361  AACTAAATCCTGCATTAGCATTATGCTCTGGAGACTCTTCGGAAAACTTAAGTGCTCTGTTGAAAAGCTCTGGGCTGTGA   3440
3441  CTCAGTCTGAGTAGACGGTATAAAAGCGAATAAAATTAGTTCTTGCTGTTCGGCAAC
```

Figure S7. Upstream sequences of piranha *gnrh3,* related to Figure 3 and Figure 4.

The upstream sequence of piranha *gnrh3,* which was used for generation of transgenic medaka and zebrafish, Tg(*pngnrh3*:GFP).



(A)
pngnrh1:RFP
Piranha gnrh1 up5kb
Pzhs
dTomato
BGHpA
Pzcmlc2
mCherry
SV40pA
pngnrh3:GFP
Piranha gnrh3 up3.6kb
Pzhs
EGFP
BGHpA
Pzcmlc2
EGFP
SV40pA
(B) Tg (pngnrh1:RFP) zebrafish
TN
pngnrh1:RFP
zebrafish gnrh3 mRNA
pngnrh1:RFP
zebrafish gnrh3 mRNA
(C) Tg (pngnrh3:GFP) zebrafish
TN
zebrafish gnrh3 mRNA
pngnrh3:GFP
zebrafish gnrh3 mRNA
pngnrh3:GFP
(D) Tg (pngnrh1:RFP; pngnrh3:GFP) zebrafish
TN
pngnrh1:RFP
pngnrh3:GFP
pngnrh1:RFP
pngnrh3:GFP
(E) Tg (pngnrh1:RFP) medaka
TN
pngnrh1:RFP
medaka gnrh3 mRNA
pngnrh1:RFP
medaka gnrh3 mRNA
(F) Tg (pngnrh3:GFP) medaka
TN
medaka gnrh3 mRNA
pngnrh3:GFP
medaka gnrh3 mRNA
pngnrh3:GFP
(G) Tg (pngnrh1:RFP; pngnrh3:GFP) medaka
TN
pngnrh1:RFP
pngnrh3:GFP
pngnrh1:RFP
pngnrh3:GFP

Figure S8. Examination of enhancer activity of piranha *gnrh1* and *gnrh3* (*pngnrh1*/*pngnrh3*) in the terminal nerve (TN) of zebrafish and medaka, related to Figure 3 and Figure 4.

(**A**) The constructs used to generate transgenic zebrafish and medaka. Both constructs examine the enhancer activity of piranha *gnrh1* or *gnrh3* 5'flanking regions by using a basal promoter (zebrafish heat shock promoter, Pzhs) and a fluorescent protein (dTomato or EGFP). For screening of embryos, cardiac myosin light chain 2 promoter of zebrafish (Pzcmlc2), mCherry or EGFP and SV40 poly(A) signal were inserted downstream of reporter construct. (**B**) In Tg (*pngnrh1*:RFP) zebrafish, *pngnrh1* enhancer-induced RFP expression is observed in GnRH3 neurons (*gnrh3* mRNA-expressing neurons) in the TN. (**C**) In Tg (*pngnrh3*:GFP) zebrafish, *pngnrh3* enhancer-induced GFP expression is observed in GnRH3 neurons in the TN. (**D**) Analysis of the double transgenic zebrafish, Tg (*pngnrh1*:RFP; *pngnrh3*:GFP). In Tg (*pngnrh1*:RFP; *pngnrh3*:GFP) zebrafish, some of the neurons in the TN express both RFP and GFP suggesting that *pngnrh1* and *pngnrh3* enhancers are activated in the same neurons. (**E**) In Tg (*pngnrh1*:RFP) medaka, *pngnrh1* enhancer-induced RFP expression is not observed in the GnRH3 neurons (*gnrh3* mRNA-expressing neurons) in the TN. (**F**) In Tg (*pngnrh3*:GFP) medaka, *pngnrh3* enhancer-induced GFP expression is observed in the GnRH3 neurons in the TN. (**G**) In double transgenic medaka, Tg (*pngnrh1*:RFP; *pngnrh3*:GFP), some of the neurons in the TN express only GFP suggesting that only *pngnrh3* enhancers are activated in the TN. Scale bars, 100 μm.

Table S1. The sequence of primers used in this study, related to Figure 1 and Figure 2.

For full-length cloning of piranha *gnrh1*

5'-CTSYCAGCAYTGGTCITWYGG-3'
5'-ACTGGTCNTWYGGICTIMGICCIGGIG-3'
5'-TACTGCGTGGTCACTTTCAGCTTTCGG-3'
5'-TGCAGCCGCCTGAAAAGTGCCTGTC-3'

For full-length cloning of piranha *gnrh3*

5'-ACCCTSTSYCARCAYTGGTCITAYGGITGG-3'
5'-AGCACTGGTCIYAYGGITGGYWNCCIGGIGG-3'
5'-CARCAYTGGTCITAYGGITGG-3'
5'-TATCTGCTGCAGTGGAGACTCCAGG-3'
5'-GGGGCAAAGCCACAACAGCATCACC-3'

For cloning of head-and-tail-light tetra *gnrh3*

5'-CTSYCAGCAYTGGTCITWYGG-3'
5'-ACTGGTCNTWYGGICTIMGICCIGGIG-3'
5'-CTACTTTAGCGCTTCAGAATCTTCCTCA-3'
5'-TCTCCAGCGTCCATCATTCTGAA-3'
5'-GTGATCTGCTGCAGTGGAGAGTT-3'

For genotyping of piranha *gnrh1* KO

5'-GGCTGTTTTCTGTTGCTCTTTTCAGGATG-3'
5'-TACCGCCTGAAAAGTGCCTGTCA-3'

Supplemental References

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