

# Primary Structure and Function of Three Gonadotropin-Releasing Hormones, Including a Novel Form, from an Ancient Teleost, Herring\*

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## ABSTRACT

The evolution of GnRH and the role of multiple forms within the brain are examined. Three forms of GnRH were purified from the brain of Pacific herring (*Clupea harengus pallasii*) and characterized using Edman degradation and mass spectrometry. Two forms correspond with the known structures of chicken GnRH-II and salmon GnRH that are found in many vertebrate species. The third form, designated herring GnRH (hrGnRH), has a primary structure of pGlu-His-Trp-Ser-His-Gly-Leu-Ser-Pro-Gly-NH<sub>2</sub>. This novel peptide is a potent stimulator of gonadotropin II and GH release from dispersed fish pituitary cells. The content of hrGnRH in the pituitary was

8-fold that of salmon GnRH and 43-fold that of chicken GnRH-II, which provides supporting evidence that hrGnRH is involved in the release of gonadotropin.

Herring is the most phylogenetically ancient animal in which three forms of GnRH have been isolated and sequenced. Our evidence suggests that the existence of three GnRHs in the brain of one species 1) is an ancestral condition for teleosts, 2) has the potential for separate regulation of the distinct GnRHs, and 3) may be an evolutionary advantage for refined control of reproduction in different environments. (*Endocrinology* 141: 505–512, 2000)

GnRH IS A PEPTIDE best known for its action in releasing gonadotropins from the pituitary of vertebrates. Other proposed functions of GnRH include neuromodulation in various parts of the central nervous system (1, 2) and local actions in the gonads (3–6) and placenta (7, 8). In fish, GnRH also releases GH (9) and PRL (10) from the pituitary.

Studies on the evolution of GnRH indicate that GnRH is present in all vertebrate species studied to date (11) as well as in some invertebrates (12–16). Twelve different forms of GnRH were initially identified by primary structure or complementary DNA (11, 17). Each identified GnRH peptide has a length of 10 amino acids and identical residues in positions 1, 4, 9, and 10. This conservation of structure is also present in the organization of the GnRH genes. Only one form of GnRH is encoded in each gene or complementary DNA isolated to date (11). The question arises of whether there have been other changes that alter the central GnRH control of reproduction and whether diversification of GnRH peptides is important in this process.

One possibility for change in the control of reproduction

is the duplication of GnRH genes with subsequent nucleotide substitutions in the GnRH-encoding region or in the regulatory flanking region. This could result in individual species having more than one GnRH form, each with a distinct function and location within the brain. We know that at least two forms of GnRH are present in the brain in both rhesus monkeys (18–20) and humans (21, 22). One of these two GnRH forms in the primate species is the most widely distributed and possibly the most ancient form of GnRH in jawed vertebrates (11); it is named chicken GnRH-II (cGnRH-II) because it was first isolated from chicken brains (23). The other GnRH form in primates is mammalian GnRH (mGnRH), which was first isolated from pigs and sheep, but is common to all mammals (24, 25); mGnRH is now considered an ancient form because it is present also in early bony fish (26, 27) and amphibians (28). Other GnRH forms that are not present in mammals include salmon GnRH (sGnRH), which is present in most teleosts studied. cGnRH-I is found only in birds (29) and reptiles (30). These two forms may have arisen as a result of nucleotide substitutions in the mGnRH gene. The mGnRH form disappears as sGnRH (in teleosts) and cGnRH-I (in reptiles) appear in the same brain location as mGnRH does in other species (31). Additional forms of GnRH have been isolated and sequenced from jawless fish (32, 33), cartilaginous fish (34, 35), catfish (36), and the more recent perciform fishes such as seabream (37).

A major problem in understanding the role of GnRH in reproduction is the functional significance of multiple GnRH forms in all vertebrates. One approach to this question is to

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examine bony fish at one of the earliest points where three forms of GnRH are expressed in the brain rather than the two forms that are detected in tetrapods. The appearance of a novel third form of GnRH in the brain is of great interest.

As a representative of an early evolving teleost group, the herring was selected as an appropriate fish in which to examine these questions. The Pacific herring is an extant representative of a basal teleost group (order: Clupeiformes) that evolved before the euteleosts; the latter group includes fish such as salmon, catfish, and seabream.

## Materials and Methods

### Brains and pituitaries

Brains were collected from 1200 adult Pacific herring (*Clupea harengus pallasii*) of both sexes about 1 week after spawning. The fish were captured by purse seine in February before completing sexual recrudescence; they were held in ocean pens at the Pacific Biological Station (Nanaimo, Canada) until they spawned in June. Brains, with and without pituitaries, were removed and frozen on dry ice. Isolated pituitaries were removed from an additional 50 unspawned mature and 140 spawned herring. All animal studies were approved by the animal care committees of University of Victoria or University of Alberta.

### Extraction of GnRH

Frozen tissues were powdered in a Waring blender with liquid nitrogen and extracted in acidified acetone as described previously (36). After filtration, lipids and most of the acetone were removed by repeated extractions of the filtrate with petroleum ether; the remaining acetone was removed from the crude extract with vacuum centrifugation.

### Purification of GnRH

Purification of GnRH was performed by HPLC and RIA in six steps for the brain extract and in four steps for pituitary extract. A HPLC pump (model 166, Beckman Coulter, Inc., Palo Alto, CA) was used for the preparative chromatography. Fractions of 1 ml were collected from each chromatography step, and aliquots were assayed for immunoreactive GnRH (irGnRH). The absence of residual contaminating GnRH on the column was confirmed before purification of GnRH by collecting and assaying fractions of blank runs.

The brain extract was applied to a column of 10 C<sub>18</sub> Sep-Pak cartridges (Waters Corp., Milford, MA) in step 1 and eluted with mobile phases A [0.5% trifluoroacetic acid (TFA)] and B [0.5% TFA, 80% acetonitrile (ACN)] in the program shown in Fig. 1A. In step 2, the immunoreactive fractions were applied to a C<sub>18</sub> HPLC column and eluted with mobile phases A [0.25 M triethylammonium formate (TEAF), pH 6.5] and B (100% ACN), as shown in Fig. 1B. Thereafter, each of the 3 immunoreactive peaks was purified separately, but in the same sequence. Step 3 was based on the procedure described by Rivier (38); fractions were applied to a C<sub>18</sub> column and eluted with 0.13 M triethylammonium phosphate (TEAP), pH 2.5 (mobile phase A), and ACN (Fig. 1C). Step 4 used a C<sub>18</sub> column and elution with 0.05% TFA and 0.05% TFA/80% ACN, whereas step 5 used a phenyl column and the same mobile phases as those in step 4 (Fig. 1, D and E). The final step (not shown) was microbore HPLC on a Vydac C<sub>18</sub> column using 0.1% TFA and 0.1% TFA/80% ACN.

The initial step of purification of the pituitary extract is shown in Fig. 1F; only the earliest eluting peak was purified further using the methods shown in Fig. 1, C and E. The final step was microbore HPLC as described for the brain extract.

### RIA

During the purification of GnRH herring extracts, 10- $\mu$ l aliquots of each 1-ml fraction were assayed for irGnRH using methods described previously (27). Antiserum GF-4 (1:25,000 final dilution) was used to detect irGnRH, with mGnRH used as the iodinated tracer and standard. The GF-4 antiserum has been shown to detect several known forms of GnRH: mGnRH, 100%; cGnRH-I, 44%; catfish GnRH, 44%; sea bream

GnRH (sbGnRH), 41%; dogfish GnRH, 5%; lamprey GnRH-III, less than 0.4%; and lamprey GnRH-I, less than 0.03% (19). The cross-reactivities of GF-4 with peptides used in this paper were herring GnRH (hrGnRH), 17%; sGnRH, 29%; and cGnRH-II, 7%; the assay detection limit was [bound/free ratio (B/Bo), 80%] 10 pg. Where tracer binding was outside the assay limits (B/Bo, <20%), aliquots were diluted serially, and the value closest to 50% B/Bo was used for analysis.

### Sequencing of peptides

Peptides in the three purified irGnRH components from herring brain extraction and one purified irGnRH component from herring pituitaries were sequenced using a model 470A PE Applied Biosystems protein sequencer (Foster City, CA). Sequencing was initially attempted on 10% of each sample. Failure of this sequencing indicated a blocked N-terminus. Subsequent sequencing was carried out on the remaining material after digestion with pyroglutamate aminopeptidase and microbore HPLC purification, as detailed previously (39).

### Mass spectrometry

The intact molecular mass of hrGnRH was determined on a Bruker Reflex time of flight instrument using an accelerating voltage of 31 kV and a reflectron voltage of 30 kV (100-MHz digitizer). The sample was applied to a thin layer of  $\alpha$ -cyano-4-hydroxycinnamic acid, allowed to dry, and rinsed with water before analysis. To establish whether the C-terminus of the peptide was amidated, esterification with methanolic HCl monitored with matrix-assisted laser desorption ionization-mass spectrometry was used (40, 41). Control reactions were carried out with bombesin and bombesin free acid, where a mass shift of 14 Da was observed only for the free acid form.

### Synthesis of peptides

The peptides for hrGnRH, sGnRH, cGnRH-II, and sbGnRH were synthesized using a solid phase method on a methylbenzhydrylamine resin as previously described (37, 42).

### Physiology

To investigate the physiological activity of GnRH peptides, the ability of synthetic hrGnRH, sGnRH, cGnRH-II, and sbGnRH to stimulate pituitary hormone release from primary cultures of dispersed goldfish pituitary cells was examined in 2-h static incubation studies. Goldfish pituitaries were used because it has been shown that multiple native GnRH forms stimulate the release of hormones (35) and because assays are available for goldfish, but not herring, pituitary hormones. Pituitary cells from both male and female goldfish were prepared by trypsinization, as previously described (43). Cells were cultured overnight at a density of  $0.25 \times 10^6$  cells/ml-well in 24-well culture plates in culture medium (medium 199 with Earle's salts, supplemented with 25 mM HEPES, 2.2 g/liter NaHCO<sub>3</sub>, 1% horse serum, 100,000 U/liter penicillin, and 100 ng/liter streptomycin, pH adjusted to 7.2 with NaOH; Life Technologies, Inc., Grand Island, NY) at 28 C under 5% CO<sub>2</sub> and saturated humidity. Before the experiment, cells were washed with 1 ml testing medium (medium 199 containing Hanks' salts, 0.1% BSA, 2.2 g/l NaHCO<sub>3</sub>, 100,000 U/liter penicillin, and 100 mg/liter streptomycin, pH 7.2) and allowed to rest in the incubator for at least 1 h. The cells were then washed again with 1 ml fresh testing medium, and concentrated GnRH solutions were added (in distilled deionized water; 1  $\mu$ l/ml to achieve the final desired GnRH concentration). All treatments were performed in triplicate or quadruplicate. Cells were then returned to the incubator for the duration of secretion testing (2 h). At the end of the testing period, 800  $\mu$ l medium were collected from each well. All experiments were repeated four times (twice each with cells prepared from fish at sexually regressed and recrudescence stages). All samples were stored at -20 C until their gonadotropin II contents were quantified by RIA validated for measurements of goldfish maturational gonadotropin (GTH-II) (44, 45) and GH (46). Results were expressed as a percentage of basal release (unstimulated controls), and pooled data (mean  $\pm$  SEM) are presented. Statistical analyses were performed using ANOVA fol-

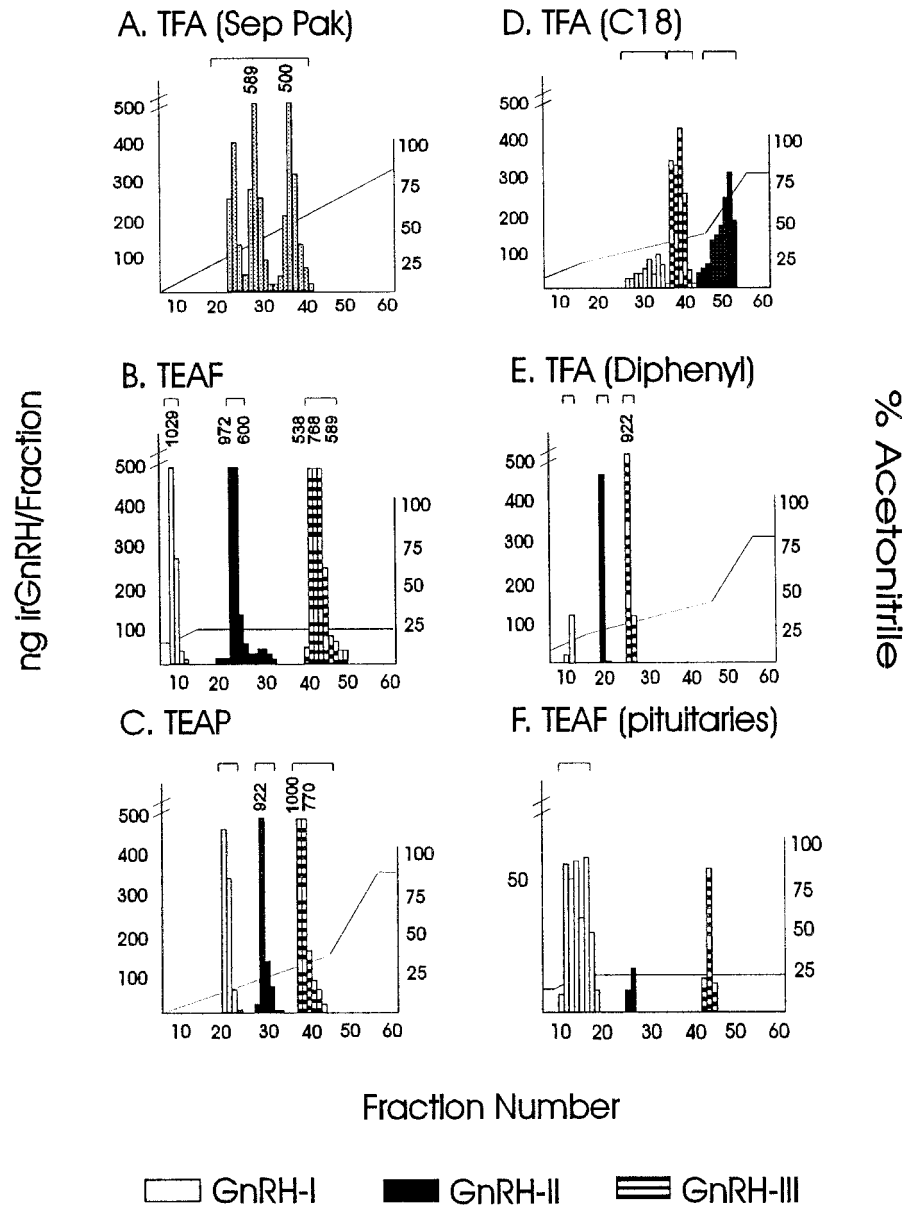


FIG. 1. Elution of irGnRH during chromatographic purification of an extract of herring brains or pituitaries. Letters indicate purification steps. GnRH immunoreactivity of fractions is indicated by bars; different bar graph patterns indicate separate HPLC purification of the three areas indicated in step B. The fractions that were further purified are indicated by horizontal brackets over the bars in each step. The first step of purification for pituitary irGnRH is shown in F (note that the vertical scale is changed); further purification was performed using the same method as that in C and E. The final step with microbore HPLC is not shown for the brain or pituitary extracts. Lines indicate the acetonitrile concentration of the mobile phase.

lowed by Fisher's least significant difference test. Differences were considered significant at  $P < 0.05$ .

**Results**

*irGnRH from herring brains*

Step A of the purification with the Sep-Pak column resulted in a broad elution of 3.8 μg irGnRH (Fig. 1A). The fractions with irGnRH were pooled and carried to step B. HPLC elution of the material in step B resulted in three distinct peaks of immunoreactive GnRH that were designated sequentially according to elution time as herring I-III (Fig. 1B) and were pooled individually. Each pool was carried through the subsequent purification steps individually (presented as composite graphs in Fig. 1, C-E) until single absorption peaks were obtained. At the end of step E, the amounts were 0.12, 0.45, and 1.04 μg herring I, II, and III irGnRH, respectively.

*irGnRH from pituitaries*

From the pituitary extract, three peaks of irGnRH eluted in positions comparable to those of the brain extract (Fig. 1F). Peaks I, II, and III contained 301, 3, and 68 ng irGnRH, respectively. Only peak I was purified further.

*Sequence and mass spectral analysis*

The following three amino acid sequences were deduced from the combination of pyroglutamyl aminopeptidase digestion and Edman degradation of the purified immunoreactive components: herring I (hrGnRH), [pGlu]-His-Trp-Ser-His-Gly-Leu-Ser-Pro-Gly; herring II (cGnRH-II), [pGlu]-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly; and herring III (sGnRH), [pGlu]-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly.

The accession numbers in SWISS PROT are P81749 (hrGnRH-I), P81750 (hrGnRH-II), and P81751 (hrGnRH-III).

Figure 2 shows the matrix-assisted laser desorption ionization mass spectrum of the untreated hrGnRH in which an intense species was observed at  $m/z$  1087.9 compared with the calculated monoisotopic  $[M+H]^+$  of 1087.51 Da for the sequence of hrGnRH (herring I) shown above. Treatment with methanolic HCl did not result in a mass shift, indicating that the C-terminus was amidated. We have named the novel structure hrGnRH in accordance with the convention of naming GnRH molecules after the organism from which the primary structure is first determined. The deduced sequences and the elution characteristics during purification of herring II and herring III corresponded to those previously described for chicken GnRH-II (23) and salmon GnRH (47), as shown in Fig. 3. Sequencing of the irGnRH form purified from herring pituitary yielded the same sequence as hrGnRH (herring I) isolated from the herring brain.

#### Physiological effects

**Gonadotropin II release.** The release of gonadotropin II from goldfish pituitary cells after the addition of the synthetic forms of hrGnRH, sGnRH, cGnRH-II, or sbGnRH is shown in Fig. 4A. sGnRH and cGnRH-II, two GnRH forms that also are native to the goldfish, stimulated gonadotropin II release in this as in previous studies (43). Similarly, hrGnRH increased gonadotropin II release from goldfish pituitary cells. For these three GnRHs, the lowest concentration of GnRH tested (0.1 nM) significantly elevated gonadotropin II secretion above basal values and the maximal gonadotropin II responses elicited were not different from one another. The efficacies of sGnRH and hrGnRH were also not different throughout the entire dose range examined. On the other hand, cGnRH-II was consistently more effective than either sGnRH or hrGnRH in the lower dose range (1–100 nM;  $P < 0.05$ ; cGnRH-II vs. sGnRH and hrGnRH). In contrast to the other GnRHs, sbGnRH failed to stimulate gonadotropin II secretion throughout the dose range tested (0.1–1000 nM).

**GH release.** sGnRH and cGnRH-II stimulated GH release from goldfish pituitary cells in a previous study (43) and in the present study (Fig. 4B). Also, hrGnRH and sbGnRH elevated GH secretion, and the maximal GH responses to

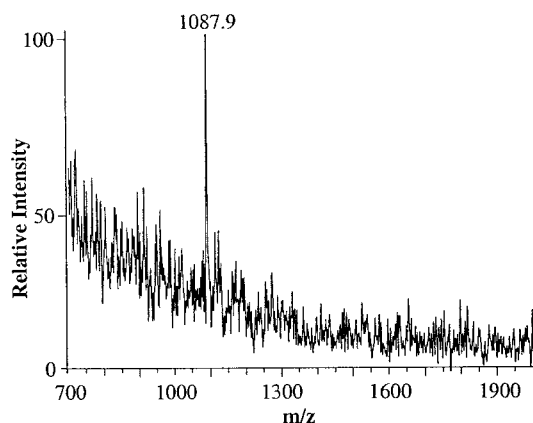


FIG. 2. Mass spectral chromatograph of purified irGnRH from herring brains. The vertical axis indicates the relative intensity of the signal; the horizontal axis indicates the mass/charge ratio. The signal from the purified peptide ( $m/z$  1087.9) is labeled.

GnRH	AMINO ACID SEQUENCES										Ref.	
	1	2	3	4	5	6	7	8	9	10		
Herring	pGlu	His	Trp	Ser	His	Gly	Leu	Ser	Pro	Gly	NH <sub>2</sub>	
Catfish	-	-	-	-	-	-	-	Asn	-	-	-	5,21
Seabream	-	-	-	-	Tyr	-	-	-	-	-	-	13
Chicken-II	-	-	-	-	-	-	Trp	Tyr	-	-	-	19
Dogfish	-	-	-	-	-	-	Trp	Leu	-	-	-	14
Chicken-I	-	-	-	-	Tyr	-	-	Gln	-	-	-	22
Mammal	-	-	-	-	Tyr	-	-	Arg	-	-	-	23,24
Salmon	-	-	-	-	Tyr	-	Trp	Leu	-	-	-	20
Lamprey-III	-	-	-	-	-	Asp	Trp	Lys	-	-	-	25
Guinea pig	-	Tyr	-	-	Tyr	-	Val	Arg	-	-	-	26
Tunicate-I	-	-	-	-	Asp	Tyr	Phe	Lys	-	-	-	4
Tunicate-II	-	-	-	-	Leu	Cys	His	Ala	-	-	-	4
Lamprey-I	-	-	Tyr	-	Leu	Glu	Trp	Lys	-	-	-	27

FIG. 3. Comparison of known GnRH structures with herring GnRH. Only amino acid residues different from those in the comparable position of the hrGnRH structure are shown.

sGnRH, cGnRH-II, and hrGnRH were similar (Fig. 4B). Both sGnRH and cGnRH-II were effective at the lowest (0.1 nM) dose tested; however, the GH responses were less sensitive to hrGnRH and sbGnRH stimulation. The minimal effective dose for hrGnRH was 100 nM, and that for sbGnRH was 1000 nM.

## Discussion

### Proof of three forms of GnRH in a single species

We have identified the primary structure of three forms of GnRH isolated from the brain of herring: one novel GnRH, one identical to cGnRH-II, and one identical to sGnRH. Both chemical sequencing and mass spectrometry were used in the characterization. The newly discovered GnRH form has the lowest mass (1088 Da) and greatest hydrophilicity of any natural GnRH yet described.

hrGnRH differs from other known forms primarily in positions 5–8 (Fig. 3), which is consistent with the idea that the C- and N-terminals are conserved because they are needed for conformation and receptor binding (48). The histidine in position 5 and the serine in position 8 are probably responsible for the high hydrophilicity compared with those of the other forms of GnRH. The forms of GnRH that have the highest sequence identity (90%) with hrGnRH are sbGnRH, which differs only in position 5 (Tyr), and catfish GnRH (cfGnRH), which differs in position 8 (Asn). Both sbGnRH and cfGnRH are also hydrophilic. hrGnRH differs from mGnRH by two residues.

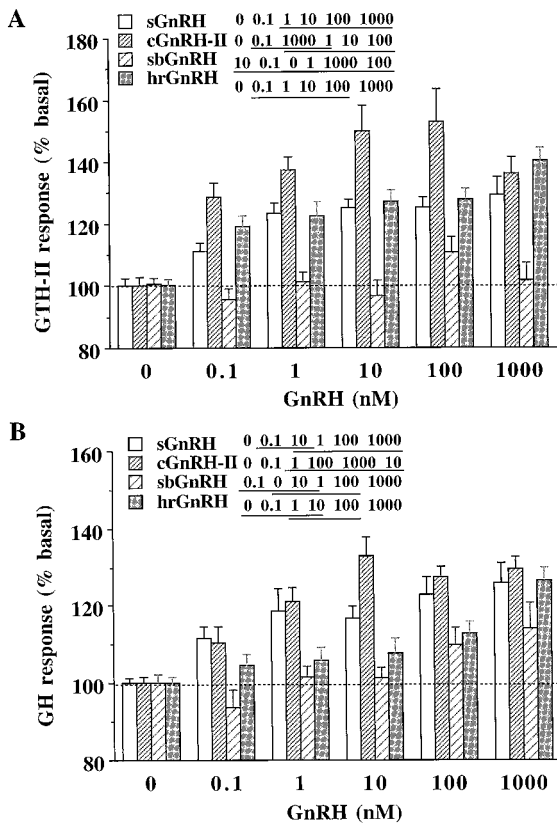


FIG. 4. Comparison of the abilities of sGnRH, cGnRH-II, hGnRH, and sbGnRH in stimulating gonadotropin-II (GTH-II; A) and GH (B) release from dispersed goldfish pituitary cells. Pooled results (mean  $\pm$  SEM) from four replicate experiments are presented. Average basal GTH-II and GH secretions were  $161 \pm 17$  and  $618 \pm 32$  ng/ml, respectively. *Inset*, For each GnRH peptide, doses resulting in responses that are not different from one another are identified by the same *underscore* (by ANOVA followed by Fisher's least significant difference test,  $P > 0.05$ ).

#### Deduction of separate location and function for GnRH forms

The function of the three forms of GnRH in the herring can be deduced in part from other studies of teleosts. Published research, including the use of both specific antisera and *in situ* hybridization, shows there are three distinct clusters of GnRH-containing cell bodies: 1) the terminal nerve ganglion (TN) at the junction between the olfactory bulb and telencephalon, 2) the preoptic area (POA) just anterior to the hypothalamus, and 3) the midbrain tegmentum (MT) (49). The TN GnRH fibers project throughout the brain and spinal cord, but usually not to the pituitary; the POA GnRH cells project to the pituitary; and the MT GnRH neurons project mainly to brain areas posterior to the hypothalamus and to the spinal cord (50). In all of the teleosts studied, only cGnRH-II was found in the MT. In teleosts such as salmon (or catfish), in which only two forms of GnRH are detected in the brain, sGnRH (or cfGnRH) was present in both the TN and POA cluster of neurons (51–53). In recently evolved teleosts (such as sea bream, cichlid, or tilapia) that have three forms of GnRH in the brain, cGnRH-II was in the MT, sGnRH was in the TN, and the new form of GnRH (sbGnRH) was in the POA (49, 54, 55). It is the new form of GnRH that is

thought to be the principal form that induces gonadotropin release from the pituitary. This conclusion is based on evidence that the new form is the most abundant GnRH in the pituitary (41, 56), and the new form of GnRH is expressed in neurons in the preoptic area of the brain, with axons terminating in the pituitary. GnRH in the olfactory region has been suggested to couple sensory information from the environment with reproduction, although the evidence is based primarily on the distribution of GnRH neurons; GnRH in the midbrain has been related to reproductive behavior.

In the present study, the highest content of GnRH in the pituitary is the herring form, which is also the newly evolved GnRH form. The level of hrGnRH was 8-fold higher than that of sGnRH and 43-fold higher than that of cGnRH-II in the HPLC fractions after each peak was corrected for relative cross-reactivity in our assay (16.7% for hrGnRH, 6.6% for cGnRH-II, and 29.1% for sGnRH). The isolation and determination of primary structure for hrGnRH from pituitary tissue confirm that this novel form exists in the pituitary and is not an artifact of immunological cross-reactivity. These data suggest that hrGnRH is located in the POA and controls the release of gonadotropin from the pituitary.

hrGnRH is clearly effective in releasing gonadotropin II and GH from fish pituitary cells, with a maximal response similar to that observed for sGnRH and cGnRH-II. hrGnRH was as effective as sGnRH in releasing gonadotropin II. On the other hand, hrGnRH is less potent than both sGnRH and cGnRH-II in increasing GH release. Surprisingly, seabream GnRH, the form responsible for gonadotropin II release in fish that are more recently evolved than herring (e.g. sea bream and cichlids) is ineffective in stimulating gonadotropin II release and is relatively ineffective in stimulating GH release in the goldfish assay. This observation is consistent with the low potency of sbGnRH compared with sGnRH and cGnRH-II when injected *in vivo* into sea bream; the concentration of gonadotropin II in sequential blood samples was measured after injection of each peptide (57). Thus, a single amino acid difference in position 5 (Tyr in sbGnRH and His in hrGnRH) results in a considerable difference in potency for activating receptors on the goldfish gonadotropes and somatotropes.

#### Phylogenetic argument for GnRH gene duplication

Herring is the most phylogenetically ancient animal in which three forms of GnRH have been identified by determination of primary structure. This evidence suggests that a GnRH gene duplication occurred in an ancestral fish that gave rise to herring and possibly to the euteleostean line that evolved after herring. We suggest that the gene encoding hrGnRH resulted from modification of one copy of a duplicated GnRH gene. Evolutionary selection of fish with more refined control of reproduction may be one factor in the success of the teleosts, the vertebrate group with the largest number of individuals and species (36% of all vertebrate species) (58).

The novelty of the hrGnRH sequence is important in considering whether a gene duplication with subsequent modification of one copy occurred. The suggestion of duplication rests on both the novel structure and the conservation of

GnRH gene organization in which only one form of GnRH is encoded in each gene in the seven forms studied to date (11).

The proof that herring is the first major group of bony fish to have three forms of GnRH has been provided by an examination of a number of species that evolved before herring. In bony fish (class: Osteichthyes), there are four major divisions of bony fish that evolved before the teleosts. The same two forms of GnRH (mGnRH and cGnRH-II) have been identified in representative fish (reedfish, sturgeon, garfish, and amia) in each division by HPLC-RIA methods (27) and by primary structure for sturgeon (26) (Fig. 5). Thereafter, the teleosts arose as a monophyletic group and as the most advanced and recent group of bony fish. The same two forms of GnRH were found by HPLC and RIA, but have not yet been found by primary structure in the earliest teleosts to evolve, the eels and one of the bonytongued fish (butterfly fish) (62). However, the mGnRH form disappeared, and the sGnRH form appeared, most likely as a substitution within the mGnRH gene. The new combination of sGnRH and cGnRH-II has been detected by HPLC-RIA methods in a number of bonytongued fish (featherfins, arawana, and elephantnose) (62).

In the next major group (Clupeomorpha) to evolve, which includes the herring, both of the two GnRH forms (sGnRH and cGnRH-II) are retained, and a new form (hrGnRH) has appeared. The presence of three forms of GnRH in a basal

teleost such as herring indicates that three GnRH genes were present early in the teleostean stem line. It is possible that a third form of GnRH will be identified in the two orders of teleosts that evolved before herring, but the crucial point is that three forms evolved early in teleosts, and this coincides with new evidence that a genomic duplication occurred in early teleosts (63). If teleosts are monophyletic (64, 65), then all teleosts evolving after the herring taxon would be expected to have three forms of GnRH. The presence of three forms is documented for euteleosts in the orders of Characiformes, Scorpaeniformes, Perciformes, and Pleuronectiformes. However, some euteleosts have only two forms of GnRH. Possible explanations are that the ancestral fish for herring did not give rise to all ancestral euteleosts or that a third GnRH is present in all euteleosts, but has not been detected in some fish. Another explanation is that in tetraploid fish (*e.g.* salmon, goldfish, catfish, and zebrafish), the gene encoding the new GnRH is lost due to deletion of the gene or chromosome. In euteleosts that have three forms of GnRH, the first two forms remain the same as in herring, but the third form (seabream GnRH) has one amino acid change compared with hrGnRH, and this is probably due to nucleotide substitution.

#### *Evolutionary advantage for separate control of expression for each GnRH form*

In herring we can deduce the brain locations of the three forms of GnRH: sGnRH is in the TN region, hrGnRH is in the POA, and cGnRH-II is in the MT. The evidence is based on the definitive identification of hrGnRH in the pituitary at 8- to 43-fold the content of the other forms. In teleosts the axons of POA-GnRH neurons are distributed to the pituitary. Expression of distinct GnRH forms in separate locations implies that at least some regions in the regulatory 5'-flanking region of each of the three genes are different.

Separate control of expression of the three GnRHs within the brain would offer an evolutionary advantage. The TN GnRH neurons connect the olfactory region with the retina and other parts of the brain. Some of these neurons synapse onto the POA neurons. The MT neurons with cGnRH-II may influence reproductive behavior, and many of their axons enter the spinal cord. Recent evidence shows that sGnRH and cGnRH-II can alter spawning behavior in goldfish (66).

Separate control of the three GnRHs is important when each form has a different location and a different function. All three GnRHs can release gonadotropin II if used in an *in vitro* assay, but *in vivo* only the GnRH form in the POA neurons is delivered in abundance to the pituitary. There are several possibilities for the advantage of separate control of the new form of GnRH, which releases gonadotropin II.

First, ovulation in herring can be separated from spawning by 1 or 2 months. When spawning does occur, it is rapid and almost simultaneous for all males and females in the school of herring. This mass spawning appears to be under the control of a male pheromone in the milt (67), and thus it may be a considerable advantage for all females in the school to hold the ovulated eggs until the best conditions for spawning have been detected. Separate control of hrGnRH for ovula-

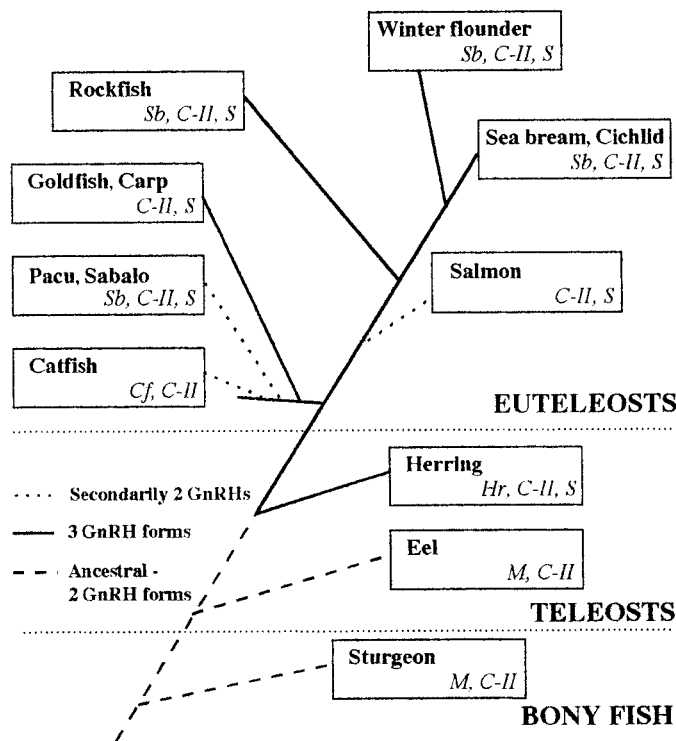


FIG. 5. Proposed evolutionary scheme for GnRH multiplicity in teleosts, showing representative fish and their forms of GnRH enclosed in boxes. Each box represents a different order of fish. See text for references other than for eels (59), pacu (56), sabalo (60), and winter flounder (61). GnRH forms in pacu are identified by primary structure; GnRH forms in eels, sabalo, and flounder are identified by HPLC-RIA methods.

tion and of sGnRH and cGnRH-II for sensory detection or behavior in spawning would be useful.

Second, many prolific and phylogenetically recent fish with three forms of GnRH release their eggs in batches, either daily or periodically, over a prolonged period of time. The advantage would be to avoid "putting all the eggs in one basket," that is in a single environmental context. For example, sea bream release batches of eggs daily for several months, and tilapia spawn many times a year. The advantage of the third GnRH (sbGnRH) found in both of these fish is that it has a low potency, as measured in sea bream (57), which may result in ovulation of only fully mature eggs. If sbGnRH were more potent, immature eggs might be released, and batch spawning lost.

Evolutionary pressure may have selected fish with three forms of GnRH provided that each form has a distinct location and/or function. Initially, the duplicated form of the GnRH gene would be a neutral change. At the point where the GnRH sequence changed in one gene and where regulation of expression was distinct from the original gene, then selection could occur. The refined control of reproduction may be a considerable advantage in fish in which spawning occurs many times over a prolonged period of time or spawning is delayed to match optimal environmental conditions. Increased survival of the offspring is likely to occur in both types of fish.

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### References

- Oka Y, Matsushima T 1993 Gonadotropin-releasing hormone (GnRH)-immunoreactive terminal nerve cells have intrinsic rhythmicity and project widely in the brain. *J Neurosci* 13:2161-2176
- Pfaff DW, Schwanzel-Fukuda M, Parhar IS, Lauber AH, McCarthy MM, Kow L-M 1994 GnRH neurons and other cellular and molecular mechanisms for simple mammalian reproductive behaviors. *Recent Prog Horm Res* 49:1-25
- Clayton RN, Harwood JP, Catt KJ 1979 Gonadotropin-releasing hormone analogue binds to luteal cells and inhibits progesterone production. *Nature* 282:90-92
- Clayton RN, Katikineni M, Chan V, Dufau ML, Catt KJ 1980 Direct inhibition of testicular function by gonadotropin-releasing hormone: mediation by specific gonadotropin-releasing receptors in interstitial cells. *Proc Natl Acad Sci USA* 77:4459-4463
- Hsueh AJW, Erickson GF 1979 Extrapituitary action of gonadotropin-releasing hormone: direct inhibition of ovarian steroidogenesis. *Science* 204:854-855
- Hsueh AJW, Erickson GF 1979 Extra-pituitary inhibition of testicular function by luteinizing hormone releasing hormone. *Nature* 281:66-67
- Khodr G, Siler-Khodr TM 1978 The effect of luteinizing hormone-releasing factor on human chorionic gonadotropin secretion. *Fertil Steril* 30:301-304
- Kelly AC, Rodgers A, Dong KW, Barrezaeta NX, Blum M, Roberts JL 1991 Gonadotropin-releasing hormone and chorionic gonadotropin gene expression in human placental development. *DNA Cell Biol* 10:411-421
- Marchant TA, Chang JP, Nahorniak CS, Peter RE 1989 Evidence that gonadotropin-releasing hormone also functions as a growth-hormone releasing factor in the goldfish. *Endocrinology* 124:2509-2518
- Weber GM, Powell JFF, Park M, Fischer WH, Craig AG, Rivier JE, Nanakorn U, Parhar IS, Ngamvongchon S, Grau EG, Sherwood NM 1997 Evidence that gonadotropin-releasing hormone (GnRH) functions as a prolactin-releasing factor in a teleost fish (*Oreochromis mossambicus*) and primary structures for three native GnRH molecules. *J Endocrinol* 155:121-132
- Sherwood NM, von Schalburg K, Lescheid D 1997 Origin and evolution of GnRH in vertebrates and invertebrates. In: Parhar IS, Sakuma Y (eds) *GnRH Neurons: Gene to Behavior*. Brain Shuppan, Tokyo, pp 3-25
- Kelsall R, Coe IR, Sherwood NM 1990 Phylogeny and ontogeny of gonadotropin-releasing hormone: comparison of guinea pig, rat and a protochordate. *Gen Comp Endocrinol* 78:479-494
- Powell JFF, Reska-Skinner SM, Prakash OM, Fischer WH, Park M, Rivier JE, Craig AG, Mackie GO, Sherwood NM 1996 Two new forms of gonadotropin-releasing hormone in a protochordate and the evolutionary implications. *Proc Natl Acad Sci USA* 93:10461-10464
- Craig AG, Fischer WH, Park M, Rivier JE, Musselman BD, Powell JFF, Reska-Skinner SM, Prakash MO, Mackie GO, Sherwood NM 1997 Sequence of two gonadotropin releasing hormones from tunicate suggest an important role of conformation in receptor activation. *FEBS Lett* 413:215-225
- Cameron CB, Mackie GO, Powell JFF, Lescheid DW, Sherwood NM 1999 Gonadotropin-releasing hormone in mulberry cells of *Saccoglossus* and *Ptychodera* (Hemichordata: Enteropneusta). *Gen Comp Endocrinol* 114:2-10
- Goldberg JL, Garofalo R, Price CJ, Chang JP 1993 Presence and biological activity of a GnRH-like factor in the nervous system of *Helisoma trivolvis*. *J Comp Neurol* 336:571-582
- Jimenez-Linan M, Rubin BS, King JC 1997 Examination of guinea pig luteinizing hormone-releasing hormone gene reveals a unique decapeptide and existence of two transcripts in the brain. *Endocrinology* 138:4123-4130
- Quanbeck C, Sherwood N, Millar R, Terasawa E 1997 Two populations of luteinizing hormone-releasing neurons in the forebrain of the rhesus macaque during embryonic development. *J Comp Neurol* 380:293-309
- Lescheid DW, Terasawa E, Abler LA, Urbanski HF, Warby CM, Millar RP, Sherwood NM 1997 A second form of gonadotropin-releasing hormones (GnRH) with characteristics of chicken GnRH-II is present in the primate brain. *Endocrinology* 138:5618-5629
- Terasawa E, Abler LA, Sherwood NM, The luteinizing hormone-releasing hormone system in the developing monkey brain. In: Matsumoto A (ed) *Sexual Differentiation and the Brain*. CRC Press, Boca Raton, in press
- Seeburg PH, Adelman JP 1984 Characterization of cDNA for precursor of human luteinizing hormone releasing hormone. *Nature* 311:666-668
- White RB, Eisen JA, Kasten TL, Fernald RD 1998 Second gene for gonadotropin-releasing hormone in humans. *Proc Natl Acad Sci USA* 95:305-309
- Miyamoto K, Hasegawa Y, Nomura M, Igarashi M, Kangawa K, Matsuo H 1984 Identification of the second gonadotropin-releasing hormone in chicken hypothalamus: evidence that gonadotropin secretion is probably controlled by two distinct gonadotropin-releasing hormones in avian species. *Proc Natl Acad Sci USA* 81:3874-3878
- Matsuo H, Baba Y, Nair RMG, Arimura A, Schally AV 1971 Structure of the porcine LH- and FSH-releasing hormone. I. The proposed amino acid sequence. *Biochem Biophys Res Commun* 43:1334-1339
- Burgus R, Butcher M, Amoss M, Ling N, Monahan M, Rivier J, Fellows R, Blackwell R, Vale W, Guillemin R 1972 Primary structure of ovine hypothalamic luteinizing hormone-releasing factor (LRF). *Proc Natl Acad Sci USA* 69:278-282
- Lescheid DW, Powell JFF, Fischer WH, Park M, Craig A, Bukovskaya O, Barannikova IA, Sherwood NM 1995 Mammalian gonadotropin-releasing hormone (GnRH) identified by primary structure in Russian sturgeon, *Acipenser gueldenstaedti*. *Regul Pept* 55:299-309
- Sherwood NM, Doroshov S, Lance V 1991 Gonadotropin-releasing hormone (GnRH) in bony fish that are phylogenetically ancient: reedfish (*Calamoichthys calabaricus*), sturgeon (*Acipenser transmontanus*), and alligator gar (*Lepisosteus spatula*). *Gen Comp Endocrinol* 84:44-57
- Conlon JM, Collin F, Chiang Y-C, Sower SA, Vaudry H 1993 Two molecular forms of gonadotropin-releasing hormone from the brain of the frog, *Rana ridibunda*: purification, characterization, and distribution. *Endocrinology* 132:2117-2120
- Miyamoto K, Hasegawa Y, Igarashi M, Chino N, Sakakibara S, Kangawa K, Matsuo H 1983 Evidence that chicken hypothalamic luteinizing hormone-releasing hormone is (Gln<sup>8</sup>)-LH-RH. *Life Sci* 32:1341-1347
- Lovejoy DA, Fischer WH, Parker DB, McRory JE, Park M, Lance V, Swanson P, Rivier JE, Sherwood NM 1991 Primary structure of two forms of gonadotropin-releasing hormone from brains of the American alligator (*Alligator mississippiensis*). *Regul Pept* 33:105-116
- Sherwood NM, Parker DB, McRory JE, Lescheid DW 1994 Molecular evolution of growth hormone-releasing hormone and gonadotropin-releasing hormone. In: Sherwood NM, Hew CL (eds) *Fish Physiology*. Academic Press, San Diego, vol 13:3-66
- Sherwood NM, Sower SA, Marshak DR, Fraser BA, Brownstein MJ 1986 Primary structure of gonadotropin-releasing hormone from lamprey brains. *J Biol Chem* 261:4812-4819
- Sower SA, Chiang Y-C, Lovas S, Conlon JM 1993 Primary structure and biological activity of a third gonadotropin-releasing hormone from lamprey brain. *Endocrinology* 132:1125-1131
- Lovejoy DA, Sherwood NM, Fischer WH, Jackson BC, Rivier JE, Lee T 1991 Primary structure of gonadotropin-releasing hormone from the brain of a holoccephalan (Ratfish: *Hydrolagus collii*). *Gen Comp Endocrinol* 82:152-161
- Lovejoy DA, Fischer WH, Ngamvongchon S, Craig AG, Nahorniak CS, Peter RE, Rivier JE, Sherwood NM 1992 Distinct sequence of gonadotropin-releasing hormone (GnRH) in dogfish brain provides insight into GnRH evolution. *Proc Natl Acad Sci USA* 89:6373-6377
- Ngamvongchon S, Lovejoy DA, Fischer WH, Craig AG, Nahorniak CS, Peter RE, Rivier JE, Sherwood NM 1992 Primary structures of two forms of gonadotropin-releasing hormone, one distinct and one conserved, from catfish brain. *Mol Cell Neurosci* 3:17-22
- Powell JFF, Zohar Y, Elizur A, Park M, Fischer WH, Craig AG, Rivier JE,

- Lovejoy DA, Sherwood NM 1994 Three forms of gonadotropin-releasing hormone characterized from brains of one species. *Proc Natl Acad Sci USA* 91:12081–12085
38. Rivier J 1978 Use of trialkylammonium phosphate (TEAP) buffers in reverse phase HPLC for high resolution and high recovery of peptides and proteins. *J Liquid Chromatogr* 1:343–367
39. Fischer WH, Park M 1992 Sequence analysis of pyroglutamyl peptides: microscale removal of pyroglutamate residues. *J Prot Chem* 11:366
40. Fischer WH, Craig AG 1994 Determination of C-terminal amidation in peptides by MALDI-MS after microscale esterification. *J Protein Chem* 13:452–453
41. Powell JFF, Fischer WH, Park M, Craig AG, Rivier JE, White SA, Francis RC, Fernald RD, Licht P, Warby C, Sherwood NM 1995 Primary structure of solitary form of gonadotropin-releasing hormone (GnRH) in cichlid pituitary; three forms of GnRH in brain of cichlid and pumpkinseed fish. *Regul Pept* 57:43–53
42. Rivier J, Porter J, Hoeger C, Thobald P, Craig AG, Dykert J, Corrigan A, Perrin M, Hook WA, Siraganian RP, Vale W, Rivier C 1992 Gonadotropin-releasing hormone antagonists with *N* omega-triazolylornithine, -lysine, or *p*-aminophenylalanine residues at positions 5 and 6. *J Med Chem* 35:4270–4278
43. Chang JP, Cook H, Freedman GL, Wiggs AJ, Somoza GM, de Leeuw R, Peter RE 1990 Use of a pituitary cell dispersion method and primary culture system for the studies of gonadotropin-releasing hormone action in the goldfish, *Carassius auratus*. I. Initial morphological, static, and cell column perfusion studies. *Gen Comp Endocrinol* 77:256–273
44. Peter RE, Nahorniak CS, Chang JP, Crim LW 1984 Gonadotropin release from the pars distalis of goldfish, *Carassius auratus*, transplanted beside the brain or into the brain ventricle: additional evidence for gonadotropin-release inhibiting factor. *Gen Comp Endocrinol* 55:337–346
45. Van Der Kraak G, Suzuki K, Peter RE, Itoh H, Kawachi H 1992 Properties of common carp gonadotropin I and gonadotropin II. *Gen Comp Endocrinol* 85:217–229
46. Marchant TA, Fraser RA, Andrews PC, Peter RE 1987 The influence of mammalian and teleost somatostatins on the secretion of growth hormone from goldfish (*Carassius auratus* L.) pituitary fragments *in vitro*. *Regul Pept* 17:41–52
47. Sherwood N, Eiden L, Brownstein M, Spiess J, Rivier J, Vale W 1983 Characterization of a teleost gonadotropin-releasing hormone. *Proc Natl Acad Sci USA* 80:2794–2798
48. Sealton SC, Weinstein H, Millar RP 1997 Molecular mechanisms of ligand interaction with the gonadotropin-releasing hormone receptor. *Endocr Rev* 18:180–205
49. White SA, Kasten TL, Bond CT, Adelman JP, Fernald RD 1995 Three gonadotropin-releasing hormone genes in one organism suggest novel roles for an ancient peptide. *Proc Natl Acad Sci USA* 92:8363–8367
50. Yamamoto N, Oka Y, Amano M, Aida K, Hasegawa Y, Kawashima S 1995 Multiple gonadotropin-releasing hormone immunoreactive systems in the brain of the dwarf gourami, *Colisa lalia*: immunohistochemistry and radioimmunoassay. *J Comp Neurol* 355:354–368
51. Zandbergen T, Kah O, Bogerd T, Peute J, Goos HJTh 1995 Expression and distribution of two gonadotropin-releasing hormones in the catfish brain. *Neuroendocrinology* 62:571–578
52. Suzuki M, Hyodo S, Kobayashi M, Aida K, Urano A 1992 Characterization and localization of mRNA encoding the salmon-type gonadotropin-releasing hormone precursor of the masu salmon. *J Mol Endocrinol* 9:73–82
53. Bailhache T, Arazam A, Klungland H, Alestrom P, Breton B, Jégo P 1994 Localization of salmon gonadotropin-releasing hormone mRNA and peptide in the brain of Atlantic salmon and rainbow trout. *J Comp Neurol* 347:444–454
54. Gothilf Y, Elizur A, Chow M, Chen TT, Zohar Y 1995 Molecular cloning and characterization of a novel gonadotropin-releasing hormone from the gilthead seabream (*Sparus aurata*). *Mol Mar Biol Biotechnol* 4:27–35
55. Parhar IS 1997 GnRH in tilapia: three genes, three origins and their roles. In: Parhar IS, Sakuma Y (eds) *GnRH Neurons: Gene to Behavior*. Brain Shuppan, Tokyo, pp 99–122
56. Powell JFF, Standen EM, Carolsfeld J, Borella MI, Gazola R, Fischer WH, Park M, Craig AG, Warby CM, Rivier JE, Val-Sella MV, Sherwood NM 1997 Primary structure of three forms of gonadotropin-releasing hormone (GnRH) from the pacu brain. *Regul Pept* 68:189–195
57. Zohar Y, Elizur A, Sherwood NM, Powell JFF, Rivier JE, Zmora N 1995 Gonadotropin-releasing activities of the three native forms of gonadotropin-releasing hormones present in the brain of gilthead seabream, *Sparus aurata*. *Gen Comp Endocrinol* 97:289–299
58. Nelson JS 1994 *Fishes of the World*, ed 3. Wiley & Sons, New York
59. King JA, Dufour S, Fontaine YA, Millar RP 1990 Chromatographic and immunological evidence for mammalian GnRH and chicken GnRH II in eel (*Anguilla anguilla*) brain and pituitary. *Peptides* 11:507–514
60. Somoza GM, Stefano A, D'Eramo JL, Canosa LF, Friedman O 1994 Immunoreactive GnRH suggesting a third form of GnRH in addition to cIIGnRH and sGnRH in the brain and pituitary gland of *Prochilodus lineatus* (Characiformes). *Gen Comp Endocrinol* 94:44–52
61. Idler DR, Everard BA 1987 Mammalian, salmon, and chicken-like LHRH's from hypothalami of winter flounder (*Pseudopleuronectes americanus*) as evidenced by chromatographic mobility and immunoreactivity. In: Idler DR, Crim LW, Walsh JM (eds) *Third Fish Symposium 1987*. Marine Sciences Research Laboratory, Memorial University, St. John's, Newfoundland, Canada, p 30
62. O'Neill DF, Powell JFF, Standen EM, Youson JH, Warby CM, Sherwood NM 1998 Gonadotropin-releasing hormone (GnRH) in ancient teleosts, the bony-tongue fishes: putative origin of salmon GnRH. *Gen Comp Endocrinol* 112:415–425
63. Amores A, Force A, Yan Y-L, Joly L, Amemiya C, Fritz A, Ho RK, Langeland J, Prince V, Wang Y-L, Westerfield M, Ekker M, Postlethwait JH 1998 Zebrafish *hox* clusters and vertebrate genome evolution. *Science* 282:1711–1714
64. De Pinna MCC 1996 Teleostean monophyly. In: Stiassny MLJ, Parenti LR, Johnson GD (eds) *Interrelationships of Fishes*. Academic Press, New York, pp 147–162
65. Lauder GV, Liem KF 1983 Patterns of diversity and evolution in ray-fined fishes. In: Northcutt RG, Davis RE (eds) *Fish Neurobiology*. University of Michigan Press, Ann Arbor, pp 1–24
66. Volkoff H, Peter RE, Effects of two forms of gonadotropin releasing hormone and a GnRH antagonist on the spawning behavior of the female goldfish, *Carassius auratus*. 6th International Symposium on the Reproductive Physiology of Fish, Bergen, Norway, 1999, p. 42 (Abstract OP-17)
67. Carolsfeld J, Scott AP, Collins PM, Sherwood NM 1996 Reproductive steroids during maturation in a primitive teleost, the Pacific herring (*Clupea harengus pallasii*). *Gen Comp Endocrinol* 103:331–348