General and Comparative Endocrinology 119, 121–131 (2000)
doi:10.1006/gcen.2000.7488, available online at http://www.idealibrary.com on IDEAL®

Gonadotropin-Releasing Hormone (GnRH) Variants in a Lizard Brain: Is Mammalian GnRH Being Expressed?[1]

Alejandro D. Montaner,* Olga Gonzalez,† Dante A. Paz,*,‡ Jorge M. Affanni,* and Gustavo M. Somoza§,[2]

**INEUCI-CONICET, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, 1428 Buenos Aires, Argentina; †Departamento de Zootecnia, Facultad de Agronomía, Universidad de Buenos Aires, 1417 Buenos Aires, Argentina; ‡Laboratorio de Embriología Animal, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, 1428 Buenos Aires, Argentina; and §Instituto de Investigaciones Biomédicas Fundación Pablo Cassará, 1440 Buenos Aires, Argentina*

Accepted February 28, 2000

In reptiles as in other vertebrates, multiple forms of gonadotropin-releasing hormone (GnRH) within a single brain have been identified. In this group the following GnRH molecular variants have been characterized either by direct or indirect methods: chicken GnRH I (cGnRH-I), chicken GnRH II (cGnRH-II), salmon GnRH (sGnRH) and several unidentified GnRH-like forms. In the present study GnRH variants were investigated in brain extracts of the lizard *Tupinambis teguixin* (= *T. merinae*) by combining high-performance liquid chromatography (RP-HPLC) followed by radioimmunoassays (RIA). Two peaks showing GnRH immunoreactivity with the elution position of synthetic mammalian GnRH (mGnRH) and cGnRH-II were detected. Both peaks were further analyzed with different radioimmunoassay systems specific for mGnRH, cGnRH-I, and cGnRH-II. Pooled fractions corresponding to the first eluting peak showed no cross-reactivity when analyzed with a cGnRH-I specific assay and logit-log displacement curves were not significantly different from those of synthetic mGnRH with homologous RIA systems. The second peak showed immunological characteristics of cGnRH-II when analyzed with a specific antiserum. The first ir-GnRH peak was selected for further RP-HPLC purification showing similar chromatographic behavior as mGnRH synthetic standard. We demonstrated the absence of cGnRH-I in this lizard using well-characterized antisera.

Key Words: gonadotropin-releasing hormone; RP-HPLC; RIA; reptiles; brain.

The presence of multiple molecular forms of gonadotropin-releasing hormone (GnRH) within a single brain appears to be a common pattern in vertebrates. Up to now, 14 GnRH variants have been sequenced from vertebrate and protochordate nervous tissue (Jimenez-Liñan *et al.,* 1997; Carolsfeld *et al.,* 2000; Fradinger *et al.,* 1999; Montaner *et al.,* personal communication).

The mammalian form of GnRH (mGnRH) was originally isolated from porcine and ovine brains (Matsuo *et al.,* 1971; Amoss *et al.,* 1971). In mammals another form has been described in guinea pig nerve tissue (gpGnRH, Jimenez-Liñan *et al.,* 1997). Two other GnRH forms were first isolated from chicken brains: cGnRH-I and cGnRH-II (King and Millar, 1982; Miyamoto *et al.,* 1983, 1984). Additional variants have been isolated from brains of salmon (sGnRH, Sherwood *et al.,* 1983), lamprey (lGnRH-I and lGnRH-III, Sherwood *et al.,* 1986; Sower *et al.,* 1993), catfish (cf-

[1] Part of this work was presented at the XIX Conference of European Comparative Endocrinologists, Nijmegen, The Netherlands, September 1998.

[2] To whom correspondence should be addressed. Fax: (+54) 11-4687-7886. E-mail: somoza@bg.fcen.uba.ar.

0016-6480/00 $35.00

GnRH, Ngamvongchon *et al.,* 1992), dogfish (dfGnRH, Lovejoy *et al.,* 1992), seabream (sbGnRH, Powell *et al.,* 1994), herring (hrGnRH, Carolsfeld *et al.,* 2000), and pejerrey (pGnRH, Montaner *et al.,* personal communication). Two further variants, tunicate I (tGnRH-I) and tunicate II (tGnRH-II), were also characterized in a protochordate (Powell *et al.,* 1996). All forms are decapeptides, with a conserved structure. Amino-terminus pGlu and carboxy-terminus NH_2 are modified and unchanged. They all share positions 1, 2, 3, 4, 9, and 10, exceptions being lGnRH-I (Tyr in residue 3) and gpGnRH (Tyr in residue 2). Furthermore, evidence based on indirect chromatographic and immunological techniques suggests the existence of novel variants (Montaner *et al.,* 1998a, 1999).

The most widely distributed GnRH variant, cGnRH-II, is present in all taxa except agnathans (King and Millar, 1997) and it has been suggested that this molecule is phylogenetically ancient and diverged early in vertebrate evolution to form a separate evolutionary branch (King and Millar, 1997).

In reptiles, evidence of the expression of cGnRH-I and cGnRH-II has been reported in the turtles *Pseudemys scripta* (Sherwood and Whittier, 1988) and *Trachemys scripta* (Tsai and Licht, 1993). These two forms have also been identified through sequencing in the American alligator *Alligator mississippiensis* (Lovejoy *et al.,* 1991). In lizards, there is evidence of the presence of cGnRH-II, sGnRH, and several other unidentified GnRH-like forms (Powell *et al.,* 1985, 1986; Lescheid *et al.,* 1997). Less is known about GnRH in snakes where just cGnRH-I was detected in the only species studied *Thamnophilis sirtalis* (Sherwood and Whittier, 1988; Smith *et al.,* 1997).

However, the expression pattern of GnRH variants in reptiles is far from being understood, due to the low number of species analyzed. In this context, it is important to note that although mGnRH has been described in early evolved fish, amphibians, and mammals (King and Millar, 1997), this molecule has not yet been reported in reptiles.

Tupinambis teguixin (= *T. merinae*) belongs to the Teiidae family, which includes nearly 40 genera distributed mainly in South America (Avila-Pires, 1995). Only the genus *Cnemidophorus* is well represented in North America and there is some controversy about the South or North American origin of the family (Estes and Baez, 1985). This family has been considered to be closely related with the Lacertidae family (Macey *et al.,* 1997). The present distribution of the lacertids in Eurasia and Africa has suggested that there was a teiid ancestor which moved from North America to Asia and gave rise to the lacertids in Asia (Gorman, 1970).

The reproductive cycle of *T. teguixin* is characteristic of lizards from seasonal and subtropical environments (Saint Girons, 1984). In the north of Argentina *T. teguixin* breeds almost exclusively in the spring, from October to the middle of November (Mercolli and Yanosky, 1989).

The aim of the present study was to characterize GnRH variants in the brain of the tegu lizard *Tupinambis teguixin,* employing an indirect method based on RP-HPLC and RIA analysis.

MATERIALS AND METHODS

Two sets of experiments were performed using animals captured in 1997 and 1998, during the late summer months of the southern hemisphere (February–March), at a sexual regression stage (Paz *et al.,* 1993). Lizards were captured in the surroundings of La Plata City (Province of Buenos Aires, Argentina) and kept according to the protocols of Yanosky and Mercolli (1993) at ECAS (Ministerio de Asuntos Agrarios, Province of Buenos Aires, Argentina). Six adult animals were used each time: 4 males and 2 females for the first set of assays and 3 males and 3 females for the second.

Animals were anesthetized with sodium pentothal (Tiopental, Abbot, Buenos Aires), 40 mg/kg, and diazepan (Diazepan Lamar, Buenos Aires), 1.25 mg/kg, in order to minimize any pain or discomfort. The brain was exposed, removed, immediately frozen on dry ice, and stored at −70°C until peptide extraction.

Tissue Extraction

Frozen pooled brains (near 10 g each time) were homogenized in acetone:1 N HCl (100:3, *v/v*) at 4°C with an Ultraturrax homogenizer. The extraction mixtures were stirred at 4°C and filtered through Whatman No. 1 filter paper. The insoluble material was reextracted in acetone:0.01 N HCl in 40% of the original volume, stirred for 5 min at 4°C, and refiltered. The combined filtrates were treated with petroleum

TABLE 1
Radioimmunoassay Characteristics

Antiserum	^{125}I-GnRH	Standard	Titer	Percentage of cross-reactivity				
				mGnRH	cGnRH-I	cGnRH-II	sGnRH	lGnRH-I
EL-15	mGnRH	mGnRH	1:175,000	100	<0.01	<0.01	<0.01	0.05
CRR11B73	mGnRH	mGnRH	1:175,000	100	<0.01	<0.01	<0.01	0.095
CII678	mGnRH	mGnRH	1:40,000	100	416	81	473	3.1
PBL No. 45	sGnRH	sGnRH	1:150,000	100	133	25.9	100	24
PBL No. 49	sGnRH	sGnRH	1:250,000	93.3	333	32.6	100	2.0
cII1458	cGnRH II	cGnRH II	1:10,000	2.5	1.9	100	0.6	0.05
cI1665	cGnRH I	cGnRH I	1:160,000	<0.01	100	<0.01	0.01	<0.01
cII741	cGnRH II	cGnRH II	1:5,000	<0.01	<0.01	100	3.5	0.2

Note. PBL No. 45, PBL No. 49, and CII678 RIA systems were used in the screening assays. The following antisera were used in homologous assays: EL-15, CRR11B73, cI1665, cII741, and cI1458. ^{125}I-GnRH: radioiodinated hormone. The percentages of cross-reactivities of EL-15 and CRR11B73 RIA systems were calculated in our laboratory. Cross-reactivities of PBL No. 45 and PBL No. 49 were reported by Yu *et al.* (1988) and retested in our laboratory and the cross-reactivities of cII678, cII741, cII1458, and cI1665 were given by Dr. J. A. King (personal communication) and retested in our laboratory.

ether (bp 30–60°C) for 5 times as previously described (Yu *et al.*, 1988).

Reverse-Phase High-Performance Liquid Chromatography

For the first characterization, extracts were evaporated in a vacuum concentrator to approximately 1 ml, filtered through a 0.45-μm Millipore HA filter, and injected via a 1-ml injection loop onto a Lichrospher 100 RP18 column (4 × 250 mm, 5-μm particle size, Merck) with a C18 guard column. A Konic liquid chromatograph was programmed as previously described (Somoza *et al.*, 1994). The filtrate was applied at the beginning of a 10-min isocratic period of 17% acetonitrile (ACN) in 0.25 M triethylammonium formate, pH 6.5 (TEAF); ACN was then increased to 24% over a 7-min period and maintained under isocratic conditions for 43 more min (Somoza *et al.*, 1994). The flow rate was kept at 1-ml/min and 1-ml fractions were collected (RP-HPLC system I). Fractions were lyophilized and resuspended in RIA buffer in order to detect GnRH immunoreactivity (ir-GnRH). Each injection of the tissue extract was preceded by a blank run. These fractions were radioimmunoassayed to ensure that the column was not contaminated. Fresh standards were chromatographed after the tissue extract run for comparison. Synthetic mGnRH, cGnRH-I, cGnRH-II, and sGnRH were obtained from Peninsula Laboratories Inc. (Belmont, CA).

In the second round of assays, the brain extract was treated and first chromatographed as described for RP-HPLC system I (TEAF 0.25 M, pH 6.5). Aliquots of 10 μl from each fraction were used for RIA to determine ir-GnRH. Eluted ir-GnRH fractions were pooled and designated as peaks I and II. The ir-GnRH peak I was selected for further purification, concentrated, and reinjected onto a Lichrospher 100 RP18 column (4 × 250 mm, 5-μm particle size, Merck). This sample was injected in three sequential steps at the beginning of a 10-min isocratic period of 10% acetonitrile in 0.13 M triethylammonium phosphate, pH 2.5. The mobile phase was increased to 17% ACN, kept at this point for 10 min, and then increased to 24% during a 7-min period and kept under isocratic conditions for 43 more min. The flow rate was kept at 1 ml/min and 1-ml fractions were collected (RP-HPLC system II). Each tissue injection was preceded by a blank run and then radioimmunoassayed under the same conditions. Mammalian GnRH and cGnRH-II synthetic standards (obtained from Peninsula Laboratories) were chromatographed after the tissue extract to detect the elution position of each peak in the RP-HPLC system II. The fractions were lyophilized and resuspended in RIA buffer for detection of GnRH immunoreactivity as already described.

Radioimmunoassays

Radioimmunoassays were performed as previously described (Montaner *et al.*, 1998a), using synthetic

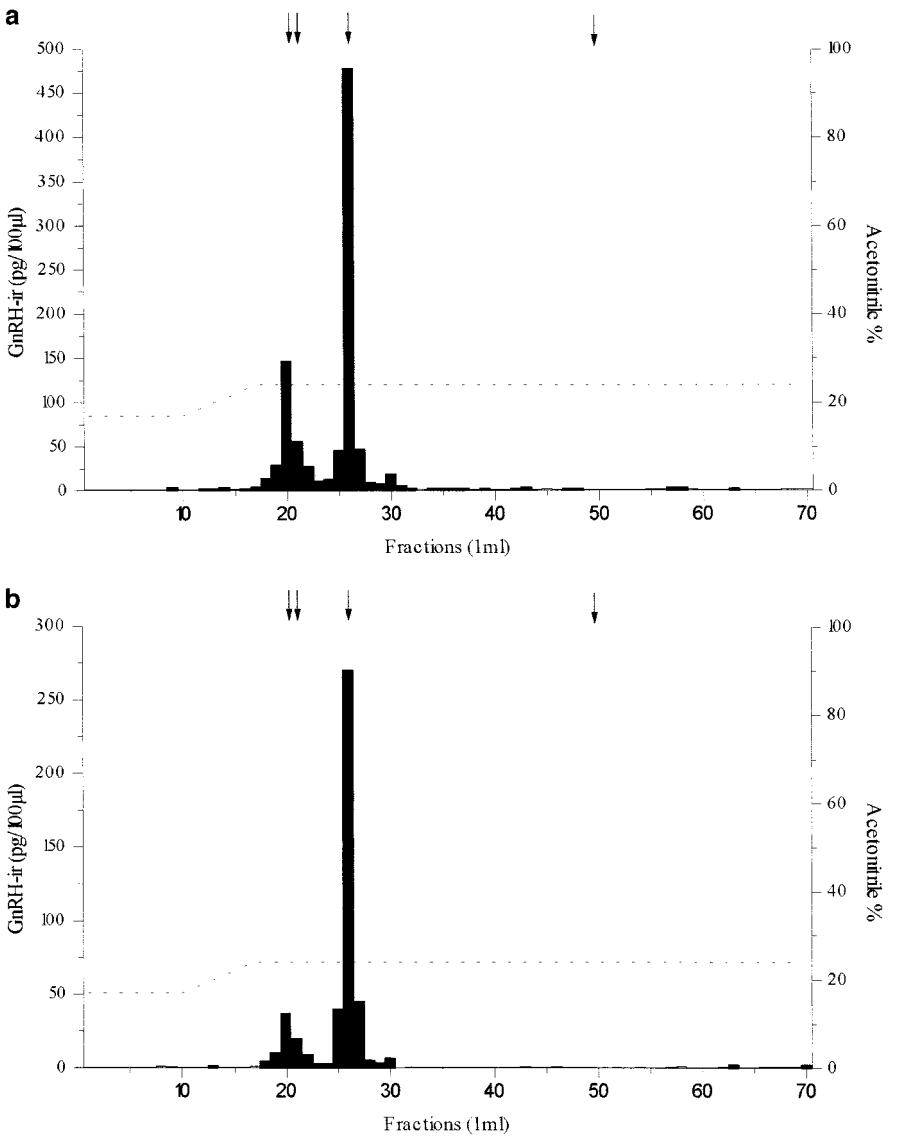


FIG. 1. RP-HPLC system I from brain extract assayed with (a) cII678 and (b) PBL No. 49 antisera in the first round of assays. Percentage of acetonitrile is represented by the dotted line. Arrows indicate the elution position of four different GnRH variants. The first two arrows to the left represent the elution position of mGnRH and cGnRH I, the third represents cGnRH II, and the fourth is sGnRH.

mGnRH, cGnRH-I, cGnRH-II, and sGnRH as radioiodinated tracers and standards. The GnRH standard was radiolabeled following established protocols (Montaner *et al.*, 1998a).

In the first set of assays, immunoreactivity to GnRH (ir-GnRH) was tested with different GnRH RIA systems using cII678 antisera (raised in rabbits against cGnRH-II) and PBL No. 49 (raised in rabbits against sGnRH). Fractions with ir-GnRH were pooled and analyzed with specific RIAs. Four homologous GnRH RIA systems were used: CRR11B73 and EL-15, specific antisera for mGnRH; cI1665, a specific antiserum for cGnRH-I; and cII741, a specific antiserum to detect cGnRH-II. Slopes of the displacement curves were compared with those of synthetic standards by ANOVA (Stefano *et al.*, 1997).

In the second set of assays, PBL No. 45, PBL No. 49, and cII1458 antisera were used on screening RIAs systems to identify the elution position of native GnRH molecules both in RP-HPLC system I and system II.

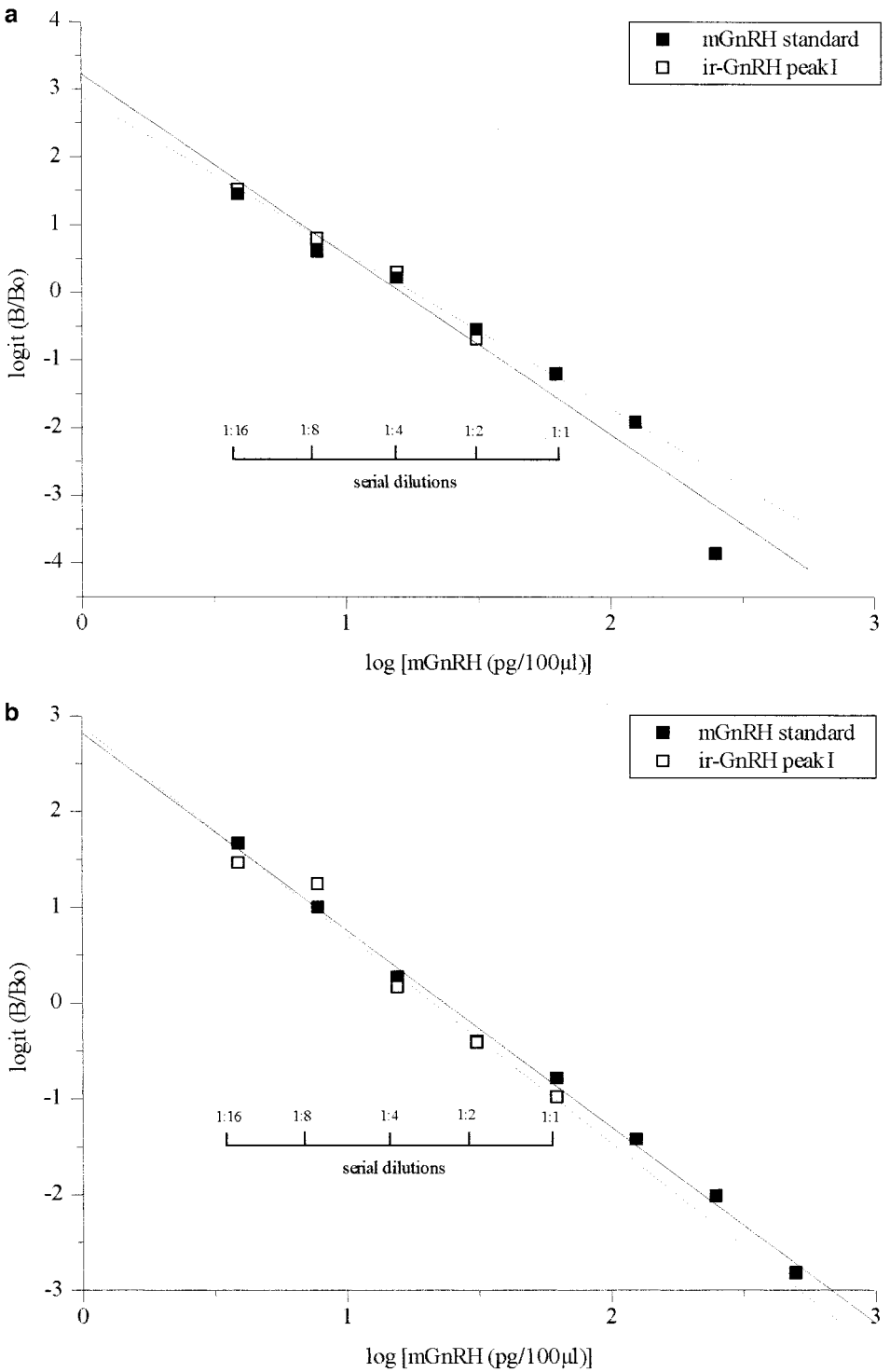


FIG. 2. Parallelism of serial dilutions of ir-GnRH peak I from RP-HPLC of brain extracts with respect to synthetic mGnRH using (a) EL-15 and (b) CRR11B73 antisera. The slopes of both curves were not significantly different from those of synthetic standard.

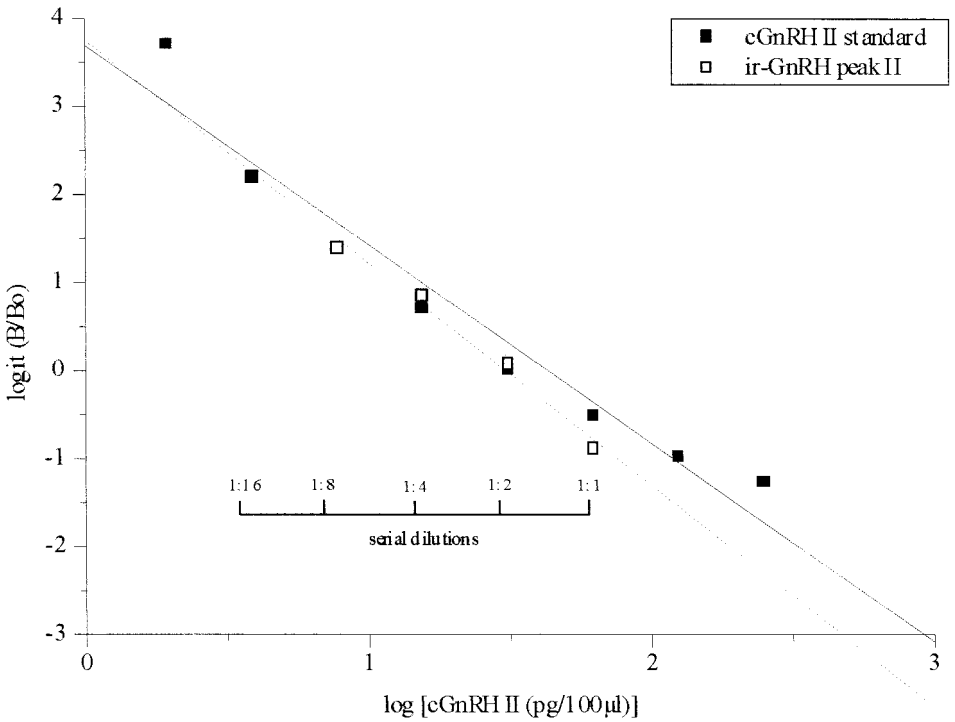


FIG. 3. Parallelism of serial dilutions of ir-GnRH peak II from RP-HPLC of brain extracts with respect to synthetic cGnRH-II using cII741 antiserum. The slope of the curve was not significantly different from that of synthetic standard.

Percentages of cross-reactivity against different synthetic GnRH standards are summarized in Table 1.

RESULTS

In the first set of assays (RP-HPLC system I), the elution position of three different GnRH synthetic standards was tested showing the following retention times: mGnRH, 20 min; cGnRH-I, 21 min; cGnRH-II, 26 min; and sGnRH, 50 min.

When brain extracts from *Tupinambis merianae* were analyzed with cII678 and PBL No. 49 RIA systems, two ir-GnRH peaks were revealed (Figs. 1a and b). The first eluting peak, fractions 19–21 (ir-GnRH peak I), was in a similar position to that of mGnRH and cGnRH-I. The second peak, fractions 25–27 (ir-GnRH peak II), eluted as synthetic cGnRH-II. No evidence of the presence of sGnRH or other forms was detected with these two systems.

Fractions corresponding to ir-GnRH peaks I and II were pooled independently. Each peak was analyzed with specific RIA systems. Due to the close elution position of mGnRH and cGnRH-I, ir-GnRH peak I was analyzed with two specific RIA systems for mGnRH (E-15 and CRR11B73) and with one specific RIA for cGnRH-I (cI1665). Peak II was analyzed with one specific cGnRH-II RIA system with a cII741 antiserum.

The logit-log displacement curves of ir-GnRH peak I were not significantly different from those of the synthetic mGnRH standard (Figs. 2a and 2b). The same ir-GnRH peak showed no immunorreactivity when tested in a cGnRH-I homologous assay (data not shown). The quantification of ir-GnRH peak I with both RIA systems specific for mGnRH showed similar results: 63.5 and 69.6 pg/100 μl for EL-15 and CRR11B73 respectively.

The logit-log displacement curve of ir-GnRH peak II was not significantly different from that of the cGnRH-II synthetic standard (Fig. 3).

In the second set of assays, the elution position of two different GnRH synthetic standards was tested. In the RP-HPLC system I, mGnRH eluted at 21.5 min and cGnRH-II at 28 min and in the RP-HPLC system II,

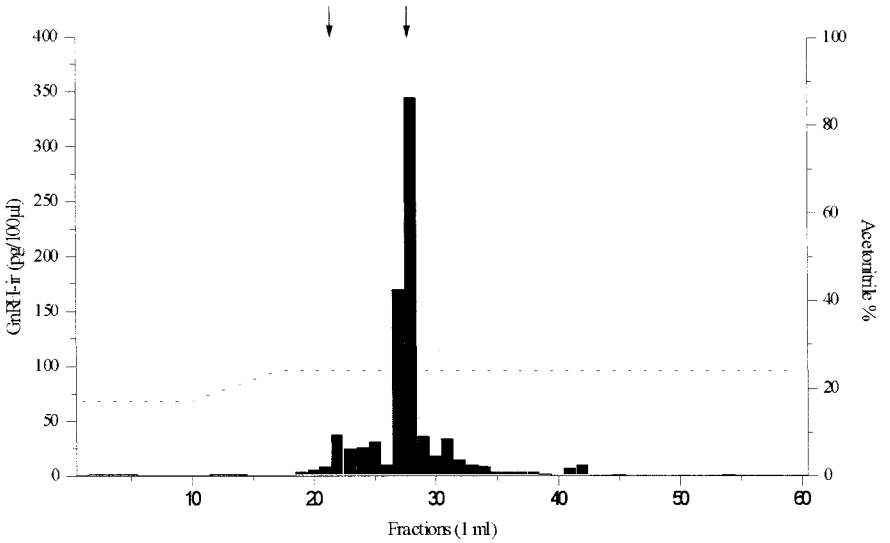


FIG. 4. RP-HPLC system I from brain extract assayed with PBL No. 49 antiserum in the second round of assays (Step I). The percentage of acetonitrile is represented by the dotted line. Arrows indicate the elution position of two different GnRH variants. The first arrow represents the elution position of mGnRH and the second is cGnRH II.

synthetic mGnRH and cGnRH-II eluted at 25 min and 32 min respectively.

After the first step of RP-HPLC purification, brain extracts were analyzed with PBL No. 49 RIA system. Two ir-GnRH peaks were revealed (Fig. 4): the first ir-GnRH peak between fractions 22–25 (ir-GnRH peak I) and the second one between fractions 25–27 (ir-GnRH peak II).

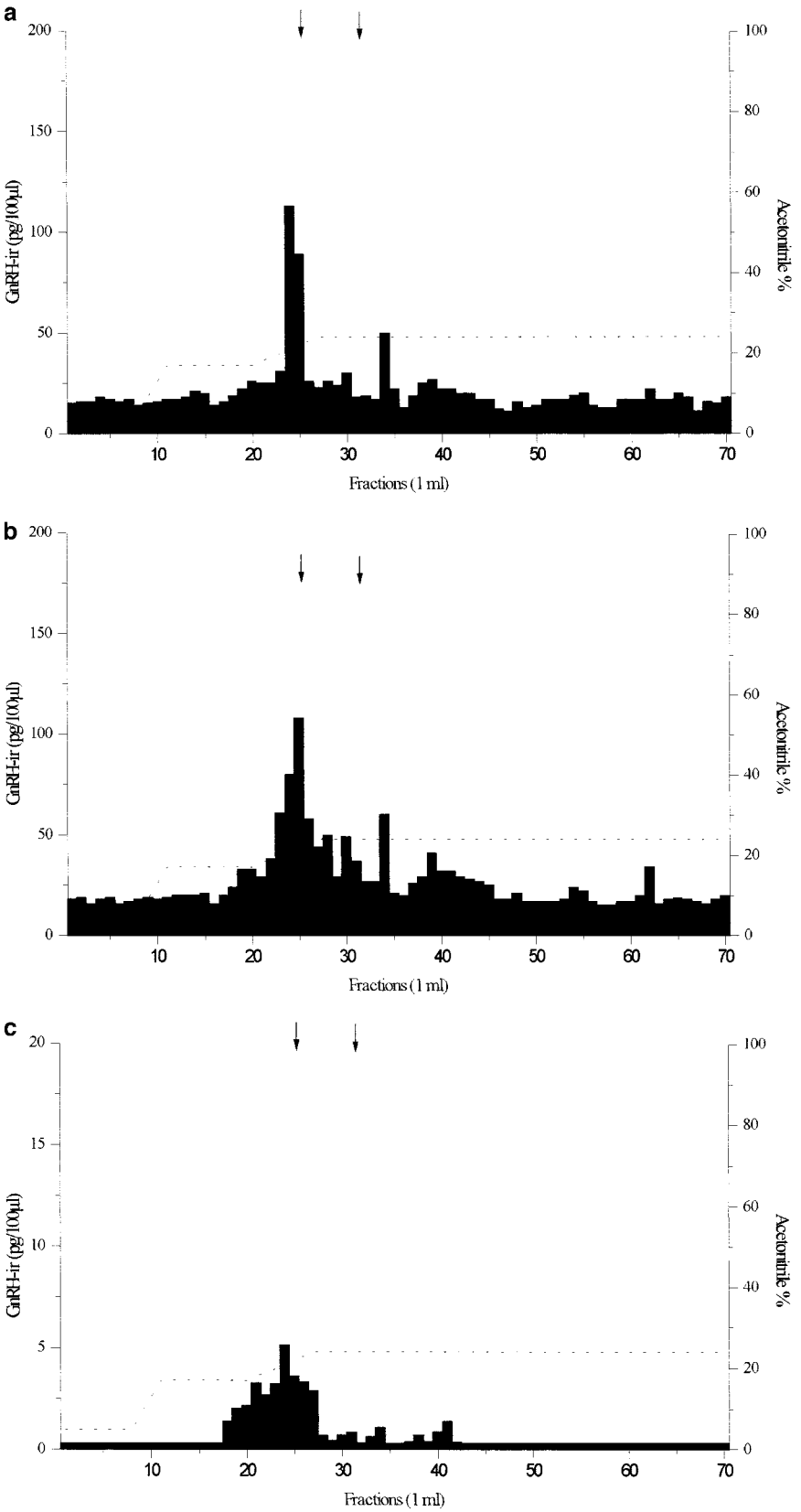
In order to further characterize ir-GnRH peak I, it was rechromatographed in RP-HPLC system II and analyzed with PBL No. 45, PBL No. 49, and cII1458 antisera. Two ir-GnRH peaks were revealed when analyzed both with PBL No. 45 and PBL No. 49 antisera. The first and major peak eluted as mGnRH (fractions 24–25) and the second minor peak eluted in fractions 34–35. (Figs. 5a and 5b). An extended ir-GnRH zone was revealed near fraction 24 using cII1458 antiserum (Fig. 5c).

DISCUSSION

In the present work, RP-HPLC and RIA data showed that two ir-GnRH forms are expressed in the brain of the lizard *T. teguixin.* Although we were not able to identify through sequencing both ir-GnRH peaks, ir-GnRH peak I has chromatographic and immunological characteristics resembling those of mGnRH and we demonstrated that in this peak, cGnRH-I is absent. However, the expression of a novel GnRH molecule should not be discarded. The second ir-GnRH peak may represent the expression of cGnRH-II.

In the first round of assays, characterization of brain extracts with two generic GnRH RIA systems showed two immunoreactive peaks corresponding to the elution position of mGnRH and cGnRH-II. Serial dilution studies with specific antisera and quantification analyses reinforced RP-HPLC and RIA data. Due to the close elution position of mGnRH and cGnRH-I, ir-GnRH peak I was analyzed not only with two specific RIA systems for mGnRH but also with one specific RIA for cGnRH-I. Using this last system, this peak showed no immunorreactivity, clearly indicating that cGnRH-I is not an endogenous brain peptide in *T. teguixin.*

A second round of assays was carried out in order to reinforce these findings. At this stage, sequential RP-HPLC analysis was performed in two different steps of purification. The goal of this experiment was to follow the elution position of the ir-GnRH peak I in comparison with mGnRH synthetic standard using different chromatographic conditions. It has already



been shown that the elution position of GnRH variants changes using different mobile phases and GnRH synthetic standards eluting together in one system can be discriminated in other conditions (Powell *et al.*, 1995; Montaner *et al.*, 1998b).

Using RP-HPLC system I, two ir-GnRH peaks were revealed corresponding to the elution positions of mGnRH and cGnRH-II. Immunoreactive fractions corresponding to ir-GnRH peak I, suspected to be caused by mGnRH, were pooled and rechromatographed. Two ir-GnRH peaks were observed using RP-HPLC system II; the major peak eluted in the same position to that of mGnRH synthetic standard and the second ir-GnRH peak was observed in fractions 34–35, which did not correspond to the elution position of any of the standard assayed in this step. At this point, we were not able to discriminate whether this peak represented another GnRH molecule which coeluted with mGnRH in RP-HPLC system I or represented some degradation product. Nevertheless, it is clear that this peak was not caused by cGnRH-II contamination from the first chromatograph because the cII1458 RIA system could not detect it (see Table 1).

Multiple GnRH molecular forms have been demonstrated in brain tissue from a single animal in all vertebrate groups. In most species cGnRH-II is expressed together with at least one of the other GnRH variants (King and Millar, 1997; Sherwood *et al.*, 1997). The neuroanatomical location of GnRH variants in the brain also shows a similar pattern in all vertebrates. Neurons expressing cGnRH-II are located in midbrain areas and the other GnRH form is expressed by cells located in the terminal nerve, septopreoptic area, and basal forebrain (Muske, 1997).

Some other studies have also demonstrated structural diversity in reptilian expression of GnRH. Basically, cGnRH-II has been described together with cGnRH-I in Chelonia and Crocodilia (Lovejoy *et al.*, 1991; Powell *et al.*, 1986; Tsai and Licht, 1993). In all species of Squamata studied so far, the expression of cGnRH-II is widely conserved (King and Millar, 1997; Lescheid *et al.*, 1997; Sherwood and Whittier, 1988). Some reports informed the presence of a sGnRH-like variant in the brain of lizards (Powell *et al.*, 1985; 1986) but none has detected the presence of cGnRH-I or mGnRH in this group (Lescheid *et al.*, 1997; Powell *et al.*, 1985, 1986). This work also supports the original hypothesis of Lescheid *et al.* (1997) about the lack of expression of cGnRH-I in lizard's brains, which makes them different from other reptiles: snakes, turtles, and alligators.

It is well known that mGnRH is a very conserved molecule among vertebrates. There are evidences of its expression from early-evolved bony fish, through amphibians to mammalian species (King and Millar, 1997). If we focus our results on the presence of a mGnRH-like variant in a reptile brain, they suggest that mGnRH is being expressed in at least one living reptile and that it might have already been expressed in primitive reptiles. This is in agreement with King and Millar's hypothesis (1997) that cGnRH-I could have derived from mGnRH and this change might have occurred in reptiles.

Although a differential distribution of GnRH molecules in this lizard has not been performed, if we follow Muske's scheme (1997), cGnRH-II-expressing neurons are expected to be located in midbrain areas while the mGnRH-like expressing cells should be in the basal forebrain, related to gonadotropin secretion.

In summary, our results support the idea of the expression of a mGnRH form together with cGnRH-II in the brain of a reptile and add data on the lack of cGnRH-I expression in lizards. However, this mGnRH-like variant may represent the expression of a novel form. The knowledge of the sequence of this molecule may help to clarify the evolutionary history of mGnRH and the origin of cGnRH-I molecule in vertebrates. In this context, since Squamata is the most diversified group within Reptilia, the analysis of GnRH expression in other species is important to determine the distribution of GnRH variants in this group.

ACKNOWLEDGMENTS

We are indebted to Dr. W. Vale (The Salk Institute, La Jolla, CA), who kindly provided PBL No. 45 and PBL No. 49 antisera. Dr. J. A. King (University of Cape Town, South Africa) generously donated cII678, cII741, cII1458, and cI1665 antisera. EL-15 antiserum was

FIG. 5. RP-HPLC system II from brain extract assayed with (a) PBL No. 45, (b) PBL No. 49, and (c) CII1458 antisera in the second round of assays (Step II). The percentage of acetonitrile is represented by the dotted line. Arrows indicate the elution position of two different GnRH variants. The first arrow represents the elution position of mGnRH and the second is cGnRH-II.

obtained from Dr. V. Ellynwood (Oregon State University) and Dr. V. Ramirez (University of Illinois) kindly provided CRR11B73 antiserum. We also thank Lic. Luis Giambellucca and Mónica Cassiaro (ECAS) for their help and Luis and Facundo Maranesi, for technical advice. The critical readings of Dr. Leandro Miranda, Dr. Victoria Lux-Lantos, Dr. Carlos Libertun, and Ms. Silvia Talía is also acknowledged. This work was supported by grants from CONICET to INEUCI and Grant No. ANPCyT 01-0442 to G.M.S.

REFERENCES

Amoss, M., Burgus, R., Blackwell, R., Vale, W, Fellows, R., and Guillemin, R. (1971). Purification, amino-acid composition and N-terminus of the hypothalamic luteinizing hormone releasing factor (LRF) of ovine origin. *Biochem. Biophys. Res. Commun.* **4,** 205–210.

Avila-Pires, T. C. S. (1995). Lizards of Brazilian Amazonia (Reptilia: Squamata). *Zool. Verh. Leiden* **299,** 3–706.

Carolsfeld, J., Powell, J. F. F, Park, M., Fischer, W. H., Craig, A. G., Chang, J. P., Rivier, J. E., and Sherwood, N. M. (2000). Primary structure and function of three gonadotropin-releasing hormones, including a novel form, from an anciente teleost, herring. *Endocrinology* **141,** 505–512.

Estes, R., and Baez, A. (1985). Herpetofauna of North and South America during late Cretaceous and Cenozoic: Evidence for interchange? *In* "Topics in Geobiology: The Great American Biotic Interchange" (F. Stahll and D. Webb, Eds.), pp. 140–197. Plenum Press, New York.

Fradinger, E. A., von Schalburg, K., and Sherwood, N. (1999). An evolutionary perspective on GnRH in fish. VIth International Symposium on the Reproductive Endocrinology of Fish, Bergen, Norway, July 4–9. (Abstract)

Gorman, G. (1970). Chromosomes and the systematics of the family Teiidae (Sauria, Reptilia). *Copeia* **1970,** 230–245.

Jimenez-Liñan, M., Rubin, B. S., and King, J. C. (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.

King, J. A., and Millar, R. P. (1982). Structure of chicken hypothalamic luteinizing hormone-releasing hormone. I. Structural determination on partially purified material. *J. Biol. Chem.* **257,** 10772–10732.

King, J. A., and Millar, R. P. (1997). Coordinated evolution of GnRHs and their receptors. *In* "GnRH Neurons: Gene to Behavior" (I. S. Parhar and Y. Sakuma, Eds.), pp. 51–78. Brain Shupan, Tokyo.

Lescheid, D. W., Rosen, G. J., Bridge, A. E., Jones, R. E., Warby, C. M., and Sherwood, N. M. (1997). Immunoreactive gonadotropin-releasing hormone (GnRH) is detected only in the form of chicken GnRH-II within the brain of the green anole, *Anolis carolinensis. Gen. Comp. Endocrinol.* **108,** 247–257.

Lovejoy, D. A., Fischer, W. H., Ngamvongchon, S., Craig, A. G., Nahorniak, C. S., Peter, R. E., Rivier, J. E., and Sherwood, N. (1992). Distinct sequence of gonadotropin-releasing hormone (GnRH) in dogfish brain provides insight into GnRH evolution. *Proc. Natl. Acad. Sci. USA* **89,** 6373–6377.

Lovejoy, D. A., Fischer, W. H., Parker, D. B., McRory, J. E., Park, M., Lance, V., Swamson, P., Rivier, J. E., and Sherwood, N. M. (1991). Primary structure of two forms of gonadotropin-releasing hormone from brains of the American alligator (*Alligator mississippiensis*). *Regul. Pept.* **33,** 105–116.

Macey, J., Larson, A., Anajeva, N., Fang, Z., and Papenfuss, T. (1997). Two novel gene orders and the role of light-strand replication in rearrangement of the vertebrate mitochondrial genome. *Mol. Biol. Evol.* **14,** 91–104.

Matsuo, H., Baba, Y., Nair, R. M. G., Arimura, A., and Schally, A. V. (1971). Structure of the porcine LH and FSH-releasing hormone. I. The proposed amino acid sequence. *Biochem. Biophys. Res. Commun.* **43,** 1334–1339.

Mercolli, C., and Yanosky, A. A. (1989). Répertoire des comportements du Téju (*Tupinambis teguixin*). Sauria: Teiidae. *Revue fr. Aquariol.* **16,** 123–130.

Miyamoto, K., Hasegawa, Y., Igarashi, M., Chino, N., Sakakibara, S., Kangawa, K., and Matsuo, H. (1983). Evidence that chicken hypothalamic luteinizing hormone-releasing hormone is [Gln^{8}]LHRH. *Life Sci.* **32,** 1341–1347.

Miyamoto, K., Hasegawa, Y., Nomura, M., Igarashi, M., Kangawa, K., and 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.

Montaner, A. D., Somoza, G. M., King, J. A., Bianchini, J. J., Bolis, C. G., and Affanni, J. M. (1998a). Chromatographic and immunological identification of GnRH (Gonadotropin-releasing hormone) variants: Occurrence of mammalian and a salmon-like GnRH in the forebrain of an eutherian mammal: *Hydrochaeris hydrochaeris* (Mammalia, Rodentia). *Regul. Pept.* **73,** 197–204.

Montaner, A. D., Affanni, J. M., King, J. A., Bianchini, J. J., Tonarelli, G., and Somoza, G. M. (1999). Differential distribution of gonadotropin-releasing hormone variants in the brain of *Hydrochaeris hydrochaeris. Cell. Mol. Neurobiol.* **19,** 635–651.

Montaner, A. D., Somoza, G. M., Affanni, J. M., and Sherwood, N. M. (1998b). Identification of multiple GnRH variants in the brain of an eutherian mammal. Jornada Interdisciplinaria de Endocrinología y Biología de la Reproducción. Sociedad Argentina de Biología, Buenos Aires, Argentina, December 3. (Abstract)

Muske, L. E. (1997). Ontogeny, phylogeny and neuroanatomical organization of multiple molecular forms of GnRH. *In* "GnRH Neurons: Gene to Behavior" (I.S. Parhar and Y. Sakuma, Eds.), pp. 145–180. Brain Shupan, Tokyo.

Ngamvongchon, S., Lovejoy, D. A., Fisher, W. H., Craig, A. G., Nahorniak, C. S., Peter R. E., Rivier J. E., and Sherwood, N. (1992). Primary structures of two forms of gonadotropin-releasing hormone, one distinct and one conserved, from catfish brain. *Mol. Cell. Neurosci.* **3,** 17–22.

Paz, D. A., Donadío, O. A., Pisanó, A., and Miranda, L. A. (1993). Annual testicular changes in *Tupinambis rufescens* (Sauria, Teiidae) from Salta, Argentina. *Rev. Esp. Herp.* **7,** 89–96.

Powell, J. F. F., Fischer, W. H., Park, M., Craig, A. G., Rivier, J. E., White, S. A., Francis, R. C., Fernald, R. D., Licht, P., Warby, C., and Sherwood, N. (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.

Powell, J. F. F., Reska-Skinner, S. M., Om Prakash, M., Fischer, W. H., Park, M., Rivier, J. E., Craig, A. G., Mackie, G. O., and Sherwood, N. (1996). Two new forms of gonadotropin-releasing hormone in a protochordate and the evolutionary implications. *Proc. Natl. Acad. Sci. USA* **93,** 10461–10464.

Powell, J. F. F., Zohar, Y., Elizur, A., Park, M., Fisher, W. H., Craig, A. G., Rivier, J. E., Lovejoy, D. A., and Sherwood N. (1994). Three forms of gonadotropin-releasing hormone characterized from brains of one species. *Proc. Natl. Acad. Sci. USA* **91,** 12081–12085.

Powell, R. C., King, J. A., and Millar, R. P. (1985). [Trp^7,Leu^8]LH-RH in reptilian brain. *Peptides* **6,** 223–227.

Powell, R. C., Ciarcia, G., Lance, V., Millar, R. P., and King, J. A. (1986). Identification of diverse molecular forms of GnRH in reptile brain. *Peptides.* **7,** 1101–1108.

Saint Girons, H. (1984). Les cycles sexueles des lézards mâles et leurs rapports avec le climat et les cycles reproducteurs des femelles. *Ann. Sci. Natur. Zool. Paris* **6,** 221–243.

Sherwood, N., and Whittier, J. M. (1988). Gonadotropin-relasing hormone from brains of reptiles: Turtles (*Pseudemys scripta*) and snakes (*Tamnophis sirtalis parietalis*). *Gen. Comp. Endocrinol.* **69,** 319–327.

Sherwood, N., Eiden, L., Brownstein, M., Spiess, J., Rivier, J., and Vale, W. (1983). Characterization of a teleost gonadotropin-releasing hormone. *Proc. Natl. Acad. Sci. USA* **80,** 2794–2798.

Sherwood, N., Sower, S. A., Marshak, D. R., Fraser, B. A., and Brownstein, M. (1986). Primary structure of gonadotropin-releasing hormone from lamprey brain. *J. Biol. Chem.* **261,** 4812–4819.

Sherwood, N., von Schalburg, K., and Lescheid, D. W. (1997). Origin and evolution of GnRH in vertebrates and invertebrates. *In* "GnRH Neurons: Gene to Behavior" (I. S. Parhar and Y. Sakuma, Eds.), pp. 3–25. Brain Shupan, Tokyo.

Smith, M. T., Moore, F. L., and Masson, R. T. (1997). Neuroanatomical distribution of chicken-I gonadotropin-releasing hormone (cGnRH-I) in the brain of the male red-sided garter snake. *Brain Behav. Evol.* **49,** 137–148.

Somoza, G. M., Stefano, A. V., D'Eramo, J. L., Canosa, L. F., and Fridman, 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.

Sower, S. A., Chiang, Y. A., Lovas, S., and Conlon J. M. (1993). Primary structure and biological activity of a third gonadotropin-releasing hormone from lamprey brain. *Endocrinology* **132,** 1125–1131.

Stefano, A. V., Canosa, L. F., D'Eramo, J. L., Fridman, O., Affanni, J. M., and Somoza, G. M. (1997). GnRH molecular variants in the brain and pituitary gland of pejerrey, *Odontesthes bonariensis* (Atheriniformes). Chromatographic and immunological evidence for the presence of a novel molecular variant. *Comp. Biochem. Physiol.* **C 118,** 335–345.

Tsai, P. S., and Licht, P. (1993). Differential distribution of chicken-I and chicken-II GnRH in the turtle brain. *Peptides* **14,** 221–226.

Yanosky, A. A., and Mercolli, C. (1993). Supression of dormancy in Tegu lizards (*Tupinambis teguixin*) culture at El Bagual Ecological Reserve (Argentine Republic). *Arch. Zootec.* **42,** 3–12.

Yu, K. L., Sherwood, N. M., and Peter, R. E. (1988). Differential distribution of two molecular forms of gonadotropin-releasing hormone in discrete brain areas of goldfish (*Carassius auratus*). *Peptides* **9,** 625–630.