Differential binding of mammalian and salmon GnRHs with rat and carp pituitary receptors

Kazimierz Kochman [1] , Alina Gajewska [1] and Krzysztof Bieniarz [2]

1. Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, Jabłonna, Poland
2. Department of Ichtyobiology and Fisheries, Agricultural University, Cracow, Poland

Correspondence to: Professor Kazimierz Kochman, Ph.D.
Dept. of Neuroendocrinology, Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences
05-110 Jablonna near Warsaw, Poland.
TEL.: (48-22) 782-44-22; FAX: (48-22) 774-20-38
E-MAIL: k.kochman@ifzz.pan.pl

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Abstract

OBJECTIVES: Receptor binding of GnRH is connected with the stimulation of pituitary gonadotropic cells leading to both the release and biosynthesis of gonadotropins. The binding is connected with the conformational changes in the receptor which induce the specific intracellular signalisation. The study of fish GnRHs and their receptors may give us new knowledge of the complex interplay of different mechanisms involved in neuroendocrine regulation of reproduction.
METHODS: Receptor binding of both mGnRH and sGnRH were compared by the study utilizing the displacement method with mGnRH or sGnRH as radioactive tracers. Incubation was performed at 2°C to avoid ligand degradation.
RESULTS: The comparative binding of mGnRH and sGnRH with GnRH receptors from the female rat pituitary and female carp pituitary was studied. At the 50% of displacement, the binding of sGnRH to the rat pituitary receptor was very small and in comparison to the binding of mGnRH (100%) was in the range 2–15%. However, the binding of mGnRH to carp pituitary receptors is small in comparison with the binding of sGnRH (100%) and was in the range 5–20%.
CONCLUSION: The results demonstrated the differences in binding of different GnRHs to the receptor in rats and carp. This suggests that the structures of GnRH and its receptor undergo co-evolution in different classes of animals.

INTRODUCTION

The knowledge and understanding of regulatory mechanisms involved in neurohormonal and neuromodulatory action may lead us to the solving of fundamental processes in their full complexity in growth, reproduction and vertebrate sex differentiation. The study of these processes in humans and mammals is relatively well advanced, but the research concerning the specific pathways in teleost and bony fish organisms offers the possibility to touch the inside of the fundamental mechanisms of endocrine regulation [3].

The two principal gonadotropins in the fish anterior pituitary are released from the proximal pars distalis, GTH-1 is structurally similar to follicle stimulating hormone (FSH), and GTH-2 is similar to luteinizing hormone (LH) [30]. There are only several publications on the neuroendocrine regulation of GTH-1, while the understanding of the secretion and action of GTH-2 is much greater [3]. It should also be mentioned that growth hormone (GH) in fish not only stimulates

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somatic growth [25] but also release of gonadotropins [25, 34, 39].

Gonadotropin releasing hormone (GnRH) is the key stimulatory neuropeptide, while the neurotransmitter dopamine (DA) inhibits the release of GTH-2 [39].

GnRH in vertebrates and protochordates exists in eleven forms [29]. The most widely present form is chicken GnRH-II, which coexists with the other molecular form in teleost fish [17, 23].

Recently, it was proven that three different forms of GnRH coexist in the brain of several fish species [6, 17, 23] and it remains unknown how gonadotropes are able to recognize and specifically respond to similar forms of GnRH.

The aim of this study was to compare the binding potency of salmon GnRH (sGnRH) to the GnRH receptor from the anterior pituitary of the female rat and the binding of the mammalian GnRH (mGnRH, GnRH) to the receptor from the pituitary of the adult female carp.

MATERIAL AND METHODS

Animals

Adult female carps (n=6) weighing 7000–9000 grams with regular reproductive potency were from the Institute of Inland Fisheries in Żabieniec. Pituitaries were prepared by Dr. Zygmunt Okoniewski and they were instantly frozen in –80°C till experiment.

Randomly cycling female rats (n=25) were the source of pituitaries, which were kindly donated by different laboratories, were collected during four months, frozen immediately and kept at –80°C till the experiment.

Chemicals and peptides

Mammalian GnRH (mGnRH, GnRH, pGlu^{1}-His- Trp-Ser-Tyr- Gly-Leu-Arg- Pro-Gly^{10}-NH_2) and salmon GnRH (sGnRH, pGlu^{1}-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly^{10}-NH_2) were purchased by Sigma-Aldrich (St. Louis, MO, USA).

Bovine serum albumin (BSA) and bovine gamma-globulin were from Sigma, polyethylene glycol 6000 (PEG 6000) was the product of Merck, and GFC filter (2.5 inches in diameter) were from Whatman (Clifton, NJ, USA).

Radioactive iodide I^{125} for iodination was purchased from Amersham.

Other chemicals and products of the highest purity were obtained from Merck or Sigma-Aldrich.

Pituitary receptor binding assays

The method was generally the same as it was described earlier [18, 20]. The GnRH or sGnRH was used as a radioactive tracer for the assay of GnRH-R binding and was iodinated by the modification of the iodogen method (specific radioactivity: mGnRH: 207 mCi/mg, sGnRH: 219 mCi/mg.

GnRH-R samples in aliquot (100 μL) of pituitary hemi-purified homogenates from the same weight of tissue by equilibration at 4°C (100 μg of tissue per tube) were taken to incubation. Incubation was performed during 16 h at 2°C in total volume 500 μL in phosphate buffered saline, pH 7.4 (PBS) containing 0.1% BSA and specific binding was measured in the presence of 1000-fold excess of unlabelled GnRH. At the end of incubation, 50 μL of 0.3% bovine gamma-globulin was added to each tube, followed immediately by 1.0 mL of 25% polyethylene glycol 6000 (PEG 6000) dissolved in PBS, pH 7.4, vortex mixed, and the mixture was left standing for 15 min at 2°C. Then 4.5 mL of 16% PEG was added. The mixture was then applied to GFC filters presoaked in 2% BSA and retained in a multi-place holder and filtered. The filters were washed four times. The radioactivity retained on the filter was measured in a gamma-spectrometer with a counting efficiency 60% for I^{125}. The separation and washing procedure of filters took less than 15s/tube.

RESULTS

The results of binding of mGnRH and sGnRH with GnRH receptor in the female rat pituitary and their competition with radioactive mGnRH as a radioactive tracer is shown in Figure 1.

The 50% of displacement of mGnRH was approximately at the concentration of cold mGnRH 10^{-8} M while sGnRH at 10^{-6} M. The binding of sGnRH to rat pituitary receptors is very small and comparable to the binding of mGnRH (100%) and is in the range 2–15%.

The results of binding of sGnRH and mGnRH with the GnRH receptor in the female carp pituitary and their competition with radioactive sGnRH as a radioactive tracer is shown in Figure 2.

The 50% of displacement of sGnRH was approximately at the concentration of cold sGnRH 10^{-6} M while mGnRH at 10^{-4} M. The binding of mGnRH to carp pituitary receptors is small in comparison with the binding of sGnRH (100%) and is in the range 5–20%.

DISCUSSION

GnRH molecules exert a stimulatory action on the pituitary gonadotropes cells leading to the release and biosynthesis of gonadotropins (LH and FSH in mammals or GTH-I and GTH-II in fish). GnRH-R activates several distinct signaling pathways by binding multiple G proteins (Gq/G11, Gs and Gi). The activation of Gs and Gi proteins was not found in all experimental systems and their importance is not known [31]. Recent results have proved that the formed complex activates the enzyme phospholipase Cβ (PLCβ) [2, 13]. The physiological consequence of this stimulation is the synthesis of several second messengers such as inositol 1,4,5-trisphosphate (IP_3) or diacylglycerol (DG). IP_3 activates

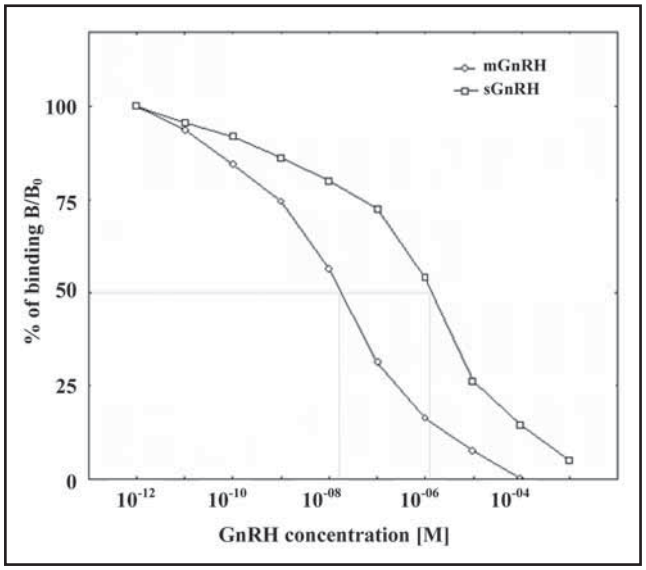

Figure 1. Competitive binding of mGnRH and sGnRH with the GnRH receptor in the female rat pituitary . The radioactive tracer was mGnRH. Significance at the 50% of displacement: P≤0.05.

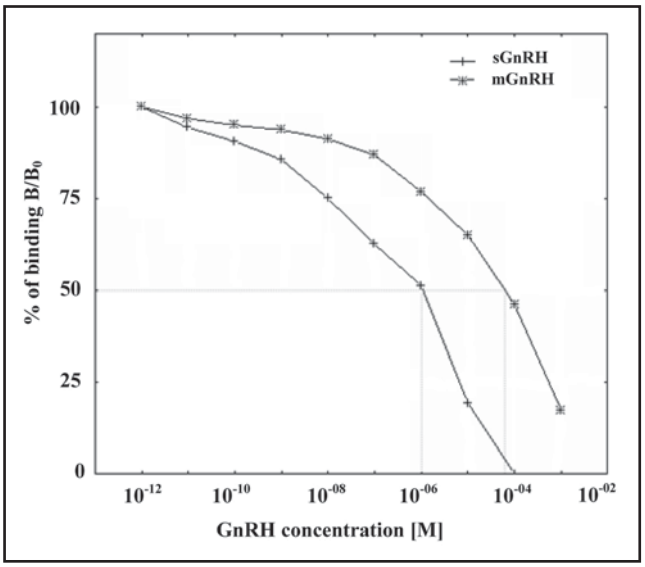

Figure 2. Competitive binding of sGnRH and mGnRH with the GnRH receptor in the female carp pituitary. The radioactive tracer was sGnRH. Significance at the 50% displacement: P≤0.05.

the release of Ca^{2+} ions from intracellular pools while DG activates the protein kinase C [24].

Studies on the structure and spatial arrangements of GnRH molecules enable us to understand more and more precisely the physiological consequences of its action on the pituitary gonadotropic cells [31].

GnRH is classified as a member of the rhodopsin-like G-protein-coupled receptor superfamily (GPCR) which consists of the N-terminal extracellular domain, seven α-helical transmembrane domains (TMD) connected by hydrophilic intra- and extracellular loops [28, 33] and the C-terminal intracellular domain [31]. The C-terminal domain mediates the binding of effectors, propagation of signaling in the cell, desensitization and internalization [19, 21].

The sequence of GnRH-Rs receptors was determined in human and several mammalian species [14, 15, 4, 5, 7, 43]. In mammalian GnRH-R the intracellular carboxyl-terminal tail is absent [37, 38]. Using the original methodology of structure research it was possible to predict the spatial structure of GnRH-R including the way how GnRH and a G protein interact with the receptor molecule [40, 41]. GnRH and agonists bind to the extracellular part of GnRH-R changing its conformation into its active state. Active conformation creates favorable conditions for transmitting the signal into the cell through associated G-proteins [8, 10, 31].

Flanagan et al. [9] established that Asp^{98} and Lys^{121} in the receptor specifically interact with His^2 of mammalian GnRH. The replacement of Asp^{98} residue with positively charged amino acid residue causes an obstacle which prevents the formation of a hydrogen bond with His^2 but does not abolish all mGnRH interactions with receptor [9, 31]. It was also demonstrated that the presence of Trp^7 and Leu^8 residues in the salmon GnRH molecule, a native peptide in goldfish, are important for the recognition of the ligand by the GnRH receptors in the goldfish pituitary. The high-affinity GnRH receptors recognize the native neurohormone sGnRH better than mGnRH [11].

Studies have shown that certain GnRH receptors other than mGnRH receptors are not selective for Arg^8 containing ligands [36, 42, 27].

When GnRH interact with the receptor it stabilizes the extracellular loop 3 which goes consequently to the changes of conformation in the transmembrane domains which encourage G-protein activation, subsequent signal induction and propagation in the cell [26].

Our present results are in agreement with previous publications by other authors mentioned above and indicate that the binding of sGnRH to the rat GnRH receptor is very small and in comparison to the binding of mGnRH (100%) is in the range of 2–15%. The binding of mGnRH to the carp pituitaty receptor, when the sGnRH was used as a radioactive tracer, was in the range of 5–20% in comparision with the displacement of the radioactive tracer by sGnRH.

GnRH stimulates the release of GTHs from the cells of the fish pituitary gland which subsequently induces the synthesis of steroids in the gonads [1]. Rodriguez et al. [32] detected certain concentrations of three forms of GnRH in the pituitary of the male European Sea Bass (*Dicentrarchus labrax*, L.) during sex differentiation and the first spawning season: sea-bream GnRH (sbGnRH), chicken GnRH-II (cGnRH-II), and salmon GnRH (sGnRH). The highest levels of these peptides were found when the gonads started to differentiate and decrease during the first spawning season, with the exception of sbGnRH which was still in a high con-

centration. The concentration of sbGnRH were nine times higher than cGnRH and 17 times higher than sGnRH. The highest levels of these peptides were found when the gonads started to differentiate and decrease during the first spawning season, with the exception of sbGnRH which remained in a high concentration [32]. It remains unclear what role the several forms of GnRH present in fish brain have in reproductive regulation [32]. Holland et al. [12] reported that only sbGnRH, mostly present in a high concentration in the pituitary of the gilthead seabream, is responsible for GTH-2 regulation but Sorbera et al. [35] showed the higher potency of sGnRH than sbGnRH in the release of GTH-2 from the sea bass pituitary.

The presence of two or more different GnRH within the same organism raises the probability that GnRH receptors (GnRH-R) may co-evolue simultaneously with their ligands [16, 22].

The study of the multiple and complex regulatory mechanisms and processes in the neuroendocrine regulation of gonadotropin release and biosynthesis in different fish species remains challenge for physiologists, biochemists, endocrinologists and genetics. The results will be important not only for fish meat production but above all for new knowledge on the variety of possible mechanisms causing similar physiological effects. The development and maturation of neuroendocrine control mechanisms can be considered as a continuum in life of an individual organism [3].

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REFERENCES

1 Amano M, Ikuta K, Kitamura S, Aida K. The maturation of the GnRH system and its regulation by gonadal steroids in masu salmon. Fish Physiol Biochem. 1997; **17**: 63–70.
2 Anderson L, Hoyland J, Mason WT, Eidne KA. Characterization of the gonadotrophin-releasing hormone calcium response in single alpha T3-1 pituitary gonadotroph cells. Mol Cell Endocrinol. 1992; **86**: 167–175
3 Blazquez M, Bosma PT, Fraser EJ, Van Look KJW, Trudeau VL. Fish as models for the neuroendocrine regulation of reproduction and growth. Comp Biochem Physiol C. 1998; **119**: 345–364. Review.
4 Brooks J, Taylor PL, Saunders PT, Eidne KA, Struthers WJ, McNeilly AS. Cloning and sequencing of the sheep pituitary gonadotropin-relasing hormone receptor and changes in expression of its mRNA during the estrous cycle. Mol Cell Endocrinol. 1993; **94**: R23–R27.
5 Chi L, Zhou W, Prikhozhan A, Flanagan C, Davidson JS, Golembo M, Illing N, Millar RP, Sealfon SC. Cloning and characterization of the human GnRH receptor. Mol Cell Endocrinol. 1993; **91**: R1–R6.
6 Elofsson U, Winberg S, Francis RC. Number of preoptic GnRH-immunoreactive cells correlates with sexual phase in a protandrously hermaphroditic fish, the dusky anemonefish (*Amphiprion melanopus*). J Comp Physiol A. 1997; **181**: 484–492.
7 Eidne KA, Sellar RE, Couper G, Anderson L, Taylor PL. Molecular cloning and characterisation of the rat pituitary gonadotropin-releasing hormone (GnRH) receptor. Mol Cell Endocrinol. 1992; **90**: R5–R9.
8 Flanagan CA, Millar RP, Illing N. Advances in understanding gonadotrophin-releasing hormone receptor structure and ligand interactions. Rev Reprod. 1997; **2**: 113–120.
9 Flanagan CA, Rodic V, Konvicka K, Yuen T, Chi L, Rivier JE, Millar RP, Weinstein H, Sealfon SC. Multiple interactions of the Asp (2.61(98)) side chain of the gonadotropin-releasing hormone receptor contribute differentially to ligand interaction. Biochemistry. 2000; **9**: 8133–8141.
10 Gundermann T, Schoneberg T, Schultz G. Functional and structural complexity of signal transduction via G-protein-coupled receptors. Ann Rev Neurosci. 1997; **20**: 399–427.
11 Habibi HR, Marchant TA, Nahorniak CS, Van der Loo H, Peter RE, Rivier JE, Vale WW.. Functional relationship between receptor binding and biological activity for analogs of mammalian and salmon gonadotropin-releasing hormones in the pituitary of goldfish (*Carassius auratus*). Biol Reprod. 1989; **40**: 1152–1161.
12 Holland MCH, Gothilf Y, Meiri I, King JA, Okuzawa K, Elizur A, Zohar Y. Levels of the native forms of GnRH in the pituitary of the gilthead seabream., *Sparus aurata*, at several stages of the gonadal cycle. Gen Comp Endocrinol. 1998; **112**: 394–405.
13 Husieh KP, Martin TFJ. Thyrotropin-releasing hormone and gonadotropin-releasing hormone receptors activate phospholipase C by coupling to the guanosine triphosphate-binding proteins Gq and G11. Mol Endocrinol. 1992; **6**: 1673–1681.
14 Kakar SS, Musgrove LC, Devor DC, Sellers JC, Neill JD. Cloning, sequencing, and expression of human gonadotropin releasing hormone (GnRH) receptor. Biochem Biophys Res Commun. 1992; **189**: 289–295.
15 Kakar SS, Rahe CH, Neill JD. Molecular cloning, sequencing, and characterizing the bovine receptor for gonadotropin releasing hormone (GnRH). Dom Anim Endocrinol. 1993; **10**: 335–342.
16 Khakoo Z, Bhatia A, Gedamu L, Habibi HR. Functional specifity for salmon gonadotropin-releasing hormone (GnRH) and chicken GnRH-II coupled to gonadotropin release and subunit messenger ribonucleic acid level in the Goldfish pituitary. Endocrinology. 1994; **134**: 838–847.
17 King JA, Millar RP. Evolutionary aspects of gonadotropin-releasing hormone and its receptor. Cell Mol Neurobiol. 1995; **15**: 5–23.
18 Kochman K, Gajewska A, Kochman H, Kozłowski H, Masiukiewicz E, Rzeszotarska B. Binding of Cu2+, Zn2+, and Ni2+-GnRH complexes with the rat pituitary receptor. J Inorg Biochem. 1997; **65**: 277–279.

19 McArdle CA, Franklin J, Green L, Hislop JN. Signalling, cycling and desensitization of gonadotropin releasing hormone receptors. J Endocrinol. 2002; **171**: 1–11.
20 Michaluk A, Blitek A, Gajewska A, Kaczmarek MM, Gromadzka-Hliwa K, Kochman H, Ziecik AJ. LH release by Cu and Ni salts and metal-GnRH complexes, *in vitro*. Neuro Endocrinol Lett. 2006; **27**: 483–486.
21 Millar RP, Lu ZL, Pawson AJ, Flanagan CA, Morgan K, Maudsley SR. Gonadotropin-releasing hormone receptors. Endocrine Rev. 2004; **25**: 235–275.
22 Moncaut N, Somoza G, Power DM, Canário VM. Five gonadotropin-releasing hormone receptors in a teleost fish: isolation, tissue distribution and phylogenetic relationships. J Molec Endocrinol. 2002; **34**: 767–779.
23 Montero M, Dufour S. Gonadotropin-releasing hormones (GnRH) in fishes- evolutionary data on their structure, localization, regulation, and function. Zool Sci. 1996; **35**: 149–160.
24 Ortmann O, Dietrich K. Pituitary and extrapituitary actions of gonadotrophin-releasing hormone and its analogues. Hum Reprod. 1999; **14 (**Suppl 1): 194–206.
25 Peter RE, Marchant TA. The endocrinology of growth in carp and related species. Aquaculture. 1995; **129**: 299–321.
26 Petry R, Craik D, Haaima G, Fromme B, Klump H, Kiefer W, Palm D, Millar RP. Secondary structure of the third extracellular loop responsible for ligand selectivity of a mammalian gonadotropin-releasing hormone receptor. J Med Chem. 2002; **45**: 1026–1034.
27 Pfleger KDG, Bogerd J, Millar RP. Conformational constraint of mammalian, chicken, and salmon GnRHs, but not GnRH II, enhances binding at mammalian and nonmammalian receptors: evidence for preconfiguration of GnRH II. Molec Endocrinol. 2002; **16**: 2155–2162.
28 Pfleger KDG, Pawson AJ, Millar RP. Changes to gonadotropin-releasing hormone (GnRH) receptor extracellular loops differentially affect GnRH analog binding and activation: evidence for distinct ligand-stabilized receptor conformation. Endocrinology. 2008; **149**: 3118–3129.
29 Powell JFF, Reskaskinner SM, Prakash MO, Fisher WH, Park M, Rivier JE, Craig AG, Mackie GO, Sherwood NM. Two new forms of gonadotropin-releasing hormone in a protochordate and the evolutionary implications. Proc Natl Acad Sci USA. 1996; **93:** 10461–10464.
30 Querat B. Molecular evolution of the glycoprotein hormones in vertebrates. In: Davey KB, Peter RE, Tobe SS, Editors. Perspectives in Comparative Endocrinology. Ottawa: National Research Council of Canada. 1994; pp. 27–35.
31 Rispoli LA, Nett TM. Pituitary gonadotropin-releasing hormone (GnRH) receptor: structure, distribution and regulation of expression. Anim. Reprod Sci. 2005; **88**: 57–74.
32 Rodriguez L, Carrillo M, Sorbera LA, Soubrier MA, Mananos E, Holland MCH, Zohar Y, Zanuy S. Pituitary levels of three forms of GnRH in the male european sea bass (*Dicentrarchus labrax*, L.) during sex differentiation and first spawning season. Gen Comp Endocrinol. 2000; **120:** 67–74.
33 Sealfon SC, Weinstein H, Millar RP. Molecular mechanisms of ligand interaction with the gonadotropin-releasing hormone receptor. Endocrine Rev. 1997; **18**: 180–205.
34 Singh H., Griffith RW, Takahashi A, Kawauchi H, Thomas P, Stegeman JJ. Regulation of gonadal steroidogenesis in in *Fundulus heteroclitus* by recombinant salmon growth hormone and prolactin. Gen Comp Endocrinol. 1988; **72**: 144–153.
35 Sorbera L, Zanuy S, Carrillo M. Gonadotropin-releasing hormone action in seabass (*Dicentrarchus labrax*, L.) pituitary primary cultures. In "Proceedings of the 3rd International Symposium on Research for Aquaculture: Fundamental and Applied Aspects", August 24–27, 1997. University of Barcelona, Spain.
36 Sun YM, Flanagan CA, Illing N, Ott TR, Sellar R, Fromme BJ, Hapgood J, Sharp P, Sealfon SC, Millar RP. A chicken gonadotropin-releasing hormone receptor that confers agonist activity to mammalian antagonists. Identification of D-Lys(6) in the ligand and extracellular loop two of the receptor as determinants. J Biol Chem. 2001; **276**: 7754–7761.
37 Tensen C, Okuzawa K, Blomenrohr M, Rebers F, Leurs R, Bogerd J, Schulz R, Goos H. Distinct efficacies for two endogenous ligands on a singlr cognate gonadoliberin receptor. Eur J Biochem. 1997; **243**: 134–140.
38 Troskie B, Illing N, Rumbak E, Sun YM, Hapgood J, Sealfon S, Conklin D, Millar RP. Identification of three putative GnRH receptor subtypes in vertebrates. Gen Comp Endocrinol. 1998; **112**: 296–302.
39 Trudeau VL. Neuroendocrine regulation of gonadotropin II release and gonadal growth in the goldfish, *Carassius auratus*. Rev Reprod. 1997; **2**: 55–68.
40 Yeagle PL, Alderfer JL, Albert AD. Three-dimensional structure of the cytoplasmic face of the G protein receptor rhodopsin. Biochemistry. 1997; **36**: 9649–9654.
41 Yeagle PL, Salloum A, Chopra A, Bhawsar N, Ali L, Kuzmanovski G, Alderfer JL, Albert AD. Structures of the intradiskal loops and amino terminus of the G-protein receptor, rhodopsin. J Pept Res. 2000; **55**: 455–465.
42 Wang L, Bogerd J, Choi HS, Seong JY, Soh JM, Chun SY, Blomenrohr M, Troskie BE, Millar RP, Yu WH, McCann SM, Kwon HB. Three types of GnRH receptor characterized in the bullfrog. Proc Natl Acad Sci USA. 2001; **98**: 361–366.
43 Weesner GD, Matteri RL. Rapid communication: nucleotide sequence of luteinizing hormone-releasing hormone (LHRH) receptor cDNA in the pig pituitary. J Anim Sci. 1994; **72**: 1911.