Gonadotropin-releasing hormone (GnRH) in fishes: A review on basic and practical findings

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Abstract

Similar to mammals, neurohormones regulate reproductive process in fish. Gonadotropin-releasing hormone (GnRH) is one of the most important factors controlling reproduction, which causes gametogenesis by regulating the amount and time of LH and FSH secretion. The highest variations in GnRH isoforms in vertebrates is related to teleost. Phylogenetic study classifies GnRH into three forms (GnRH1, GnRH2, GnRH3), and all fishes have at least two forms. In fish that lack GnRH1, GnRH3 plays an alternative role. Unlike mammals, it seems that GnRH is not the main gate of reproduction, at least in zebrafish and medaka. However, farming fishes in captivity leads to a decrease in the level of GnRH and LH, which causes reproductive impairment. Therefore, the synthesis of GnRH analogs that have prolonged stability is important for the aquaculture industry. Over the last decade molecular techniques have been well developed and the role of other neuropeptides on the production of LH hormone have confirmed which raised new challenges of the relationship between novel peptides and GnRH. The present study is centered on the most important applied and fundamental findings on GnRH in fishes.

Keywords: GnRH, Decapeptide, Analogue, Phylogeny tree, Paracrine, Autocrine

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Introduction

The 1977 Nobel Prize in Physiology was jointly awarded to the research of Roger Guillemin and Andrew Schally for the identification and sequencing of gonadotropin-releasing hormone (GnRH). The theory of control of pituitary gonadotropin secretions by brain neuroendocrines was controversial for a long time (Benoit and Assenmacher, 1952; Donovan and Harris, 1954). Finally, in the early 1970s, two independent but simultaneous research teams succeeded in isolating luteizing hormone (LH)-releasing factors from the pig and sheep hypothalamus (Amoss *et al*., 1971; Matsuo *et al*., 1971). At first, the isolated peptide (LH-releasing hormone) was named LHRH, but over time it was found that the peptide is also effective in follicle-stimulating hormone (FSH) secretion. Therefore, the name LHRH was replaced by GnRH, which is still used today. It is worth mentioning, in the same year (1971), it was found that carp hypothalamus extract causes the release of gonadotropin by the pituitary gland *in vitro*. Soon, Breton and collegues (1971) studies of reproductive induction confirmed the previous results *in vivo*. Since then, GnRH has always played an important role in the aquaculture industry.

The aim of the present study is to review fundamental and state-of-the-art findings in GnRH as one of the most important ports of fish reproduction**.**

The anatomy of the brain and pituitary gland in fish

The brain of fish and mammals have a significant difference which make the reproductive function of brain and pitutitrary different with mammals. In mammals, the pituitary gland and the brain is connected via circulatory system and capillary network (median eminence) (Kah *et al*., 1983). More specifically, GnRH is pulsatilely released into the pituitary portal system to stimulate the biosynthesis of FSH and LH from the anterior pituitary. This median eminence system is absent in fish but instead the axons of GnRH neurons connect directly to synapses in the pituitary (Fig. 1) (Peter *et al*, 1990). However, some fish species are different such as coelacanths, gilled fish (Ball, 1981; Gorbman, 1995) and cartilaginous fish (Gaillard *et al*., 2018) and have a circulatory system. Unlike mammals, fish have distinct FSH/LH gonadotrope cells (Machluf *et al*., 2011). Although brain of teleost are structurally different from mammals, fish brains have the types of hypothalamic cells identified in mammals.

Classification and structure of GnRH

The amino acid sequence of GnRH is highly conserved in most mammalian which is the reason that for more than a decade after the discovery of GnRH decapeptide was considered to be the same in all vertebrates (Okubo and

Nagahama, 2008). In other words, 4 amino acids (positions 1, 4, 9, and 10) of GnRH decapeptide are identical in all vertebrates (Roch *et al*., 2011). Sherwood *et al*. (1983) found that the GnRH decapeptide in salmon (now known as GnRH3), with the LHRH peptide identified in mammals (now known as GnRH1), at amino acids in position 7 (Trp) and position 8 (Leu) are different (Fig. 2).

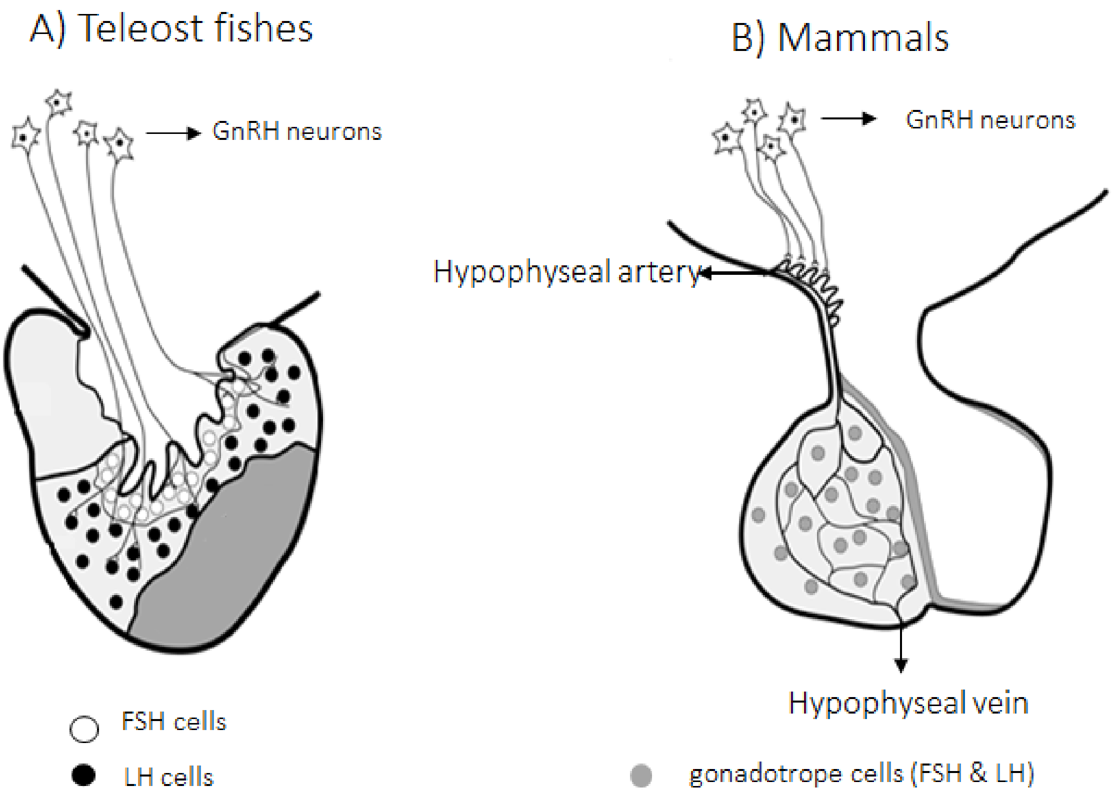

Figure 1: Hpothalamus-pituitary in the brain of teleost (a) and mammals (b). Teleost has distinct gonadotrope cells.

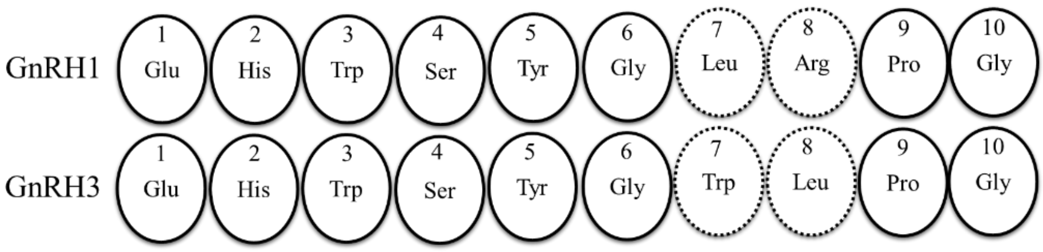

Figure 2: The active region of GnRH peptide consists of 10 amino acids (decapeptide) which is conserved in most fishes. GnRH1 and GnRH3 decapeptides differ only in amino acids 7 and 8.

The structure of *gnrh* is similar in all vertebrates and includes 4 exons and 3 introns. The main structure of GnRH protein consists of signal peptide in the N-terminal domain (including 28 amino acids), the active region of the peptide which has 10 amino acids (decapeptide) and is conserved in most fishes. Then three amino acids glycine, lysine and arginine which are conserved in all fish species. Finally, the C-terminal domain that contains GnRH-associated peptides (GAP) and contains 60 amino acids. The GAP region is less conserved than other regions which is responsible for peptide stability (Fig. 3).

For a long time, different forms of GnRH was named based on the name of the species in which they were first found. For example, salmon GnRH was first identified in salmon, therefore it was named sGnRH.

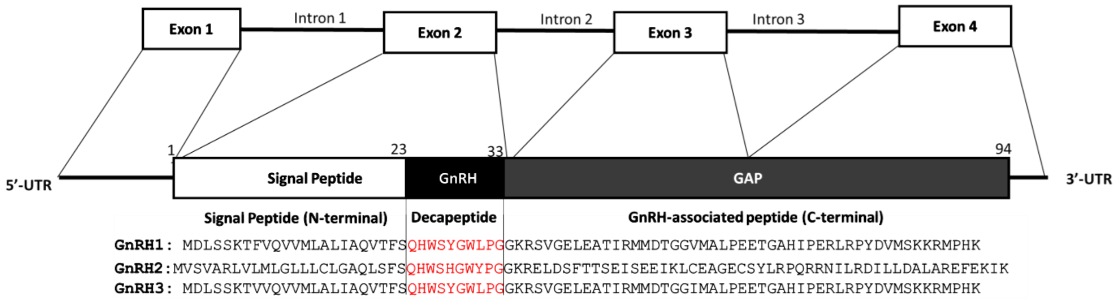

Figure 3: GnRH gene and peptides in *Oncorhynchus mykiss*

A long with development of molecular techniques different GnRH isoforms were identified so that at least 16 isoforms have been identified in vertebrates (Whitlock *et al*., 2019). With the increasing number of identification of GnRH isoforms and with the aim of unification and standardization to prevent confusion of scientists, a new classification of GnRH forms was established based on the phylogeny tree and their place of expression (Fig. 4) (Kah *et al.*, 2007). Phylogenetic analyzes of different vertebrates, divided GnRH into three branches: GnRH type 1 (GnRH1), GnRH type 2 (GnRH2), GnRH type 3 (GnRH3), (White *et al*., 1998; Fernald and White, 1999). The most similarity among vertebrates is related to the GnRH1. According to Figure 4, the characteristics of GnRH are different in teleost and cartilaginous fish. For example GnRH2 in lamprey has considerable distance with Roho and goldfish.

GnRH1 is produced in the hypothalamus which exhibits at least 12 isoforms and sequence includes: His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2 Glu-. GnRH1 has been observed in most vertebrates, except for a few species of cyprinids (Steven *et al*., 2003) and salmonids (Okuzawa *et al*., 1990) (Okubo and Nagahama, 2008; Chang and Pemberton, 2018). The role of GnRH1 in maturation and induction of gonadotropins is demonstrated (Kah *et al*., 2007; Okubo and Nagahama, 2008).

GnRH2, formerly named chicken GnRH II, is present in most vertebrates from jawed fish to humans. GnRH2 is produced in the midbrain and contains at least 2 isoforms. The s GnRH2 is fully conserved in vertebrates and its sequence includes: Glu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH2. Unlike other forms of GnRH, the role of GnRH2 is not well defined.

GnRH3 has been found only in teleost (Kah *et al*., 2007; Okubo and Nagahama, 2008), lampreys (Decatur *et al.*, 2013) and Coelacanth (Roch *et al*., 2011). It is produced in the ventral telencephalon and has at least 4 isoforms and its sequence includes Glu-His-Trp-Ser-Try-Gly-Trp-Leu-Pro-Gly-NH2 (Sherwood *et al*., 1983; Chang and Pemberton, 2018).

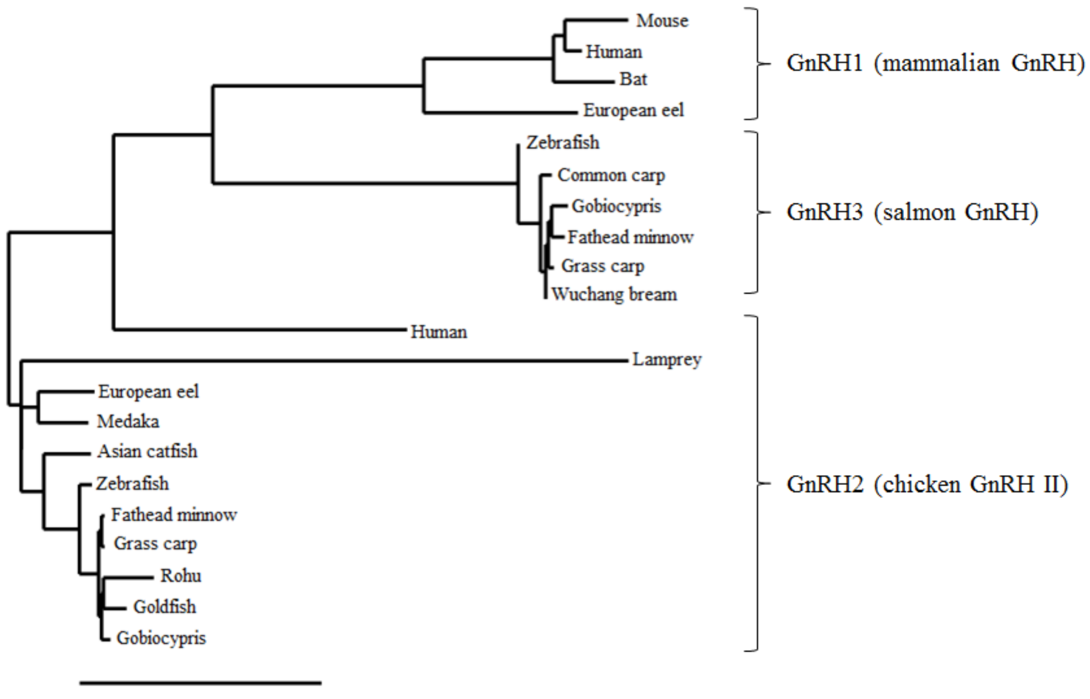

Figure 4: Classification of GnRH based on the phylogeny of amino acids. Genebank accession numbers for GnRH1: *Molossus molossus* (KAF6480186.1), (KAI4009998.1) *Homo sapiens*, (ADD92012.1) *Anguilla anguilla*, *Mus musculus* (EDL35964.1), for GnRH2: *Pimephales promelas* (ABV45417.1), *Petromyzon marinus* (ABE66462.1), *Labeo rohita* (RXN32401.1), *Oryzias latipes* (BAD02403.1), (AYE88915.1) *Carassius auratus*, (ADD92005.1) *Anguilla anguilla*, (AFJ44819.1) *Gobiocypris rarus*, *Ctenopharyngodon idella* (UXW61381.1), *Homo sapiens* (AAI15400.1), *Heteropneustes fossilis* (AUR53242.1), (AAI62951.1) *Danio rerio*, for GnRH3: *Pimephales promelas* (XP_039526578.1), *Cyprinus carpio* (XP_018926565.1), *Gobiocypris rarus* (AFJ44820.1), *Ctenopharyngodon idella* (UXW61382.1), *Megalobrama amblycephala* (XP_048046801.1), *Danio rerio* (AAU43785.1).

All three reported forms have a role in the reproductive process but are not limited to reproduction. In addition, their mechanism of action is not well defined (Okubo and Nagahama, 2008; Golshan *et al.*, 2022). There are at least 2 forms of GnRH in the vertebrate brain (GnRH2 and GnRH1 or GnRH3). In most vertebrates from humans to fishes, the highest abundance of GnRH in the midbrain is related to GnRH2. However, in most mammals, such as cattle, mice, and monkeys, GnRH2 is inactive or deleted and they only have GnRH1 (Morgan and Millar, 2004; Millar *et al.*, 2004). It is worth mentioning that in some fish all three forms of GnRH have been observed (Decatur *et al*., 2013). Among vertebrates, teleost has the most forms of GnRH with eight GnRH isoforms (Sherwood *et al*., 1983; Adams *et al*., 2002; Podhorec and Kouril, 2009).

Mechanism of action of GnRH

a) Direct effect on the pituitary gland

The hypothalamus-pituitary-gonadal axis is stimulated by GnRH leading to the synthesize of steroid hormones in the gonads and subsequently gametogenesis (Roch *et al*., 2011; Golshan and Alavi, 2019). Ontology processes show that GnRH activates receptors on the surface of cells. Gene ontology analysis showed that they directly or indirectly play a role in the emergence of various traits such as reproductive and immune behaviors. Based on the ontology of identified genes, among biological processes, regulation of biological processes, metabolic processes, and molecular functions, catalytic, binding, as well as transfer activities were the most important. Some of the genes involved in GnRH pathways include FSH, LH, thyroid-stimulating hormone (TSH), and prostaglandin receptor (ptger 1), and they play an important role in sexual maturation, gonadal development, and reproduction (Fig. 5).

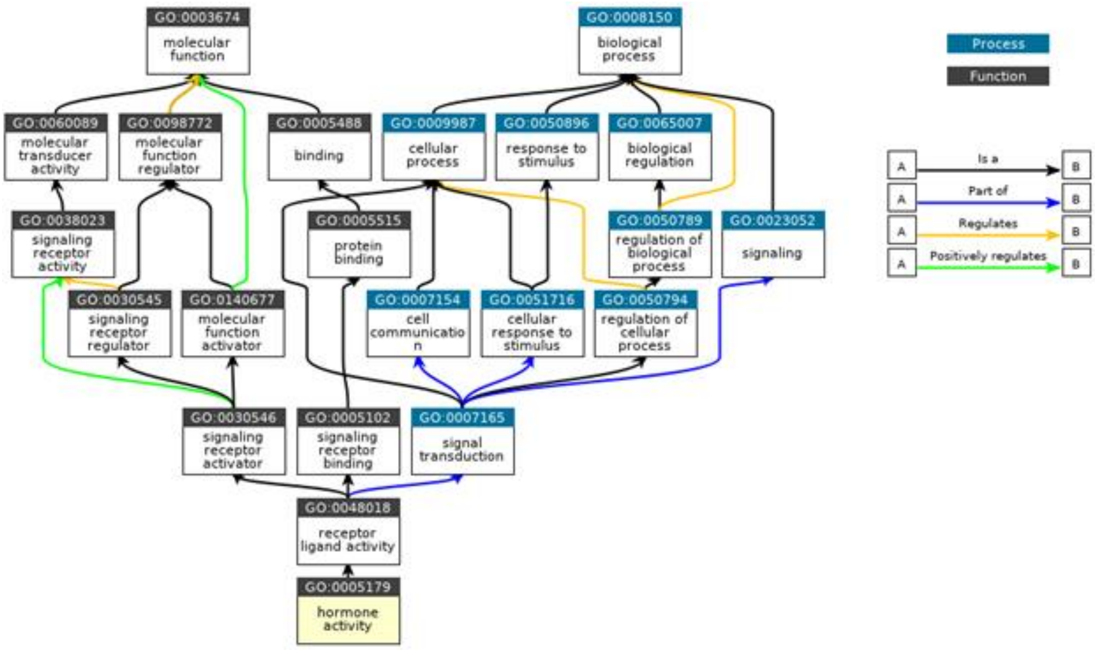

Figure 5: Ontology processes for GnRH. The recorded Go number for each biological and molecular activity is given in the corresponding rectangle. (www.uniprot.org/uniprot).

The highest amount of GnRH in the pituitary gland is in anterior part. Current knowledge about the role of GnRH isoforms in reproduction is limited. For example, in zebrafish, the level of GnRH3 in the pituitary is 3-4 times higher than GnRH2 (Steven *et al*., 2003). In addition, a positive correlation between GnRH3 gene expression and blood LH level has been reported in goldfish (Golshan *et al*., 2014). In species with three forms of GnRH, such as gilthead seabream, GnRH2 is observed in the first year, but its amount is very low (Holland *et al*., 2001). In this species, at the time of reproduction, GnRH1 is significantly higher in both males and females, and these levels is consist with the levels of LH in the blood plasma. Interestingly, *gnrh* gene expression levels during the reproductive season reveals diurnal cycles of GnRH gene expression that are closely related to daily gamete release activity (Gothilf *et al*., 1997). The maximum mRNA transcript of all three isoforms of GnRH was observed eight hours before ovulation, which coincides with the increase in the blood LH levels. In addition, the highest mRNA transcript of *gnrh* has been observed for GnRH3, followed by GnRH2 and GnRH1, respectively (Okuzawa *et al*., 2003). Although all three isoforms of GnRH have been found in the pituitary in male European sea bass, only GnRH1 increases during the spawning season (Rodriguez *et al*., 2000). Taken together, a close relationship between GnRH1 gene expression and reproductive activity is observed in European seabass, but the role of GnRH2 and GnRH3 on pituitary function is still not well defined. It seems that GnRH3 is responsible for regulating gonadotropes where GnRH1 deleted (Zohar *et al*., 2010).

b) Autocrine/paracrine regulation

Since teleost does not have a median eminence to transfer GnRH from the hypothalamus to the pituitary gland, the amount of GnRH in the blood is very low and even undetectable. On the other hand, GnRH and its receptors are not limited to the brain and pituitary and have been observed in peripheral tissues as gonads (Pati and Habibi, 1998). Similar to the pituitary, the frequency of distribution of GnRH isoforms in the gonad varies. For example, in zebrafish gonads, GnRH3 is more abundant than GnRH2 (Fallah *et al*., 2020). GnRH2 has been observed in biological germ cells (spermatogonia cells), sertoli cells and leydig's membrane (Desaulniers *et al*., 2017). Therefore, the gonads, as hypothalamus, are capable of producing GnRH. Also, the autocrine/paracrine role of GnRH has been proven in different fish species (Pati and Habibi, 1998; Fallah *et al*., 2020). Several studies indicate a direct stimulating or inhibiting effect of GnRH on gonadal hormones. For example, GnRH2 and GnRH3 stimulate testosterone secretion in the zebrafish testis. Recent study shows that if testicular tissue exposed to

GnRH3 in combination with flutamide which is known as an androgen receptor agonist, the amount of testosterone production is affected (Fallah *et al*., 2020).

c) Effects on reproductive behaviors

GnRH function is not limited to control the level of reproductive hormones and regulates reproductive behaviors as well. In female medaka fish, GnRH3 pulses are small (2-3 Hz), which causes reluctance to mate. The visual acquaintance of the female sex with the male increases GnRH3 pulses (Hz 5-6) and increases the desire of the female sex to mate. In other words, females prefer to mate with familiar males. Interestingly, female fish in which GnRH3 was deactivated showed a decrease in the desire to mate with familiar and unfamiliar males (Okuyama *et al*., 2017). Also, in goldfish, injection of GnRH2 and GnRH3 peptides of 0.5/ng/g increases spawning behaviors. However, the injection of 1 ng/g of the GnRH peptides causes a decrease in spawning behaviors (Volkoff and Peter, 1999), which is probably due to the activation of the negative feedback mechanism of hormones (Golshan *et al*., 2016). Besides, the connection between GnRH2 cells with the pineal and melatonin secretion has been domenstated (Servili *et al*., 2010). Therefore, it may transmit optical signals to the reproductive axis (Munoz-Cueto *et al*., 2020). In addition, GnRH2 is significantly increased in zebrafish under food deprivation indicating an important link between reproduction and physical condition (Marvel *et al*., 2019). Also, the role of GnRH in fish migration through olfactory memory in migration to the spawning place in *Oncorhynchus keta* has been determined. The migration of adult fish for reproduction is accompanied by the development of gonads. There is a positive correlation between gonadal development and GnRH expression. An increase in sGnRH gene expression level in forebrain neurons is observed in migration for spawning and at the same time as the final stage of gonadal development (Onuma *et al*., 2005). In addition, GnRH analog implantation in adult salmon helps to detect the river migration route (Ueda *et al*., 2016) and also significantly accelerates homing to the mother river (Kitahashi *et al*., 1998).

Is GnRH the main reproductive gate of fish?

In mammals, the role of GnRH as the main gate of reproduction is well documented. Studies show that hypothalamus-lesioned monkeys or sheep exposed to GnRH antagonists have reduced GnRH as well as FSH/LH secretions (Pohl *et al.*, 1983; Campbell *et al.*, 1998). Also, natural mutation in GnRH decreases the level of FSH and LH hormones in both male and female mice (Cattanach *et al*., 1977). In fish, reproductive processes such as spermatogenesis, folliculogenesis, ovulation and fertility were evaluated in the absence of GnRH. To this end, laboratory model fish, zebrafish and medaka were used in which GnRH was deactivated using the TALEN method. The results showed that the phenotype does not change in fish. In female and male zebrafish, inactivation of GnRH3 (Spicer *et al*., 2016) and GnRH2 (Marvel *et al.*, 2019) had no effect on fish fertility. However, in the fish deactivated GnRH2, the size of the eggs decreased significantly and the death rate of larvae was higher than the control group. In medaka fish, inactivation of GnRH1 using Talen method did not affect male fertility or female folliculogenesis, but ovulation was completely stopped (Takahashi *et al*., 2016). These unexpected results were completely contrary to the results obtained from mammals, which show that reproduction, at least in male zebrafish and medaka, cannot be completely dependent on GnRH secretion. In fact, reproductively effective peptides alone or in networks are alternatives for reproductive control in fish (Trudeau, 2018). Therefore, future studies should examine gene networks and causal relationships (Mortazavi *et al*., 2022).

Application of GnRH in aquaculture

The farm conditions has caused a reproductive impairement as disruption of final maturation of eggs or spawning (Zohar and Mylonas, 2001). The mentioned disorder can be due to the low level of circulatory LH which itself is affected by the decrease in GnRH production (Mylonas *et al*., 2010). In natural environment, the level of LH increases from the early stages of vitellogenesis to the final maturation of the egg and spawning, while mainly in farmed fish, the level of LH does not increase significantly. Therefore, induction of LH secretion can be an ideal for aquaculture industry. Reproductive stimulants not only help in solving the reproductive process disorder of breeders in farms, but also help in the simultaneous maturation of male and female (Zohar *et al*., 2021). The pituitary extract of adult fish (usually carp or salmonidea) as a reproductive stimulant to induce ovulation of fish has been common for many years. Using synthesized stimuli compared to fish pituitary has more advantages. The stimulants eliminate the risk of transmitting infectious diseases as well as calculating accurate doses. GnRH analogs are capable of significantly increasing LH levels even at very doses. Also, due to the similarity of GnRH in fish species, it is possible to use GnRH peptides to induce LH in many fish species. The use of human chorionic gonadotropin (hCG) is similar to LH and directly affects the gonads while GnRHa increases the LH level by stimulating the release of LH hormone from the pituitary. In other words, GnRH is upstream of other sex hormones and enables gametogenesis by directly or indirectly stimulating the relevant downstream hormones (Le Gac *et al.,* 1993). On the other hand, ovulation induction using pituitary extract (natural GnRH peptides) has a relatively low ovulation rate, even at high doses. A major problem is the interaction of dopamine with LH secretion, and most attempts to induce ovulation using pituitary extracts alone are not fully successful (Weil *et al*., 1980; Sokolowska *et al*., 1984). Therefore, the use of simultaneous combination of GnRH analog and dopamine antagonist was developed (Peter *et al*., 1988). Dopamine receptor antagonists decrease the effects of dopamine and enhance cell secretion of gonadotropin, which is essential for the preovulatory LH. Although several dopamine analogues have been prepared, the most common and cheapest dopamine analogue used in the aquaculture industry is domperidone (Hoga *et al*., 2018). Another problem is the stability of GnRH peptide against enzymatic degradation by liver, kidney and pituitary proteases. The half-life of normal GnRH is less than 2-4 minutes (Zohar *et al*., 1989). Although GnRH analogs help increase its stability, their half-life is still short. The need of most female fish to inject hormones twice for spawning can be due to the instability of GnRH. In other words, the bond between Tyr5-Gly6 and Trp3-Ser4 amino acids is very vulnerable to enzymes. The use of recombinant hormones with a longer and more stable half-life can help the reproduction of fish and the aquaculture industry. One of the ways to increase the stability of proteins is the substitution of peptide amino acids (Blomenrohr *et al*., 2002). For example, replacing the amino acid at the 6th position and stabilizing the end of the peptide chain by replacing the amino acid at the 10th position with the ethyl amide group not only helps to make it more stable, but also increases the binding affinity of GnRH to its receptor (Zohar and Mylonas, 2001). Mohammadzadeh *et al*. (2020) by replacing less conserved and non-stable amino acids with positions 1, 5, 6, 7, 8 respectively with Glutamic acid, His, Serine, Leu and Tyr and using the GAP region to produce a peptide with better efficiency. They found that after 17 hours, the level of progestin hormones increased significantly compared to the control group. Among the GnRH analogs used in aquaculture, the compound (pGlu1-His2-Trp3-Ser4-Tyr5-DAla6-Leu7-Arg8-Pro9-Net10) mGnRHa with a dopamine analog (metaclopramide) under the commercial name Ovopel and the compound (pGlu1 -His2-Trp3-Ser4-Tyr5- DArg6-Trp7-Leu8-Pro9-Net10) sGnRHa and the dopamine homologue (domperidone), known as ovaprim, are the most widely used (Hoga *et al.,* 2018). Although mammalian GnRH analogs can be used to induce fish reproduction, sGnRH analogs have obtained better results compared to mammalian GnRH analogs to induce ovulation in goldfish (Peter *et al*., 1985). For a better effectiveness of the sGnRH analogue in goldfish is probably due to the fact that sGnRH is a hypophysiotropic isoform of GnRH naturally present in the carp family.

Conclusion and future directions

One of the most important neurohormones controlling reproduction is GnRH, which controls not only the gametogenesis but also reproductive behaviors. Previous studies show that GnRH is sinthesize in hypothalamus as well as gonads but autocrine/paracrine regulation is domesntrated new role. Farm conditions causes reproductive impairement in broodstock via reduction of GnRH and consequently LH levels. Therefore, GnRH analogues is important for the aquaculture industry. Over the last decade, the full sequence of laboratory model fish has been identified. So that the reproductive role of some other brain peptides has been identified. So far, more than twenty neuropeptides that play a role in reproduction of fish have been identified. Some of the identified peptides have a reproductive stimulating role (Kisspeptin, Serotonin, Ghrelin, MCH, Leptin, GABA, NPY) and others have an inhibitory role (GnIH, nesfatin-1, Neuropeptide-Y). Some of the mentioned neuroendocrines affect reproduction via GnRH, and some directly affect the secretion of gonadotropins or even gonadal hormones. The noteworthy point is the presence of these peptides or their receptors on the level of gametes, which domenstrated their direct effect on gonadal hormones. Future studies using genome editing methods (CRISPR-cas9 method) can reveal a clearer the role of effective neuropeptides in fish reproduction.

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