Induction of final oocyte maturation in Cyprinidae fish by hypothalamic factors: a review

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ABSTRACT: Gonadotropin-releasing hormone in Cyprinidae as in other Vertebrates functions as a brain signal which stimulates the secretion of luteinizing hormone from the pituitary gland. Two forms of gonadotropin-releasing hormone have been identified in cyprinids, chicken gonadotropin-releasing hormone II and salmon gonadotropin-releasing hormone. Hypohysiotropic functions are fulfilled mainly by salmon gonadotropin-releasing hormone. The only known factor having an inhibitory effect on LH secretion in the family Cyprinidae is dopamine. Most cyprinids reared under controlled conditions exhibit signs of reproductive dysfunction, which is manifested in the failure to undergo final oocyte maturation and ovulation. In captivity a disruption of endogenous gonadotropin-releasing hormone stimulation occurs and sequentially that of luteinizing hormone, which is indispensible for the final phases of gametogenesis. In addition to methods based on the application of exogenous gonadotropins, the usage of a method functioning on the basis of hypothalamic control of final oocyte maturation and ovulation has become popular recently. The replacement of natural gonadotropin-releasing hormones with chemically synthesized gonadotropin-releasing hormone analogues characterized by amino acid substitutions at positions sensitive to enzymatic degradation has resulted in a centuple increase in the effectiveness of luteinizing hormone secretion induction. Combining gonadotropin-releasing hormone analogues with Dopamine inhibitory factors have made it possible to develop an extremely effective agent, which is necessary for the successful artificial reproduction of cyprinids.

Keywords: reproductive dysfunction; ovulation; luteinizing hormone; gonadotropin-releasing hormone; gonadotropin; dopamine; dopamine antagonist; cyprinids

List of abbreviations

DA = dopamine; **DI** = dopamine antagonist; **EU** = European Union; **GnRH** = gonadotropin-releasing hormone; **GnRHa** = gonadotropin-releasing hormone analogue; **cGnRH-II** = chicken gonadotropin-releasing hormone II; **mGnRH** = mammalian gonadotropin-releasing hormone; **sGnRH** = salmon gonadotropin-releasing hormone; **GPCR** = G-protein coupled receptor; **LH** = luteinizing hormone; **MRL** = minimum residual limit; **IM** = intramuscular injection; **IP** = intraperitoneal injection; **PCC** = pericardial cavity injection; **IV** = intravenous injection

Contents

Supported by the Ministry of Education, Youth and Sports of the Czech Republic (Grant No. MSM 6007665809) and the Ministry of Agriculture of the Czech Republic (Grant No. QH91310).

1. Introduction

The family Cyprinidae, which includes 2 010 species classified in 210 genera, is one of the most important groups of freshwater fish found in North America, Africa and Eurasia (Nelson, 2006). For sustainable cyprinidae fish production, both from the point of view of conservation programmes (Kaminski et al., 2004) or aquaculture production (Mikolajczyk et al., 2004), the basic requirement is to successfully manage all phases of artificial reproduction by providing a sufficient amount of fry. Many fish species reared in captivity exhibit some form of reproductive dysfunction (Peter et al., 1988; Brzuska, 1999; Kouril et al., 2008). In the case of cyprinids this dysfunction mostly manifests itself in the absence of final oocyte maturation (Sokolowska-Mikolajczyk and Mikolajczyk, 1991; Yaron, 1995; Mananos et al., 2009). After successfully completing vitellogenesis fish are not capable of undergoing the next steps of gametogenesis and subsequent ovulation (Mylonas and Zohar, 2007). The reason for this lies in the conditions on fish farms (Svobodova and Kolarova, 2004; Kroupova et al., 2005; Sudova et al., 2007), which are diametrically different from those brood fish are exposed to in the natural habitat of rivers and lakes. Artificial environments lack natural spawning stimuli (spawning substrate, stream hydraulics, nutrition, water quality, depth etc.) are not able to induce appropriate endogenous responses from the fish; the final result is reproductive dysfunction of FOM (Abraham, 1988). The discovery of the primary structure of mammalian GnRH neurodecapeptide (Burgus et al., 1971) in the early 1970s was significant also with regard to possibilities of hormonal therapy of reproductive dysfunctions. The possibility of direct stimulation of gonadotropin cells secreting the fish's own luteinizing hormone (Lam et al., 1975) was added to a previously used type of hormonal therapy, which replaced the insufficient production of endogenous luteinizing hormone with exogenous luteinizing hormone (von Ihering, 1937). Along with the identification of the LH inhibition factor (Peter et al. 1986) – dopamine – and use of DA antagonists, effective stimulation methods of LH secretion, the so-called hypothalamic approach (Peter et al., 1988), were developed, which can be applied to a wide range of fish species.

2. Stimulation factor of LH secretion – GnRH

In the family Cyprinidae as well as in other species of Teleostei the neurodecapeptide GnRH is the central regulator of the reproductive hormonal cascade regulating the synthesis and release of LH secretion from the pituitary gland (Somoza et al., 2002; Yaron et al., 2003; Millar et al., 2004; Kah et al., 2007). The hypophysiotropic GnRH is processed in the hypothalamic neurons by enzymatic cleavage of a precursor polypeptide and packaged in storage granules (Yaron and Sivan, 2006). The precursor polypeptide of all GnRH (prepro-GnRH) forms consists of: (a) a signal peptide, (b) the biologically active GnRH decapeptide, (c) proteolytic processing site (Gly-Lys-Arg) and (d) the GnRH associated peptide (GAP), (Lethimonier et al., 2004; Okubo and Nagahama, 2008). Due to the absence of the hypothalamic-hypophyseal portal system in teleost fish, the storage granules of GnRH are transported along nerve fibres through the pituitary stalk to the nerve ending in close proximity to the adenohypophyseal cells (Van der Kraak et al., 1998).

GnRH was first isolated from the mammalian hypothalamus as mammalian GnRH with the following amino acid structure: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly.NH_2 (Burgus et al., 1971). The first GnRH form identified in teleost fish was a salmon GnRH in chum salmon (*Oncorhynchus keta*) whose stucture was similar to that of mGnRH, differing only in amino acids at positions 7 (Trp) and 8 (Leu), (Sherwood et al., 1983). Among vertebrates Teleostei are the group where the highest number of GnRH forms occur (Chen and Fernald, 2008). A total of eight GnRH forms have been identified until now (Matsuo et al., 1971; Sherwood et al., 1983; Yu et al., 1988; Bogerd et al., 1992; Powell et al., 1994; Carolsfeld et al., 2000; Montaner et al., 2001; Adams et al., 2002).

Research up until now using diagnostic methods like RIA, HPLC, *in situ* hybridization, etc., has confirmed the occurrence of only two GnRH formes (GnRH2, GnRH3) in the members of the family Cyprinidae, e.g. goldfish (*Carassius carassius*), (Peter et al., 1991), roach (*Rutilus rutilus*), (Penlington at al., 1997) and zebra danio (*Danio rerio*), (Powell et al., 1996; Steven et al., 2003; Palevitch et al., 2007). However, in some species the three GnRH forms were detected simultaneously, e.g., gilthead seabream (*Sparus aurata*), (Powell et

al., 1994). Based on the classification proposed by Fernald and White (1999), the determined GnRH forms are divided into three branches. Into the GnRH1 line belong types such as mGnRH (Matsuo et al., 1971), seabream GnRH (Powell et al., 1994), catfish GnRH (Bogerd et al., 1992). They fulfil hypophysiotropic functions (Pham et al., 2006) and are found in the ventral telencephalon, the preoptic area, the basal hypothalamus and the pituitary gland (Dubois et al., 2002). Despite great effort, the occurrence of the GnRH1 line has not been detected in the cyprinids. It seems that it is mainly GnRH3 line, which compensates for the LH inducing role of the missing GnRH1 line in Cyprinidae. The projection of pre-optic GnRH3 neuronal axons into the pituitary (Kobayashi et al., 1997) and the fact that it is the more abundant form in the goldfish pituitary (Powell et al., 1996; Steven et al., 2003) confirm this assumption. Line 3 comprises only the sGnRH form (Sherwood et al., 1983) found only in Teleostei. The spacial distribution of sGnRH includes olfactory bulbs, the terminal nerve, the forebrain (Kim et al., 1995) while only one report of expression in the hindbrain is known from zebra danio (Steven et al., 2003). Line 2 is represented by the highly conserved GnRH form- cGnRH-II- occurring in all tested teleostes, with expression only in the midbrain region (Kah et al., 2007). The exception to this rule is goldfish, which also express cGnRH-II mRNA in the forebrain and hindbrain (Lin and Peter, 1997). After the application of exogenous cGnRH-II, its effect on sexual behaviour (Volkoff and Peter, 1999) and its inhibitory effect on food intake in goldfish (Matsuda et al., 2008) has been demonstrated. In terms of LH secretion stimulation cGnRH-II is more effective as compared to the hypophysiotropic sGnRH form (Illing et al., 1999), but with regard to the low cGnRH-II content in the pituitary (Powell et al., 1996; Steven et al., 2003) its impact on the LH level in plasma is minimal. A wide conservation of cGnRH-II in vertebrate species suggests an important role, although it has not been elucidated clearly until now.

GnRH exerts its regulatory role through recognition and binding by specific membrane associated receptors belonging among the members of the rhodopsin-like G-protein coupled receptor (GPCR) family (Millar et al., 2004; Blomenrohr et al., 2005). The typical structure of GPCR members consists of three main functional domains: an N-terminal extracellular domain and an intracellular C-terminal cytoplasmic domain linked by seven transmembrane domains, which are joined by three extracellular loops and three intracellular loops (Parhar, 2003). The extracellular and transmembrane domains are involved in ligand-recognition, whereas the cytoplasmic domains interact with G-proteins (Blomenrohr et al., 1997; Sealfon et al., 1997). Unlike the mammalian type, the fish GnRH receptor contains an intracellular C-terminal tail and has Asp residues in TM 2 and 7, which influences the cell-surface expression (higher in comparison with the mammalian type), ligand binding, agonist-induced receptor phosphorylation and desensitization by decreasing the rate of its internalization (Blomenrohr et al., 2005). Several types of GnRH receptors have been identified in fish species belonging to the family Cyprinidae: two in goldfish (Illing et al., 1999) and four types in zebra danio (Tello et al., 2008). In goldfish, GnRH receptors undergo seasonal variation with the highest pituitary content during the late stages of gonadal recrudescence. The observed changes in pituitary GnRH receptor content correlate closely with responsiveness to a GnRH agonist *in vivo* in terms of serum gonadotropin levels (Habibi et al., 1989).

3. Inhibition factor of LH secretion – dopamine

Dopamine, one of the catecholamine neurotransmitters (Dufour et al., 2005), is the only known factor having an inhibitory effect on LH secretion in the family Cyprinidae (Peter et al., 1991; Trudeau, 1997; Popesku et al., 2008). The preoptic area is the place of origin of DA cell bodies innervating the pars proximal distalis of the adenohypophysis (Kah et al., 1984). Dopamine exerts its inhibitory activity via receptors belonging to members of seven transmembrane domain GPCRs, which are separated into D_1 and D_2 receptor classes (Missale et al., 1998). Secretion of dopamine from nerve terminals in the pituitary and its binding to D_2 receptors localized on gonadotrophs results in inhibition of basal and GnRH-stimulated release of LH (Omeljaniuk et al., 1987; Van der Kraak et al., 1998). With regard to time course both acute and long-term inhibitory effects of DA occur. The acute direct effect of DA induces the disruption of intracellular GnRH signal transduction pathways (Chang et al., 1993), whereas the long-term effects account for a reduction in the number of GnRH receptors on the sur-

face of LH tropic cells (De Leeuw et al., 1989) and a reduction in GnRH peptide release from nerve terminals in the pituitary (Yu and Peter, 1992). DA inhibitory effects are reflected also in the preoptic region, where it disrupts GnRH peptide synthesis in GnRH neurons (Yu and Peter, 1990). Moreover, treatment with a DA antagonist causes an increase in the numbers of LH-like gonadotrophs and is directly proportional to time and the dose of the antagonist (Osornio et al., 2004). The inhibitory effect of DA on LH secretion changes over the course of the reproductive cycle, with the maximum DA inhibition occurring during the final stages of gametogenesis. This feature is utilised in aquaculture of Cyprinidae by using dopamine antagonists in ovulation-inducing therapies, e.g., domperidon, pimozide, reserpin, metoclopramide, haloperidol, isofloxythepin (Peter et al., 1988; Brzuska, 1999; Kouril et al., 2006, 2007).

4. Nature of endocrine dysfunction of final oocyte maturation

Due to the artificial environmental conditions on fish rearing farms (Svobodova and Kolarova, 2004; Kroupova et al., 2005; Sudova et al., 2007), Cyprinidae exhibit reproductive endocrine dysfunctions, mostly at the level of the final oocyte maturation (Yaron, 1995). This is caused by insufficient LH secretion from the pituitary (Mananos et al., 2009), which is necessary for the activation of steroidogenesis and FOM (Yaron and Levavi-Zermonsky, 1986; Drori et al., 1994). One of the first proofs testifying to this fact was the capability of the fish pituitary (containing LH) to induce ovulation in mature females of different fish species (Kouril and Chabera, 1976). It has been proved definitely by comparing LH levels during the spawning period between fishes in captivity and those in open water bodies in, e.g., gilthead sea bream (*Sparus aurata*), (Zohar, 1988) and striped bass (*Morone saxatilis*), (Mylonas et al., 1997). In the blood circulation of wild fishes, an increase in LH level was observed from early phases of vitellogenesis through the final oocyte maturation and ovulation, while fish in captivity showed no signs of LH increase and after vitellogenesis was completed, oocytes started to undergo atresia (Zohar, 1989; Mylonas and Zohar, 2001a). Measuring the levels of LH, LH mRNA, and the mRNA of LH receptors in the pituitary revealed no differences between wild striped bass and striped bass individuals in captivity (Steven, 2000), which confirms a presupposition of dysfunction at the level of LH secretion rather than LH synthesis. However, there were differences between GnRH measured in the pituitary and the same values of GnRH mRNA in the brain of wild fishes and farmed organisms (Steven et al., 2000). These data suggest that GnRH synthesis in the hypothalamus is not disrupted, but that the problem concerns GnRH secretion from nerve terminals in adenohypophysis.

5. Hypothalamic hormone therapy in aquaculture

The first publications documenting the use of GnRH peptide in aquaculture appear in the 1970s (Breton and Weil, 1973). Using GnRH-based synthetic preparations has more advantages, as compared with gonadotropin-based preparations (fish pituitary, choriogonadotropins). The most significant is an inherent correction of endocrine dysfunction represented by the stimulation of gonadotropin cells of adenohypophysis secreting endogenous LH. In hormone therapy, the position of the hypothalamic GnRH factor on the higher steps of the hormone cascade enables the involvement of co-operating endocrine factors of gametogenesis by direct or indirect stimulation of their secretion, e.g., growth hormone (Le Gac et al., 1993), insulin-like growth factor (Negatu et al., 1998), prolactin (Weber et al., 1995), and thyroid hormones (Cyr and Eales, 1996). Chemical GnRH synthesis eliminates the risk of the transmission of infectious diseases and also allows the possibility of applying exact doses of GnRH. Another important factor is the high degree of interspecies similarity between GnRH peptides (Chen and Fernald, 2008) allowing one preparation to be used for more than one fish species.

Initial experiments with induction of ovulation by means of natural GnRH peptides were characterized by a need to use high doses of GnRH and a relatively low rate of successful ovulation (Kouril and Barth, 1981). The problem was the low resistance of natural GnRH peptide to enzymatic degradation by proteases localised in kidneys, liver and hypophyses (Zohar et al., 1990). A solution was found by synthesizing GnRH analogues with amino acid substitutions at easily degradable positions of the original GnRH chain (Schally et al., 1980). Bonds

between amino acids Tyr^5–Gly^6 and Pro^9–Gly^{10}. NH_2 (Peter and Yu, 1997) have been identified to be the least resistant to enzymatic cleavage. Amino acid substitution in position 6 for dextrorotatory amino acid and the stabilization of the C end of the peptide chain in the form of amino acid substitution in position 10 for ethylamide group resulted in a rapid increase in GnRHa effectiveness (Karten and Rivier, 1986). In particular, the modification of amino acids in position 6 led to a significantly higher resistance of neuropeptide to enzymatic degradation (Zohar, 1988). Substitution of amino acids also modified polarity and tertiary structure of the GnRHa, which results in an improvement receptor binding affinity (Zohar and Mylonas, 2001). The unsatisfactory potency of natural GnRH peptides was improved by synthesising a superactive GnRHa, which is able to induce a significant increase in LH levels even at centuple smaller doses than with the use of natural GnRH forms (Table 1), (Kouril et al., 1986, 2007). The range of effective doses of GnRHa varies from 5–100 μg/kg and in the case of DI from 5–20 mg/kg of effective matter (Kouril et al., 1986; Drori et al., 1994; Brzuska, 1999; Szabo et al., 2002; Glasser et al., 2004; Mikolajczyk et al., 2004; Rutaisire and Booth, 2004; Kucharcyzk et al., 2005; Heyrati et al., 2007). Among the GnRHa forms most often used to eliminate reproductive dysfunction in fishes are: [D-Ala^6, Pro^9, NEthylamide]-mGnRH, [D-Tle^6, Pro^9, NEthylamide]-mGnRH, [D-Arg^6, Pro^9, NEthylamide]-sGnRH.

Due to the strong dopaminergic inhibition of LH secretion, typical for the family Cyprinidae, a majority of trials with ovulation induction using only GnRHa failed (Weil et al., 1980; Sokolowska et al., 1984). According to the results of other authors (Peter et al., 1988; Yaron, 1995; Heyrati et al., 2007) and our own, the only exceptions to the strong dopaminergic activity in Cyprinidae we know are tench (*Tinca tinca*), (Kouril et al., 1986) and rudd (*Scardinius erythrophthalmus*), (Hamackova et al., 2001) in which even a dose of 1 μg/kg mGnRHa was able to stimulate ovulation in a small number of females. As a consequence of the identification of DA's role in LH inhibition in Cyprinidae, Peter et al. (1988) developed the so-called LinPe method using the simultaneous administration of GnRHa and effective dopamine D_2 receptor antagonist. DI disinhibitis dopaminergic effect and strengthens the gonadotropin cell stimulation critical for induction of the preovulatory surge of LH.

As far as GnRHa or DI use are concerned, several combinations are currently available on the market. Into a group of preparations containing sGnRHa we classify for example, an Israeli preparation Dagin (sGnRH + metoclopramide), Canadian preparation Ovaprim (sGnRH + domperidone) and into a group of preparations containing mGnRHa, are included the Hungarian preparation Ovopel (mGnRH + metoclopramide), a Dutch preparation Gonazon (mGnRH), and a Czech preparation Supergestran (mGnRH). The use of salmon GnRHa has resulted in obtaining better results for ovulation induction

Table 1. Amino acid composition of naturally occurring GnRH forms and GnRH analogues used in hormonal therapies in Cyprinidae

GnRH forms	Amino acid sequences									
	1	2	3	4	5	6	7	8	9	10
Native forms										
sGnRH	pGlu – His – Trp – Ser – Tyr – Gly						– Trp – Leu – Pro – Gly-NH_2			
cGnRH-II	pGlu – His – Trp – Ser – His – Gly						– Trp – Gln – Pro – Gly-NH_2			
Synthetic analogues										
mGnRHa	pGlu – His – Trp – Ser – Tyr – D-Ala						– Leu – Arg – Pro – Net			
	pGlu – His – Trp – Ser – Tyr – D-Tle						– Leu – Arg – Pro – Net			
	pGlu – His – Trp – Ser – Tyr – D-Trp						– Leu – Arg – Pro – Net			
	pGlu – His – Trp – Ser – Tyr – [D-Nal(2)]						– Leu – Arg – Pro – aza-Gly			
	pGlu – His – Trp – Ser – Tyr – [D-Ser(t-Bu)]						– Leu – Arg – Pro – Net			
sGnRHa	pGlu – His – Trp – Ser – Tyr – D-Arg						– Trp – Leu – Pro – Net			

in goldfish (Peter et al., 1985), as compared to the use of mammalian GnRHa. The higher effectivity of sGnRHa in the stimulation of ovulation in goldfish is likely to be partly based on the fact that the sGnRH decapeptide is the hypophysiotropic form of GnRH naturally occurring in cyprinids. It is worth mentioning also the high effectivity of GnRHa preparations in ovulation induction of broodstock fish at the end of the spawning period (Alok et al., 1997). The use of GnRHa with DI has resulted in successful stimulation of ovulation in many cyprinids (Table 2).

Over the last years, the use of DI has been hampered due to the EU veterinary legislation which requires the determination of a minimum residual limit (MRL) (Directive of the European Parliament and of the Council, 2004) for every veterinary preparation applied to food animals. Since the MRL in DI is not determined, it is prohibited to use it as a drug for food animals. A reflection of the EU restriction measures with regard to the use of DI are the works of Mikolajczyk et al. (2003, 2004) verifying the effectiveness of the only certified preparation in the EU, which contains GnRHa without DI (Gonazon). The application of relatively high doses of GnRHa in a range of 40–80 μg/kg has stimulated ovulation in up to 60% common carp females.

Table 2. Summary of trials carried out in Cyprinidae using hypothalamic factors to induce final oocyte maturation

Species	Type of GnRHa	Type of DI	References
Bighead carp (*Aristichthys nobilis*)	A	Dom	Fermin, 1991
Black carp (*Mylopharyngodon piceus*)	A	Pim, Res	Peter et al., 1988
Bream (*Abramis brama*)	A	Met	Kucharcyzk et al., 2005
Chub (*Leuciscus cephalus*)	A	Met	Krejszeff et al., 2008
Common carp (*Cyprinus carpio*)	B	Met	Drori et al., 1994
	F	Hal	Arabacı et al., 2004
	D	Pim	Mikolajczyk et al., 2004
	A, C	Met	Brzuska, 2006
Goldfish (*Carassius auratus*)	A, E	Pim	Sokolowska et al., 1984
Grass carp (*Ctenopharyngodon idella*)	B	Pim	Glasser et al., 2004
Gudgeon (*Gobio gobio*)	E	Pim	Kestemont, 1988
Kutum (*Rutilus frisii kutum*)		A	Dom
Lake minnow (*Eupallasella perenurus*)	A	Met	Kaminski et al., 2004
Large mouth buffalo (*Ictiobus cyprinellus*)	A	Iso	Kouril et al., 1999
Nase (*Chondrostoma nasus*)	A	Dom	Szabo et al., 2002
Ningu (*Labeo victorianus*)	B	Met	Rutaisire and Booth, 2004
Pearl mullet (*Chalcalburnus tarichi*)	F	Hal	Arabaci and Sari, 2004
Rainbow shark (*Epalzeorhynchos frenatum*)	B	Dom	Hill et al., 2005
Rudd (*Scardinius erythrophthalmus*)	A, C		Hamackova et al., 2001
Silver carp (*Hypopthalmichthys molitrix*)	A	Pim	Brzuska, 1999
Tench (*Tinca tinca*)	A, C		Kouril et al., 1986
Thai carp (*Puntius gonionotus*)	F	Dom, Met	Sukumasavin et al., 2000
White amur bream (*Parabramis pekinensis*)	A	Pim	Lin et al., 1986

A = [D-Ala^6, Pro^9, NEt]-mGnRH; **B** = [D-Arg^6, Pro^9, NEt]-sGnRH; **C** = [D-Tle^6, Pro^9, NEt]-sGnRH; **D** = [D-Nal(2)^6, aza-Gly^{10}]-mGnRH; **E** = [D-Trp^6, Pro^9, NEt]-mGnRH; **F** = [D-Ser(t-Bu)^6,Pro^9, NEt]-mGnRH

Dom = domperidone; Hal = haloperidol; Iso = isofloxythepin; Met = metoclopramide; Pim = pimozide; Res = reserpine

6. Methods of hypothalamic factor administration

Methods of application are primarily based on the type of ovarian development of the target fish species (Zohar and Mylonas; 2001; Mananos et al., 2009). For the purpose of hormonal therapy applications, fish are separated into two classifications: single-time spawners (synchronous and single-batch group-synchronous) and multiple spawners (multiple-batch group-synchronous and asynchronous), (Mylonas and Zohar, 2007). The main difference between groups consists in a different time of action in the fish body of the stimulator inducing a short-term or long-term LH secretion that is necessary for obtaining and undergoing FOM. For FOM induction and ovulation in single-time spawned species or species spawning under inappropriate climatic conditions just once per reproductive season, it is sufficient to induce one preovulatory LH surge, e.g., in the form of an injection of GnRHa (Kouril et al., 1986; Brzuska, 2006). On the other hand, in species with repeated ovulation (multiple spawners), it is necessary to ensure increased LH during the whole spawning period, e.g., in the form of GnRHa sustained release delivery systems (Mylonas and Zohar, 2001b). Although constantly elevated LH in plasma is not the natural profile of fish, in the case of gilthead seabream, treatment with various types of GnRHa-delivery systems induce typical OM and spawning for many weeks (Zohar et al., 1995).

In aquaculture of the cyprinids, injection application (Szabo et al., 2002; Mikolajczyk et al., 2004) is the most often used delivery route for hormonal stimulation of broodfish. In this case the hormonal agent is dissolved in physiological saline solutions (max. volume 1 ml/kg) and administrated in one or two separate doses. When the method using two doses of GnRHa is applied, these are administred in a span of 8 to 24 hours, 10% and 90% of the total GnRHa dose being injected (Glasser et al., 2004). DI can be administered either with the first GnRHa dose or with both of them. From the viewpoint of labour reduction and mainly for elimination of stress of broodstock, however, it is much more advantageous to administer one combined dose of GnRHa with DI (Kouril et al., 1999), which is also one of the advantages of GnRHa preparations, as compared with the carp pituitary. Based on the published literature we can distinguish four main sites of hormone administration: (a) intraperitoneal injection (IP) – into the abdomen wall 2 cm above the ventral fin (Kouril et al., 2006), (b) intramuscular injection (IM) – penetration of the dorsal muscle 2–3 cm below dorsal fin beginning (Kouril et al., 2007), (c) pericardial cavity injection (IPP) – into the pericardial cavity (Kouril et al., 1986), (d) intravenous injection (IV) – puncture of the caudal vein at the level of the anal fin (Mikolajczyk et al., 2003). Unlike in IP and IPP, in IM administration an effluence of the injected preparation from tissue can occur, which has a negative impact on successful FOM.

Among the prospective methods of GnRHa administration currently requiring further research are topical gill application and oral application. Application through the gill lamellae (Sherwood and Harvey, 1986) would surely find its utilization in the case of stimulation of small fishes, e.g., tropical ornamental fish (Hill et al., 2005), where injection application is problematic and there is a big risk of organism damage. In oral application, prospective results such as an LH level increase in plasma and a reduction in GnRHa effective dose were achieved after mutual administration of GnRHa with intestinal absorption enhancers and protection against enzymatic digestion (Breton et al., 1998; Vertommen and Kinget, 1998; Roelants et al., 2000; Mikolajczyk et al., 2001).

The main importance of using sustained delivery systems for GnRH analogues lies in a long-term release of GnRH analogue stimulating gonadotrops, thus ensuring long term elevated levels in circulating LH plasma levels essential to induce multiple ovulations and spawnings over a prolonged period (Zohar and Mylonas, 2001; Mylonas and Zohar, 2007). In comparison with multiple injections of GnRHa, the use of a sustained delivery system for GnRHa offers a reduction in stress ratio, decreased possibility of injury of rare broodfish and less demand for expensive labour. There are three basic types of GnRHa delivery systems: cholesterol pellets, ethylene-vinyl acetate implants and biodegradable microspheres (Mylonas and Zohar, 2001b). They can be applied subcutaneously or by making a small cut in the abdomen, where they release GnRHa over a long period. One of a few works dealing with the use of slow release GnRHa in pelletized form in Cyprinidae is the study of Linhart et al. (1995). The application of slow release GnRHa in pelletized form reached lower levels of spermiation in tench, as compared with injection application.

7. Determining a suitable period for hypothalamic factor application

A successful induction of ovulation in the broodstock should be preceded by the determination of readiness for spawning based on the examination of secondary sex characteristics (plumpness and softness of the abdomen, swelling of genital papilla, fish maximal circumference) and particularly the assessment of oocyte maturation. Fish have to complete the vitellogenesis phase of oocyte growth and it must be evident that migration of the nucleus towards the oocyte periphery has already started. A sample of oocytes can be obtained by ovarian biopsy performed either by inserting a needle through the abdominal wall cavity (Sokolowska-Mikolajcyzk and Mikolajczyk, 1991) or by catheterization using flexible plastic tubing introduced through the genital pore into the ovary (Garcia, 1989; Alvarez-Lajonchere et al., 2001). The obtained oocyte sample may be evaluated on the basis of: (a) measuring of oocyte diameter (Mylonas and Zohar, 2001a), (b) identifying the onset of coalescence of the lipid droplets (Mylonas et al., 1997), (c) *in vitro* hormonal stimulation of germinal vesicle breakdown of biopsied oocytes (Weber et al., 2000), (d) assessment of germinal vesicle position (Drori et al., 1994). In cyprinids, assessment based on the position of the nucleus in the oocyte is mostly used. A sample of oocytes is cleared in a solution of ethanol, formalin, and acetic acid (6 : 3 : 1), (Levavi-Zermonsky and Yaron, 1986) in which oocytes become translucent after a few minutes and the identification of the position of the nucleus becomes possible. Successful ovulation stimulation only occurs if 66–70% oocytes show eccentric germinal vesicle or migrating germinal vesicle towards the periphery (Yaron, 1995). In the case of hormone induction delay, a low dose of hormone preparation or sub-optimal factors of external environment oocyte atresia usually take up (Mylonas et al., 1997), which drastically decrease the chances for obtaining good results.

8. Conclusions

Hormonal stimulation of final oocyte maturation and ovulation have, for decades now, been an important aid in the effective reproduction of a majority of economically important species of the cyprinids. The development of hormone stimulators first took in gonadotropic hormones found in carp pituitary, choriogonadotropins, through to currently preferred synthetic GnRH analogs applied together with DI. The development of methods using hypothalamic factors was only possible when both stimulation and inhibition mechanisms of neuroendocrinne LH regulation were known and understand in detail. The effectiveness of using GnRH analogues with or without a DA inhibitor consists not only in direct elimination of hormonal dysfunction but also in associated stimulation of a spectrum of supporting hormone factors contained in adenohypophysis. A significant contribution is also a high degree of versatility of GnRH preparations within a big spectrum of the carps, which together with easy availability and a relatively low price creates excellent conditions for use in aquaculture. Further research aimed at the identification and synthesis of more potent GnRHa along with a detailed search for the reasons of reproductive dysfunction should contribute to future progress in the area of artificial stimulation of final oocyte maturation and ovulation in Cyprinidae.

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Received: 2009–03–17
Accepted after corrections: 2009–03–31

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