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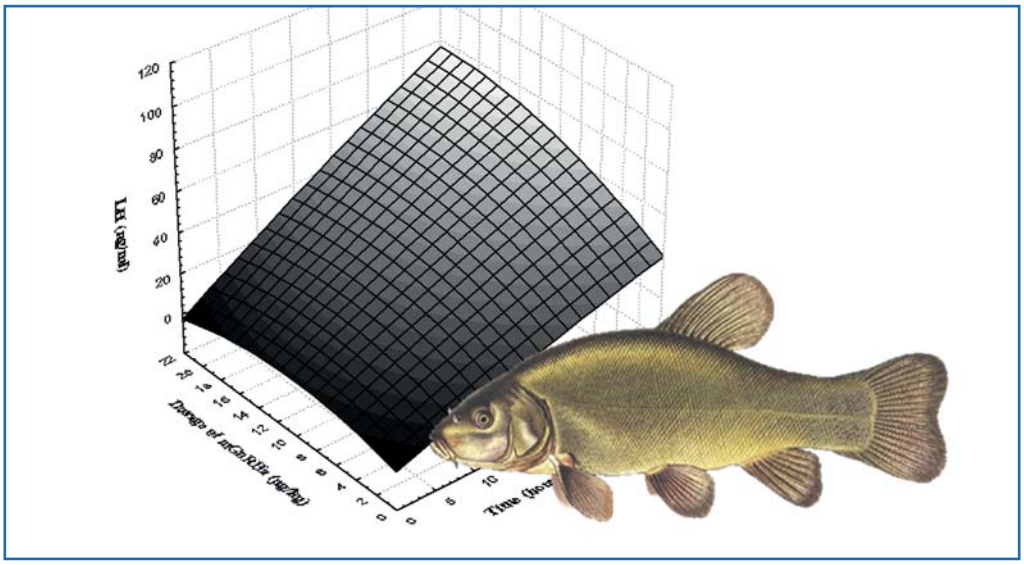

Artificial reproduction of tench (*Tinca tinca* L.), with an emphasis placed on hormonal induction of ovulation

Umělá reprodukce lína obecného (*Tinca tinca* L.), s důrazem kladeným na hormonální indukci ovulace

Peter Podhorec

Czech Republic, Vodňany, 2011

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I thereby declare that I wrote the Ph.D. thesis myself using results of my own work or collaborative work of me and colleagues and with help of other publication resources which are properly cited.

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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1. INTRODUCTION

Tench (*Tinca tinca*) is valuable food, sport, and ornamental fish (Rothbard et al., 2010) belonging into the largest freshwater family, the Cyprinidae (Nelson, 2006). Tench is commercially cultured in a broad area of Europe (Fernandez San Juan, 1995; Gela et al., 2007) and Asia (Wang et al., 2006). Tench is commonly produced through controlled natural reproduction in spawning ponds (Perez-Regadera and Velasco Gemio, 1995) or mainly through capture of mature broodstock from earth ponds shortly before spawning and holding for artificial reproduction in hatcheries (Steffens, 1995). Unfortunately, the artificial environment of fish hatcheries lacks natural spawning stimuli, e.g. spawning substrate and appropriate water quality which, in combination with captivity-induced stress, adversely affect final oocyte maturation (FOM) and subsequent ovulation in tench (Kouril, 1998). It is generally accepted that this is caused by absent of luteinizing hormone (LH) surge from the pituitary that stimulates steroidogenesis and FOM (Zohar and Mylonas, 2001). A common solution, which acts directly on the ovary, is administration of carp pituitary extract (CPE) to create an artificial LH surge (Yaron et al., 2009). Another solution based on direct stimulation of pituitary gonadotrophs includes gonadotrophin-releasing hormone analogue (GnRHa) treatment (Kouril et al., 2008). Two forms of GnRH, cGnRH-II and sGnRH, have been identified in cyprinids (Steven et al., 2003), but native forms of GnRH have limited use in aquaculture due to their intense enzymatic degradation in fish (Zohar et al., 1990). Amino acid substitutions in the original GnRH chain have been shown to markedly improve enzymatic resistance of synthetic GnRH analogues (GnRHa) and facilitate their use in fish reproductive therapies (Peter and Yu, 1997). Gonadotrophin-releasing hormone analogs vary in potency with respect to induction of LH release and ovulation. However LH secretion is influenced not only by stimulatory hypothalamic factors, but is also under inhibitory hypothalamic control represented by the dopaminergic system (Peter et al., 1986). Dopamine has a high capacity to block LH synthesis and release through disruption of intracellular GnRH signaling pathways (Chang et al., 1993), down-regulation of GnRH receptors (De Leeuw et al., 1989), and inhibition of GnRH peptide synthesis (Yu and Peter, 1990) and release (Yu and Peter, 1992), as well as interference with other LH-stimulatory systems, e.g. the GABAergic system (Popesku et al., 2008). The discovery of DA inhibition meant a significant breakthrough in artificial reproduction of cyprinids, which do not undergo final oocyte maturation (FOM) and ovulation after administration of GnRHa alone (Mikolajczyk et al., 2004; Sokolowska et al., 1984; Szabo et al., 2002). A combined treatment with a GnRHa and a DA D2-receptor antagonist (DI) has been developed to eliminate the impact of DA on the reproductive axis and augment the stimulatory effect of the exogenous GnRHa (Peter and Yu, 1997). The potency of a combined treatment to stimulate LH surge and ovulation, even at unfavourable temperatures, was demonstrated in some cyprinids (Sokolowska et al., 1985; Glasser et al, 2004). Although cyprinids are considered typical of a fish group with decisively demonstrated DA inhibition of LH release and ovulation, it appears that this does not apply to tench. For successful hormonal induction of FOM and ovulation in tench, a low dose of GnRHa is sufficient (Fernandez San Juan, 1995; Kouril et al., 1986; Rodriguez et al., 2008) contrary to what has been reported for all other cyprinids (Peter et al., 1988).

The overall aim of this thesis was to optimalize the hormonal induction of ovulation in tench

The specific objectives were to:

1) Elaborate specialized study reviewing basic and applied knowledge about hormonal induction of ovulation in Cyprinidae with an emphasis placed on hypothalamic factors.
2) Determine the optimal type and dose of GnRH analogue to stimulate a pre-ovulatory LH surge followed by high ovulation rate in tench.
3) Clarify whether LH release, and thus ovulation, in tench is under dopaminergic control.
4) Identify the optimal method of hormonal induction of ovulation under suboptimal temperature conditions in tench and especially evaluate the need of dopamine antagonists addition in hormonal therapies under these conditions.

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CHAPTER 2

INDUCTION OF FINAL OOCYTE MATURATION IN CYPRINIDAE FISH BY HYPOTHALAMIC FACTORS: A REVIEW

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Veterinarni Medicina, 54, 2009 (3): 97–110 Review Article

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

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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

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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

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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

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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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CHAPTER 3

EFFECTIVE DOSE OF mGnRHa FOR INDUCTION OF OVULATION IN TENCH (*Tinca tinca* L.)

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Effective dose of mGnRHa for induction of ovulation in tench (*Tinca tinca* L.)

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ABSTRACT

The present study was designed to investigate the *in vivo* dose-response of mammalian GnRH analogue [D-Ala^6, Pro^9, NEthylamide]-mGnRH in stimulating a pre-ovulatory LH surge and subsequent ovulation in female tench. Five doses of mGnRHa (1, 2.5, 5, 10, and 20 μg kg^{-1}) and a control group (0.9% NaCl) were tested under hatchery conditions. Concentration of plasma LH and ovulation success were evaluated. All mGnRHa treatments yielded significantly higher LH levels and ovulation success compared to control. The only difference in LH levels was a higher level with 10 μg kg^{-1} at 24 h post-treatment. No differences were observed in ovulation success (≥60% of females) among mGnRHa treatments. However, non-linear regression analyses indicated increasing LH concentration and ovulation rate with increasing mGnRHa dosage. This trend implies that dosages in the range of 10–20 μg kg^{-1} of mGnRHa can be considered reliable and effective concentrations.

1. Introduction

Tench (*Tinca tinca* L.) is a valuable food, sport, and ornamental fish (Steffens, 1995) belonging into the largest freshwater family, the Cyprinidae (Nelson, 2006). Tench is commercially cultured in a broad area of Europe (Fernandez San Juan, 1995; Gela et al., 2007) and Asia (Wang et al., 2006). Increasing demand for tench fry for aquaculture and restocking has driven research aimed at managing all aspects of their artificially controlled reproduction.

Tench is commonly produced through controlled natural reproduction in spawning ponds (Perez-Regadera and Velasco Gemio, 1995) or through capture of mature broodstock from earthen ponds shortly before spawning and holding for artificial reproduction in hatcheries (Steffens, 1995). Unfortunately, the environment of fish hatcheries lacks natural spawning stimuli, e.g. spawning substrate and appropriate water quality which, in combination with captivity-induced stress, adversely affect final oocyte maturation (FOM) and subsequent ovulation in tench (Kouril, 1998).

The first attempt to resolve this, and to facilitate synchronisation of ovulation, was so-called hypophysation, the injection of fish pituitary extract (von Ihering, 1937). In the early 1970s, the discovery of gonadotropin-releasing hormone (GnRH) (Burgus et al., 1971) and its effects introduced a significant shift in addressing reproductive dysfunction in cultured fish (Breton et al., 1975; Mylonas et al., 2010). Subsequent synthesis of superactive GnRH analogues (GnRHa), with amino acid substitutions at readily degradable positions in the original GnRH amino acid sequence, has resulted in a marked increase of GnRHa effectiveness in stimulation of LH secretion and ovulation (Zohar and Mylonas, 2001).

In contrast to other cyprinids (Peter et al., 1988; Mikolajczyk et al., 2004; Heyrati et al., 2007), tench undergo FOM and ovulation after treatment with low doses of GnRHa without the necessity of applying dopamine inhibitors (Kouril et al., 1986). A broad range of effective GnRHa doses (1–125 μg kg^{-1}) has been used in previous studies (Linhart and Billard, 1995; Barth et al., 1997; Kouril, 1998; Kouril et al., 2007; Carral et al., 2003; Rodriguez et al., 2008); however, the specification of an effective GnRHa dose based on LH profile and ovulation success is lacking. Hormone therapy applied to excess can result in needlessly increased production costs and have detrimental impacts on broodstock fecundity and the quality of eggs (Mylonas et al., 1992; Taranger et al., 1992), while an insufficient dose will not induce the desired response. Furthermore, the anticipated restriction in the use of neuroactive pharmaceuticals for veterinary practise in farm animals requires a study of spawning induction by GnRHa without such additives.

The aim of this *in vivo* experiment was to test the dose-response of the most commonly used GnRH analogue [D-Ala^6, Pro^9, NEthylamide]-mGnRH in stimulating a pre-ovulatory LH surge with subsequent ovulation in tench.

2. Material and methods

The study comprised two identical trials conducted in succession and differing only by the lack of blood sampling in trial 2. The purpose

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of the second trial was to eliminate the possible influence of stress caused by blood sampling on ovulation success.

Sexually mature 5 year old female tench (504 ± 21 g body weight [BW]) were harvested from ponds located in the south of the Czech Republic. Selection was based on external appearance (soft and distended abdomen) and the progress of germinal vesicle migration in oocytes (Levavi-Zermonsky and Yaron, 1986). Only females with ≥60% of oocytes in the peripheral position were selected (Yaron et al., 2009). Fish (trial 1, $n = 8$; trial 2, $n = 10$) were placed in six flow-through tanks with water volume 700 l, under the following conditions: photoperiod 16:8 L:D, water temperature 21.0 ± 0.5 °C, and dissolved oxygen 7.8 ± 0.4 mg l^{-1}.

mGnRHa ([D-Ala^6, Pro^9, NEthylamide]-mGnRH) was purchased from Bachem AG (Switzerland) and physiological saline solution (0.9% NaCl) from Braun Melsungen AG (Germany). Following a 36 h acclimatisation period, fish in each of the five groups were intraperitoneally injected with mGnRHa dissolved in physiological saline solution at a dose of 1, 2.5, 5, 10, or 20 μg kg^{-1} BW. The sixth (control) group was intraperitoneally injected with the physiological saline solution.

In trial 1, blood samples (0.25 μl) were taken at 0, 6, 12, 24, and 33 h post-injection by caudal venipuncture with a 21 gauge heparinised needle attached to a heparinised syringe. Plasma was withdrawn after centrifugation and stored at −20 °C until assayed.

Levels of LH in tench plasma were determined using a heterologous enzyme-linked immunosorbent assay (ELISA) (Kah et al., 1989). To determine the accuracy of the heterologous assay, serial dilutions of tench plasma and pituitary extract were conducted in the same assay with a common carp standard. All were found to be parallel. Sensitivity of the performed ELISA was in the range 0.6–100 ng ml^{-1} with the intra- and inter-assay coefficients of variance at 5% and 9%, respectively.

Fish were checked for ovulation every 2.5 h, beginning 24 h after injection. Ovulation was indicated by the release of ripened translucent ova following application of slight pressure to the abdomen. The following data were recorded: ovulation success (number of ovulated females within 48 h), latency period (time from injection to ovulation), relative fecundity (total number of eggs/kg body weight of treated females), and LH levels. All manipulations were carried out under clove oil anaesthesia (0.033 ml l^{-1}).

One-way ANOVA followed by Tukey's HSD test was used to evaluate differences in plasma LH concentrations among treatment groups. Ovulation rate was evaluated by a χ^2 test. Non-linear regression analyses were performed to determine the relationship between independent and dependent variables. The results are expressed as means ± standard errors of the means (SEM).

3. Results

No differences in basal plasma LH concentrations were found before injection (0 h) (Fig. 1). Administration of mGnRHa significantly influenced LH concentrations and induced a gradual rise of LH levels in all treated groups significantly different from the control group ($P<0.01$), which showed unchanged LH levels throughout the experiment. No differences in LH concentrations among mGnRHa groups were observed at any sampling time with the exception of 24 h for the group treated with 10 μg kg^{-1} mGnRHa, which showed significantly higher LH levels from all other groups ($P<0.05$) except 20 μg kg^{-1}. However, non-linear three dimensional regression analysis of LH concentration (dependent variable) against mGnRHa dosage and time post-injection (independent variables) was significant ($R^2 = 0.78$; $P<0.05$) and indicated increasing LH concentration with increasing mGnRHa dose and time (Fig. 2).

Administration of mGnRHa significantly influenced ovulation rate ($P<0.001$) with ovulation rates significantly higher in mGnRHa groups compared to controls ($P<0.05$; Table 1). No significant differences were recorded between mGnRHa groups in any measured parameter (Table 1). Non-linear regression analysis showed a high positive correlation between dosage level of mGnRHa and ovulation rate ($r^2 = 0.70$; ($P<0.05$); Fig. 3).

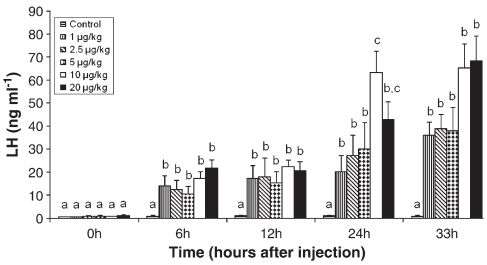

Fig. 1. Concentration of plasma LH in tench after injection of five mGnRHa doses (1, 2.5, 5, 10, 20 μg kg^{-1}). Significant differences between groups of the same sampling time are shown by different letters ($P<0.05$).

4. Discussion

The results of the current study were consistent with our earlier results (Kouril et al., 1986; Kouril et al., 2008) demonstrating the high efficacy of low doses of mGnRHa in inducing ovulation in tench. All tested doses of mGnRHa stimulated at least 60% of females to ovulate. Similar to results in common carp (Drori et al., 1994), injection of mGnRHa induced a gradual rise in circulating plasma LH, with the peak (over 100 ng kg^{-1}) reached close to ovulation. No plasma LH increase was measured in the control group despite optimal water temperature, which is the primary stimulus for cyprinid spawning (Gillet and Quetin, 2006). Stimulation of reproduction by water temperature increase alone, successful in some cyprinids (Krejszeff et al., 2009), does not induce tench to complete FOM and ovulate under hatchery conditions.

The demonstrated high sensitivity of tench to low doses of mGnRHa is an interesting characteristic of this fish. The stimulation potential of mGnRHa was sufficient to produce results with no statistical differences

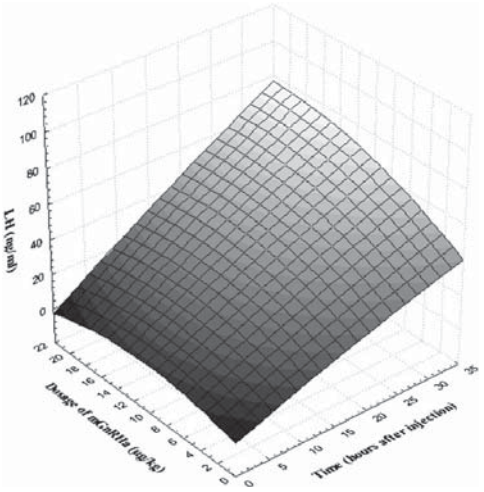

Fig. 2. Three dimensional analysis of the dependent variable LH concentration and independent variables mGnRHa dosage and time post-injection.

Table 1
In vivo dose response trials of effects of five mGnRHa doses (1, 2.5, 5, 10, 20 μg kg^{-1}) on tench ovulation success, latency period, and relative fecundity (trial 1 and 2).

Treatment of mGnRHa	Ovulation rate (%)			Latency period (h)			Relative fecundity (10^3 eggs kg^{-1})		
	Trial 1	Trial 2	Mean	Trial 1	Trial 2	Mean	Trial 1	Trial 2	Mean
1 μg kg^{-1}	62.5	60	61.1	32.5 ± 0.9	34.5 ± 0.4	33.5 ± 0.7	129 ± 20	138 ± 30	134 ± 25
2.5 μg kg^{-1}	62.5	80	72.2	33.2 ± 1.7	33.1 ± 0.9	33.1 ± 1.3	171 ± 32	120 ± 28	146 ± 30
5 μg kg^{-1}	62.5	80	72.2	32.7 ± 0.5	34.9 ± 1.3	33.8 ± 0.9	146 ± 22	180 ± 30	163 ± 26
10 μg kg^{-1}	87.5	80	83.3	31.9 ± 0.7	32.5 ± 0.4	32.2 ± 0.6	185 ± 18	147 ± 24	166 ± 21
20 μg kg^{-1}	87.5	90	88.9	32.3 ± 0.5	34.2 ± 0.6	33.3 ± 0.6	136 ± 40	153 ± 20	145 ± 30
0.9% NaCl	0	0	0	–	–	–	–	–	–

among concentrations ranging from 1 to 20 μg kg^{-1}. However, when regression analyses were employed, a positive correlation of LH concentration with spawning rate was observed to be associated with higher doses of mGnRHa. This implies that dosages in the range of 10–20 μg kg^{-1} of mGnRHa (ovulation rate ≥ 80%) can be considered reliable and effective, and that higher doses may represent needless overdosing. The potency of mGnRHa alone in inducing spermiation in tench was shown by Caille et al. (2006) when 20 μg kg^{-1} significantly increased milt production. The ovulation and spermiation in tench after receiving a low dose of GnRHa is exceptional within the Cyprinidae. The minimal dose of superactive GnRHa effective in stimulating ovulation in 40% to 60% of common carp (*Cyprinus carpio*) has been shown to be 40–80 μg kg^{-1} (Mikolajczyk et al., 2004), which is several-fold the dose effective in stimulating ovulation in a similar percentage of tench. In other cyprinids, e.g. goldfish (*Carassius auratus*), and nase (*Chondrostoma nasus*), an even smaller influence of GnRHa alone on LH profile and ovulation has been reported (Chang and Peter, 1983; Szabo et al., 2002). The failure of low doses of GnRHa to induce ovulation in cyprinids has been explained by dopamine inhibition (Peter et al., 1986), which prevents the LH surge that is a prerequisite for ovulation. This has been addressed through the simultaneous administration of GnRHa and a dopamine antagonist. Such a treatment is not necessary for successful induction of ovulation in tench (Kujawa et al., 2011). It is desirable to eliminate the use of dopamine inhibitors as veterinary drugs, since a maximum residue limit in meat animals has not been established in the European Union.

Similar effectiveness of low mGnRHa concentrations in stimulating LH release from the pituitary and inducing ovulation has been determined in fresh and salt water members of Perciformes including silver perch (*Bidyanus bidyanus*) (Levavi-Sivan et al., 2004), sea bream (*Sparus aurata*) (Zohar et al., 1989), sea bass (*Dicentrarchus labrax*) (Prat et al., 2001), and Atlantic croaker (*Micropogonias undulatus*) (Copeland and Thomas, 1989), all species in which dopamine inhibition is not essential.

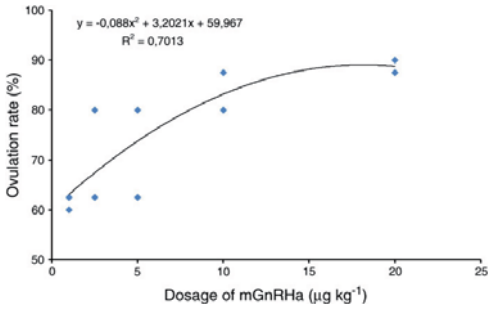

Fig. 3. Non-linear regression analysis of dosage of mGnRHa and ovulation rate (trial 1 and 2) in female tench.

Tench are shy and sensitive fish that react negatively to anthropogenic stressors, such as extended periods in tanks before the reproductive season (Kucharczyk et al., 2007). This was not reflected in an impact of blood sampling on ovulation success of sampled females. It is important to note that the treatments were applied after only 36 h of acclimatisation.

Based on the LH levels and ovulation success, we demonstrated high effectiveness of all tested mGnRHa doses in inducing ovulation in tench, with a reliable dosage range of 10–20 μg kg^{-1} without addition of any neuroactive drug.

Acknowledgements

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CHAPTER 4

DETERMINATION OF DOPAMINE CONTROL OF LUTEINIZING HORMONE RELEASE IN TENCH (*Tinca tinca*)

Podhorec, P., Socha, M., Sokolowska-Mikolajczyk, M., Policar, T., Svinger, V.W., Drozd, B., Kouril, J., 2011. Determination of dopamine control of luteinizing hormone release in tench (*Tinca tinca*). General and Comparative Endocrinology. (submitted)

DETERMINATION OF DOPAMINE CONTROL OF LUTEINIZING HORMONE RELEASE IN TENCH (*Tinca tinca*)

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ABSTRACT

Tench (*Tinca tinca*) is apparently the only known member of the Cyprinidae in which ovulation is stimulated following administration of a low dose of GnRH analogue (GnRHa) without a dopamine inhibitor. This study evaluated LH release effectiveness of the most commonly used GnRHa and clarified whether LH secretion followed by ovulation is subject to inhibitory dopaminergic control in tench. Fish were intraperitoneally injected with three types of GnRHa, GnRHa with dopamine inhibitor metoclopramide (combined treatment), or the dopamine inhibitor metoclopramide alone. LH concentrations at five sampling times (0, 6, 12, 24, and 33 h) together with ovulation success and fecundity index were recorded. The combined treatment triggered an almost immediate LH release peak with a gradual decline, and resulted in a high ovulation rate. In contrast to the combined treatment, an application of GnRHa alone induced gradual increase of LH concentrations with peaks close to ovulation time, and with high ovulation success. Significant differences in LH concentrations at 6 and 12 h and no differences in ovulation success were found between the combined and the GnRHa alone treatments. Metoclopramide alone induced a small increase in LH with no ovulation. The study presents clear evidence of dopaminergic control of LH release in tench, with a high ovulation rate obtained after application of GnRHa alone or in combination with dopamine inhibitor.

Keywords: *cyprinid, dopamine, GnRHa, reproduction, tench*

1. INTRODUCTION

The dual neuroendocrine control of luteinizing hormone (LH) secretion and ovulation is well established within the family Cyprinidae (Peter et al., 1986). The main stimulatory and inhibitory factors of LH secretion from pituitary gonadotrophs are the gonadotrophin-releasing hormone (GnRH) and the catecholamine neurotransmitter dopamine (DA), respectively (Trudeau, 1997). Gonadotrophin-releasing hormone regulates the synthesis as well as the release of LH required for stimulation of steroidogenesis with subsequent final oocyte maturation and ovulation (Nagahama and Yamashita, 2008). Two forms of GnRH, cGnRH-II and sGnRH, have been identified in cyprinids (Steven et al., 2003), but native forms of GnRH have limited use in aquaculture due to their intense enzymatic degradation in fish (Zohar et al., 1990). Amino acid substitutions in the native GnRH chain have been shown to markedly improve enzymatic resistance of synthetic GnRH analogues (GnRHa) and facilitate their use in fish reproductive therapies (Peter and Yu, 1997). Gonadotrophin-releasing hormone analogues vary in potency with respect to induction of LH release and ovulation (Fornies et al., 2003).

Variable degrees of dopaminergic inhibition of LH release have been demonstrated in teleost fish irrespective of taxonomic position, from a suggested role of DA as a puberty gatekeeper in juvenile eel (Vidal et al., 2004), through less pronounced DA inhibition of final steps of gametogenesis in salmonids (Park et al., 2007), to strong inhibition of a pre-ovulatory LH surge and ovulation in cyprinids (Sokolowska et al., 1985a). DA inhibitory tone changes over the course of the cyprinid reproductive period, with the maximum inhibition of LH secretion at the final stages of gametogenesis (Sokolowska et al., 1985b). In captivity, the natural pre-ovulatory decrease in dopaminergic inhibition is often disrupted by artificial conditions (temperature, water chemistry, spawning substrate, etc.), which leads to blocking of oocyte maturation and ovulation (Mylonas et al., 2010). DA has a high capacity to block LH synthesis and release through disruption of intracellular GnRH signaling pathways (Chang et al., 1993), down-regulation of GnRH receptors (Leeuw et al., 1989), and inhibition of GnRH peptide synthesis (Yu and Peter, 1990) and release (Yu and Peter, 1992), as well as interference with other LH-stimulatory systems, e.g. the GABAergic system (Popesku et al., 2008). The discovery of DA inhibition meant a significant breakthrough in artificial reproduction of cyprinids, which do not undergo final oocyte maturation (FOM) and ovulation after administration of GnRHa alone (Sokolowska et al., 1984; Szabo et al., 2002; Mikolajczyk et al., 2004). A combined treatment with a GnRHa and a DA D2-receptor antagonist (DI) has been developed to eliminate the impact of DA on the reproductive axis and augment the stimulatory effect of the exogenous GnRHa (Peter et al., 1988; Yaron et al., 2009). Although cyprinids are considered typical of a fish group with decisively demonstrated DA inhibition of LH release and ovulation, it appears that this does not apply to tench (*Tinca tinca*). For successful hormonal induction of FOM and ovulation in female tench, a low dose of GnRHa is sufficient (Kouril et al., 1986; Fernandez San Juan, 1995; Rodriguez et al., 2004; Podhorec et al., 2011), contrary to what has been reported for all other cyprinids (Podhorec and Kouril, 2009).

The present *in vivo* study was design to clarify whether LH release, and thus ovulation, in tench is under inhibitory dopaminergic control as has been documented in other cyprinid species. Effectiveness of three GnRHa in inducing LH release and ovulation in tench was also evaluated.

2. MATERIAL AND METHODS

2.2. EXPERIMENTAL ANIMALS

Mature female tench (924 ± 46 g body weight) were collected in the end of May, 2010 from a local fish producer and housed at the research fish facility at South Bohemia University in Vodnany. Prior to

the experiment, females with soft and distended abdomens were catheterized to determine the stage of oocyte development. Females with ≥ 60% of the oocytes possessing migrating germinal vesicles were selected (Drori et al., 1994) and randomly divided into 6 groups (n = 10). Each group was kept separately in a well aerated flow-through plastic tank (700 L) and acclimated to conditions 21.8 ± 0.4 °C and a 16L:8D photoperiod.

2.3. EXPERIMENTAL DESIGN

All tested chemicals were dissolved in 0.9% NaCl solution and administered as a single intraperitoneal injection. The following treatments were applied after two days acclimatization: (1) control treatment with 0.9% NaCl alone (Braun Melsungen AG); (2) [D-Ala^6, Pro^9, NEt]-mGnRH (10 μg kg^{-1}, Bachem AG); (3) [D-Leu^6, Pro^9, NEt]-mGnRH (10 μg kg^{-1}, Bachem AG); (4) [D-Arg^6, Pro^9, NEt]-sGnRH (10 μg kg^{-1}, Bachem AG); (5) water soluble DA D2 receptor antagonist metoclopramide (20 mg kg^{-1}, Sigma-Aldrich); and (6) combined treatment [D-Ala^6, Pro^9, NEt]-mGnRH (10 μg kg^{-1}) + metoclopramide (20 mg kg^{-1}). Serial blood samples (400–500 μL) were collected by caudal venipuncture immediately prior to injection (0 h) and at 6, 12, 24, and 33 h post-injection. Plasma was separated by centrifugation and stored at -20 °C until determination of LH levels. Females were monitored for ovulation 24 h after injection and subsequently at 3 h intervals. Ovulation success (number of ovulated females within 48 h), latency period (time from injection to ovulation), fecundity index ([weight of stripped eggs/body weight before stripping] x 100), and LH concentrations were recorded.

Fish were anesthetized (0.03 mL L^{-1} clove oil) before manipulation. The experiment was conducted in accord to the principles of the Ethical Committee for the Protection of Research Animals at the University of South Bohemia.

2.4. LH DETERMINATION

Plasma samples were assayed by heterologous ELISA previously established for common carp LH (Kah et al., 1989). To validate the assay, serial dilutions of tench pituitary homogenate and plasma were made together with a common carp standard. All were found to be parallel with the sensitivity of the assay in the range 0.6–100 ng mL^{-1} and the intra- and inter-assay coefficients of variance at 5 and 9%, respectively.

2.5. STATISTICAL ANALYSIS

For multiple comparisons among treatment groups, serum LH data were log-transformed prior to testing with one-way ANOVA followed by Tukey's HSD test. A χ^2 test was used to compare ovulation rates among the experimental groups. Significant differences were accepted with a P value of <0.05. All values are presented as means ± standard errors of the means (SEM).

3. RESULTS

With the exception of the control group, all groups showed significantly increased LH concentrations 6 h post-treatment compared to pre-treatment basal levels ($P < 0.05$). The highest mean LH value at this

sampling time was 45.42 ± 7.89 ng mL^{-1} recorded for the combined treatment, which was the highest concentration reached in this group over the course of the experimental period. Gonadotrophin-releasing hormone analogue treatments potentiated elevation of LH concentration with similar effectiveness; however the effect was significantly less pronounced than in the combined treatment group ($P < 0.05$) but significantly greater than the metoclopramide and control groups ($P < 0.05$). At 12, 24, and 33 h a progressive rise of all GnRHa treatments was observed, in contrast to a moderate decline in LH concentrations in the combined group, which however remained high. The highest mean LH concentration (47.45 ± 5.07 ng mL^{-1}) for any group over the entire experimental period was measured 33 h post-injection in the group treated with [D-Ala^6, Pro^9, NEthylamide]-mGnRH. Significant differences were detected in LH concentrations between the GnRHa groups and the combined treatment at 6 and 12 h ($P < 0.05$), all GnRHa containing treatments reached significantly higher LH levels than did the metoclopramide and control groups ($P < 0.05$). Metoclopramide treatment induced a slight LH increase differing from the control group at 6 and 12 h post injection ($P < 0.05$). Mean values for LH concentrations are presented in Figure 1.

High ovulation success was obtained after combined (90%) and GnRHa (60–70%) treatments, with similar latency periods and fecundity indices, without significant differences between groups. No ovulation was recorded in the metoclopramide and control groups (Table 1).

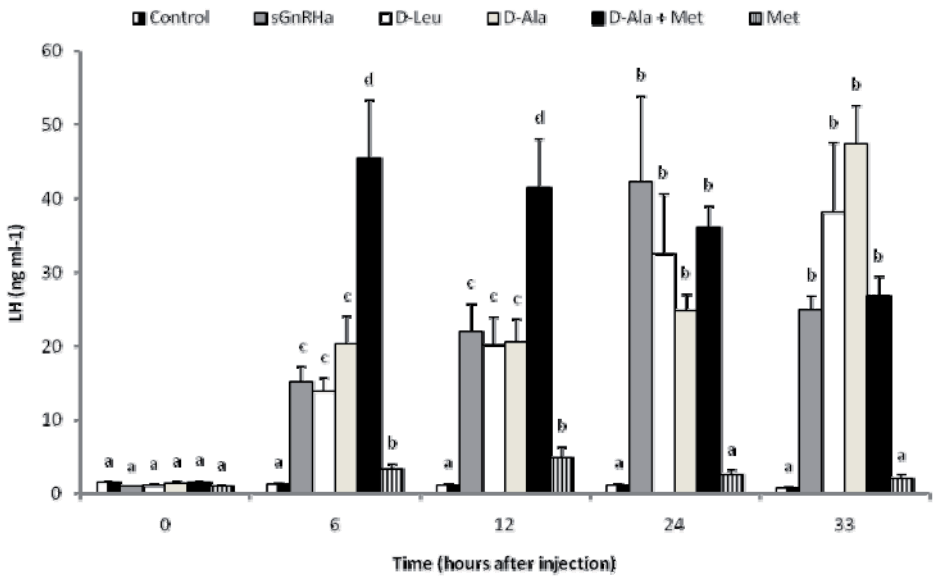

Figure 1. *Plasma levels (means ± SEM) of luteinizing hormone in female tench after 0.9% NaCl (control); [D-Arg^6, Pro^9, NEt]-sGnRH (sGnRHa); [D-Leu^6, Pro^9, NEt]-mGnRH (D-Leu); [D-Ala^6, Pro^9, NEt]-mGnRH (D-Ala); metoclopramide; and combined (D-Ala + Met) treatment. Different letter superscripts indicate significant differences ($P < 0.05$).*

Table 1. *Ovulatory response of tench following treatment with saline solution, metoclopramide, GnRHas, or a combination. Body weight, latency period, and fecundity index are expressed as means ± SEM. Different letter superscripts indicate significant differences (P < 0.05).*

Group	Treatment	Body weight (g)	Ovulation ratio	Latency period (h)	Fecundity index (%)
1	Physiological saline solution (0.9% NaCl)	977 ± 55	10 / 0 [a]		
2	Metoclopramide (20 mg kg^{-1})	930 ± 67	10 / 0 [a]		
3	[D-Arg^6, Pro^9, NEt]-sGnRH (10 µg kg^{-1})	945 ± 43	10 / 6 [b]	35.2 ± 2.1	6.1 ± 0.6
4	[D-Leu^6, Pro^9, NEt]-mGnRH (10 µg kg^{-1})	885 ± 38	10 / 7 [b]	34.5 ± 1.8	5.3 ± 0.9
5	[D-Ala^6, Pro^9, NEt]-mGnRH (10 µg kg^{-1})	915 ± 54	10 / 7 [b]	36.7 ± 1.6	6.6 ± 0.4
6	[D-Ala^6, Pro^9, NEt]-mGnRH (10 µg kg^{-1}) + metoclopramide (20 mg kg^{-1})	897 ± 45	10 / 9 [b]	34.9 ± 2.4	6.8 ± 1.1

4. DISCUSSION

Hormonal induction of ovulation in tench is well-established in aquaculture production using a low dose of GnRHa alone (Linhart and Billard, 1995; Kouril, 1998). To date there has been no investigation of DA inhibition on LH release in tench in spite of the apparent inhibitory tone in other cyprinid species. In cyprinids GnRHa alone generally does not induce an ovulatory response (Arabaci and Sari, 2004), with the exception of a minimal ovulation rate when administered at high doses (Mikolajczyk et al., 2003; Heyrati et al., 2007). The sensitivity of tench to hormonal treatment is similar to that of several marine fish species in which a low dose of GnRHa stimulates ovulation and which do not demonstrate dopaminergic control of LH release (Berlinsky et al., 1996; Prat et al., 2001; Levavi-Sivan et al., 2004). In some marine fish (e.g. Atlantic croaker; *Micropogonias undulatus*) the addition of a DA antagonist to hormonal treatment suppresses the effects of GnRHa on LH release while DA agonists potentiate it (Copeland and Thomas, 1989). However, in tench the combined treatment composed of [D-Ala^6, Pro^9, NEt]-mGnRH and the DA D2-receptor antagonist metoclopramide triggered an almost immediate LH release peak with high LH concentrations throughout the experimental period inducing a high ovulation rate. This clear confirmation of DA inhibition in tench is surprising; especially in the light of GnRHa effectiveness in ovulation (60–70%) and the gradually elevated LH profile with values peaking close to ovulation. These data are consistent with our earlier results showing that as little as 1 µg kg^{-1} of mGnRHa induced gradual elevation of LH with 63% of females ovulating (Podhorec et al., 2011). Few studies have compared effects of GnRHa with DA antagonist versus GnRHa alone treatments in tench, although Pinillos et al. (2002) reported an enhanced effect of GnRHa alone on secretion of 17,20β-dihydroxy-4-pregnen-3-one, 17,20α-dihydroxy-4-pregnen-3-one, and testosterone and ovulation success compared to a combined treatment. Although in the present study approximately 70% of females ovulated after GnRHa alone treatment (10 µg kg^{-1}) which is the mean ovulation rate obtained after combined treatment by Kujawa et al. (2011), our and other studies have more commonly reported ovulation rates of 80% or more with GnRHa treatment (Kouril et al., 1986; Podhorec et al., 2011). The relatively lower LH mean concentrations in compare with our previous study (Podhorec et al., 2011) were detected in the current trial, although LH concentrations in some ovulated females exceeded detection limits of the assay (> 100 ng mL^{-1}). The combined and GnRHa treatments were both associated with high ovulation rates, but GnRHa treatment led to a gradual LH release, while an instant LH surge was seen following the combined treatment. Peter et al. (1986) suggested that the ovulatory

response in goldfish (*Carassius auratus*) is dependent on both the magnitude of the LH concentration and the rate of increase in circulating LH levels. Immediate LH surge after the combined treatment was more effective in inducing ovulation than GnRHa-stimulated gradual increase despite comparable LH concentrations (Chang and Peter, 1983; Sokolowska et al., 1984). In tench we cannot confirm this, as an application of GnRHa alone induced completion of germinal vesicle migration and ovulation in the majority of females after a gradual increase of LH levels. Continuously elevated LH levels and high ovulation rates induced by a combined treatment have also been detected in common carp (Drori et al., 1994; Mikolajczyk et al., 2003; Mikolajczyk et al., 2004) and grass carp (Glasser et al., 2004).

The most widely used synthetic GnRHa in tench artificial reproduction were tested for induction of pre-ovulatory LH surge and ovulation. In goldfish sGnRH is considered to be the main hypophysiotropic form based on its high abundance in the pituitary (Steven et al., 2003). However no difference in the effectiveness of [D-Arg^{6}, Pro^{9}, NEt]-sGnRH compared to [D-Ala^{6}, Pro^{9}, NEt]-mGnRH or [D-Leu^{6}, Pro^{9}, NEt]-mGnRH were detected. Superiority of [D-Ala^{6}, Pro^{9}, NEt]-mGnRH previously shown in sea bass (*Dicentrarchus labrax*) (Fornies et al., 2003) was not confirmed for the tench, although it induced the maximum LH peak in the trial. All tested GnRHa induced similar LH concentrations and ovulation success and can be equally recommended for hormonal therapies in tench.

The great diversity of the family Cyprinidae has attracted scientific attention and resulted in many comprehensive phylogenetic studies (Briolay et al., 1998; Hanfling et al., 2000; Saitoh et al., 2011). However, despite considerable effort using morphological characters and molecular methods, the systematic position of the genus Tinca is still unclear (He et al., 2008). Chen and Mayden (2009) proposed that Tinca is a member of the terminal clade of cyprinids, the monophyly of which is highly supported. In conclusion, it seems that tench may be representative of ancient cyprinids with several primitive family features conserved including neuroendocrine regulation of LH.

This study represents the first report of dopaminergic control of LH release in tench, which showed no difference in ovulation rates after administration of GnRHa alone and in combination with a DA inhibitor.

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CHAPTER 5

THE EFFECTS OF WATER TEMPERATURE AND VARIOUS HORMONAL TREATMENTS ON LUTEINIZING HORMONE RELEASE AND OVULATION IN TENCH (*Tinca tinca*)

Podhorec, P., Socha, M., Sokolowska-Mikolajczyk, M., Policar, T., Svinger, V. W., Kouril, J., 2011. The effects of water temperature and various hormonal treatments on luteinizing hormone release and ovulation in tench (*Tinca tinca*). Comparative Biochemistry and Physiology – Part A: Molecular & Integrative Physiology. (submitted)

THE EFFECTS OF WATER TEMPERATURE AND VARIOUS HORMONAL TREATMENTS ON LUTEINIZING HORMONE RELEASE AND OVULATION IN TENCH (*Tinca tinca*)

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ABSTRACT

The effectiveness of three hormone treatments commonly used for artificial reproduction of tench was evaluated under three thermal regimes, with focus on the need for dopamine antagonist. Mature females were divided into nine groups (n = 8) and gradually exposed, over a 24 h period, to three temperature regimes: cold (18.1 ± 0.02 °C), optimal (22.05 ± 0.03 °C), and warm (26.3 ± 0.01 °C). Each temperature regime comprised three experimental groups injected with one of three hormone treatments: carp pituitary extract (CPE); [D-Arg^6, Pro^9, NEt]-sGnRH; and [D-Arg^6, Pro^9, NEt]-sGnRH + metoclopramide (combined treatment). No differences were found between ovulation induction (ovulation rate ≥ 75%) with sGnRHa alone and with the combined treatment; whereas CPE at cold and warm water temperatures was significantly less effective ($P < 0.05$). Administration of sGnRHa alone induced a gradual increase in luteinizing hormone (LH) levels with LH peaks close to ovulation, in contrast to the immediate LH surge with high LH levels throughout the entire study observed with the combined treatment under all thermal regimes. LH levels induced by GnRHa alone were significantly lower ($P < 0.05$) at all temperatures compared to the combined treatment, with the exception of the final sample at 26 °C, when no difference was recorded. Based on these results we recommend the application of sGnRHa without the addition of dopamine antagonist as a reliable method for inducing ovulation in tench under suboptimal temperature conditions.

Keywords: *dopamine, cyprinid, reproduction, aquaculture, GnRHa*

INTRODUCTION

Tench (*Tinca tinca*) is the second most important fish species in carp production and has a long tradition in European countries (Steffens, 1995). The nutritional value of tench meat, together with consumer interest in local foods produced by traditional methods has increased interest in tench culture (Gasco et al., 2010). Tench aquaculture relies mainly on fry supplied from artificial reproduction, the success of which is significantly influenced by external factors. The key environmental parameters regulating reproduction of cold-water cyprinids, and particularly tench, are photoperiod and temperature (Horoszewicz, 1983), with the influence of temperature predominating in the final stages of gametogenesis and ovulation (Breton et al., 1980). Tench reproduction is characterized by group synchronous pattern of oocyte development with the concurrent presence of several generations of oocytes each at a distinct developmental stage, and the multiple spawning at favourable temperatures during the season; May – August (Rodriguez et al., 2008). Spawning activity in tench starts when the mean daily temperature reaches 20 °C and decreases after the temperature rises above 26 °C (Linhart and Billard, 1995). The optimal spawning temperature range for both natural and artificial reproduction is considered to be 22–24 °C (Kouril et al., 1986). Fish hatcheries do not always provide optimal temperature for tench reproduction, a situation that, together with other stress factors, may result in reproductive dysfunction at the stage of final oocyte maturation (FOM) and ovulation (Kouril, 1998). It is generally accepted that this is due to a deficiency in luteinizing hormone (LH) surge from the pituitary that stimulates the synthesis of the maturation inducing steroid (MIS) and final oocyte maturation (FOM) (Zohar et al., 2001). A common solution, which acts directly on the ovary, is administration of carp pituitary extract (CPE) that releases its LH into the circulation creating an artificial LH surge (Yaron et al., 2009). Another indirect solution is the stimulation by GnRH of endogenous LH release from the pituitary gonadotrophs (Peter and Yu, 1997). However, LH secretion is not only regulated positively by hypothalamic stimulation, but is also under inhibitory hypothalamic control by dopamine (Popesku et al., 2008). Dopaminergic inhibition of basal and GnRHa-induced LH release is especially prominent in cyprinids, peaking at the end of vitellogenesis (Peter et al., 1986). A combined treatment comprising dopamine D2 receptor antagonists and GnRH superactive analogue (GnRHa) has been documented to stimulate FOM and ovulation in cyprinids (Sokolowska et al., 1984). The potency of a combined treatment to stimulate LH surge and ovulation, even at unfavourable temperatures, was demonstrated in several cyprinid fishes (Sokolowska et al., 1985; Glasser et al., 2004). In contrast to results in other cyprinids, early attempts to induce ovulation in tench with GnRHa alone were successful in stimulating a high proportion number of fish to ovulate (Kouril et al., 1986; Podhorec and Kouril, 2009). However, we have recently confirmed the presence of dopaminergic inhibition in tench (Podhorec et al., 2011b), but no evaluation has yet been conducted to assess whether suboptimal temperatures reduce the efficiency of treatment consisting of GnRHa only in inducing ovulation in tench and at what instances the addition of a dopamine inhibitor is required.

The objective of the present *in vivo* study was to establish optimal methods for hormone induction of ovulation in tench as well as evaluation of the intensity of dopamine inhibition of LH secretion under suboptimal temperature conditions.

MATERIAL AND METHODS

Sexually mature female tench were harvested in June 2010 when water temperature in the pond had reached 20 °C and remained at this or higher level for three days. Females with a distended and soft abdomen were selected, and oocyte samples were withdrawn with polyethylene tubing and the

position of the germinal vesicle was determined in 50 post-vitellogenic oocytes after clearing with Serra's solution. Only females possessing over 60% oocytes with a migrating germinal vesicle were taken for the experiment (Yaron et al., 2009).

Fish were transported to the experimental facility of the Faculty of Fisheries and Protection of Waters in Vodnany, Czech Republic and randomly divided into nine groups (eight fish into each group). Fish were kept under natural photoperiod in 700 L flow-through tanks supplied with water from the Blanice River. Dissolved oxygen was maintained at an average of 7.6 ± 0.7 mg L^{-1}. After 24 h acclimation at 20 °C fish were exposed gradually, over a 24 h period, to three temperature regimes: cold (18.1 ± 0.02 °C), optimal (22.05 ± 0.03 °C), and warm (26.3 ± 0.01 °C). Temperature control was achieved through use of a heating system (T-Computer Set, AB Aqua Medic, Germany) combined with cooling by addition of cold river water. Water temperature was registered on data loggers (Minikin, Environmental Measuring Systems, Brno, Czech Republic). Prior to any manipulation, fish were anesthetized with clove oil applied at 0.033 ml L^{-1}.

Each temperature regime comprised three experimental groups subjected to one of three hormone treatments dissolved in 0.9% NaCl and administered in a single intraperitoneal injection: (1) carp pituitary extract (3 mg kg^{-1}; FROV Vodnany); (2) [D-Arg^6, Pro^9, NEt]-sGnRH (10 µg kg^{-1}, Bachem AG); and (3) [D-Arg^6, Pro^9, NEt]-sGnRH (10 µg kg^{-1}) combined with metoclopramide (20 mg kg^{-1}, Sigma-Aldrich). In order to efficiently determine and accurately compare LH releasing potency of the treatments, blood sampling was scheduled based on the projected latency period at each temperature according to previous experience (Barth et al., 1997). The first sample was taken prior to injection, the second at 30% of projected time to ovulation, and the third sample time was at 70% of projected time from injection until ovulation. Blood samples (500 µL) were taken from the caudal vein with a heparinized 21-gauge needle attached to a heparinized 1 mL syringe. Blood samples were centrifuged (4000 g for 10 min at 4 °C) and plasma was stored at -80 °C until analysis.

Luteinizing hormone concentrations in plasma samples were assayed by heterologous enzyme-linked immunosorbent assay (Kah et al., 1989). The use of the heterologous assay for tench plasma was validated by demonstrating parallel displacement curve of serially diluted tench plasma with standard common carp curve. The assay sensitivity was in the range 0.1–1000 ng mL^{-1} and the intra- and inter-assay coefficients of variance were 5% and 9%, respectively.

Ovulation was checked every 4 h from 24 h until 48 h post-injection for optimal and warm temperature groups and every 6 h from 34 h post-injection until 84 h post-injection for low temperature groups. Fish that expressed eggs in response to gentle abdominal pressure were considered as ovulated fish.

The work has been reviewed and approved by an institutional animal care and use committee in accordance with EC Directive 86/609/EEC for Animal Experiments.

Student's t-test was used to compare mean LH values among groups. Ovulation rate was analyzed by the χ^2 analysis. The latency period, fecundity index, and fertilization rate were subjected to one-way analysis of variance (ANOVA), followed by Tukey's HSD test. Differences were considered significant at $P < 0.05$. All data are presented as means ± standard errors of the means (SEM).

RESULTS

No differences were found in ovulation-inducing potency among hormone treatments at 22 °C. Both low (18 °C) and high (26 °C) water temperatures negatively. At both low (18 °C) and high (26 °C) temperatures, the potency of CPE to induced ovulation was lower than all other treatments ($p < 0.05$), with only exception of no difference in case of the combined treatment at 26 °C. No differences were found in the ovulation rate in fish injected only with GnRHa and those treated with GnRHa combined with metoclopramide [($p > 0.05$) ovulation ratio ≥ 75%, Table 1)].

Injection of CPE under all thermal regimes was characterized by high LH levels at the second sampling time following with LH decrease at the final sampling time (Fig. 1). Significant LH releasing ability of GnRHa alone or in combination with metoclopramide was observed for all thermal regimes. Administration of the combined treatment induced a fast increase in LH concentrations with peaks over 100 ng L^{-1} that remained elevated throughout the sampling period for all thermal regimes. The treatments of GnRHa only were associated with a gradual LH increase under all thermal regimes that were significantly lower ($P < 0.05$) than the LH concentrations induced by the combined treatment at all sampling times and all temperatures except for the third sampling time at 26 °C (Fig. 2).

Table 1. *Effects of carp pituitary extract (CPE), sGnRHa, and a combined treatment (sGnRHa + Met) on reproduction characteristics of tench maintained at three thermal regimes. Data are expressed as the mean ± SEM. Different superscripts denote statistical differences between groups ($P < 0.05$, $n = 8$).*

Temperature (°C)	Treatment	Body weight (g)	Ovulation ratio (%)	Latency period (h)	Fecundity index (%)
18.1 ± 0.02	CPE	1092 ± 81	25^{a}	49^{c}	5.25 ± 2.6
	sGnRHa	1100 ± 52	$75^{b,c}$	49 ± 4.1^{c}	7.8 ± 0.6
	sGnRHa + Met	1122 ± 73	$87.5^{b,c}$	52.3 ± 4.5^{c}	6.5 ± 0.8
22.1 ± 0.03	CPE	1212 ± 68	$62.5^{b,c}$	27.8 ± 0.9^{b}	6.1 ± 0.8
	sGnRHa	1244 ± 52	$75^{b,c}$	31.2 ± 1^{b}	7.6 ± 1.2
	sGnRHa + Met	1080 ± 91	$87.5^{b,c}$	35 ± 2.8^{b}	5.45 ± 1.1
26.3 ± 0.01	CPE	1091 ± 57	$50^{a,b}$	20.8 ± 3.5^{a}	5.4 ± 0.6
	sGnRHa	1066 ± 99	100^{c}	29.1 ± 1.6^{b}	7.5 ± 0.7
	sGnRHa + Met	1112 ± 59	$87.5^{b,c}$	$25 \pm 2.2^{a,b}$	6.7 ± 0.8

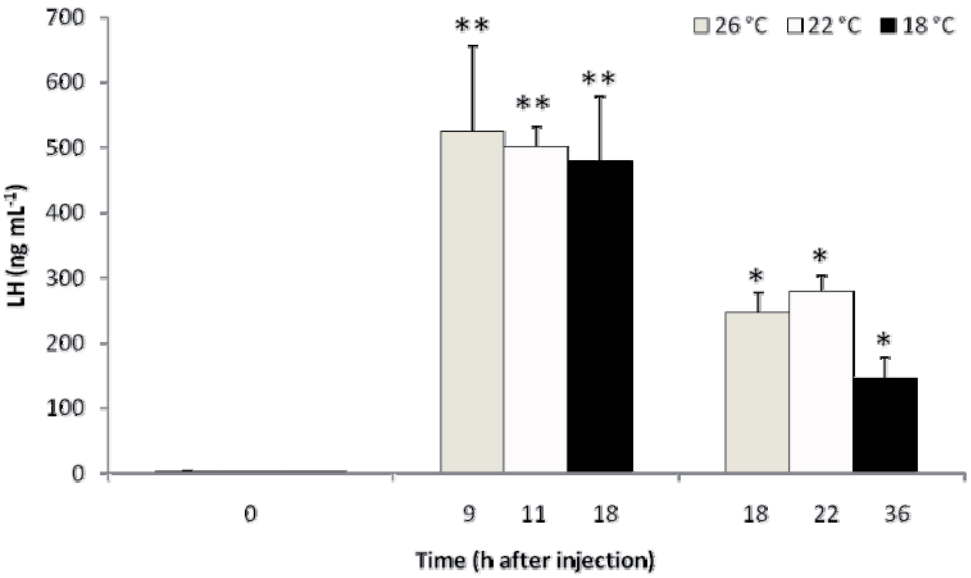

Figure 1. *Luteinizing hormone concentrations after carp pituitary extract injection in tench maintained at three temperature regimes. Values are presented as means ± SEM ($n = 8$). Asterisk denotes statistical differences between groups ($P < 0.05$).*

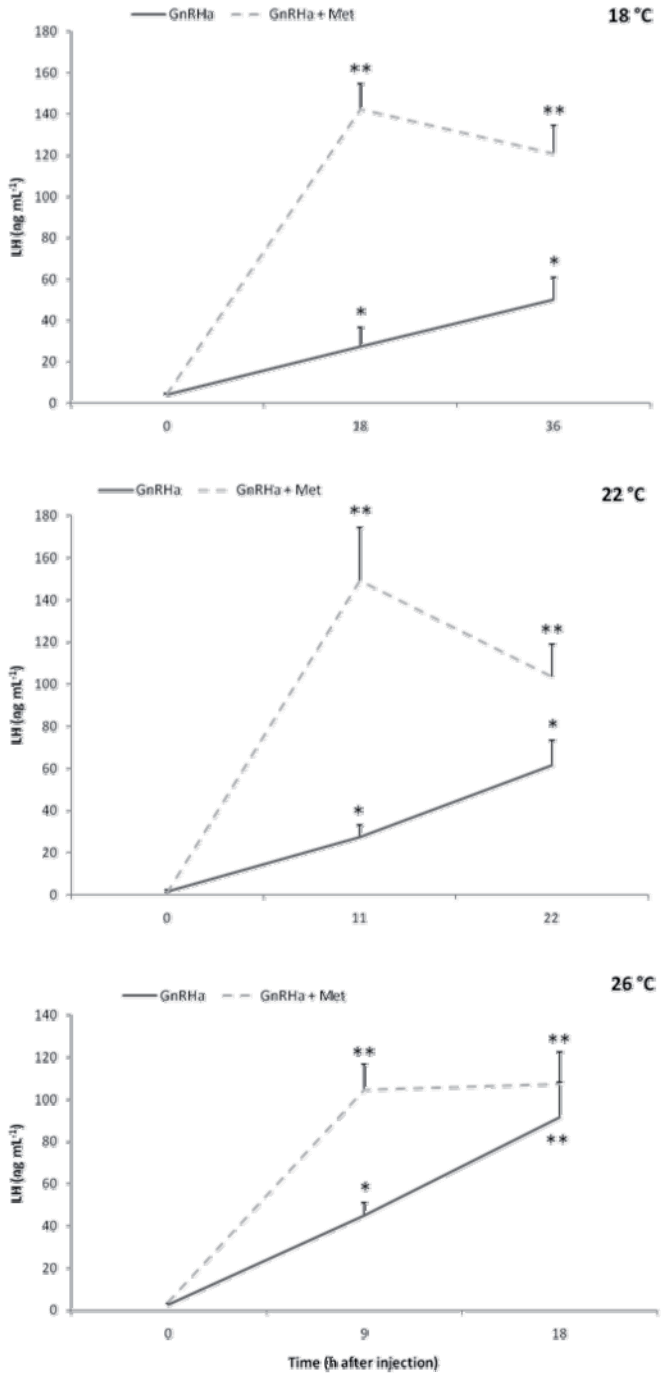

Figure 2. *Effect of sGnRHa and combined treatment [sGnRHa + metoclopramide (Met) injections] on plasma LH concentrations in tench maintained at three temperature regimes. The figure compares within cohort differences between treatments (mean ± S.E.M., n = 8). Asterisk denotes statistical differences (P < 0.05).*

DISCUSSION

Tench females do not ovulate spontaneously in culture even after environmental stimulation by a temperature increase sufficient for induction of final oocyte maturation in other cyprinids (Podhorec et al., 2011a). Development of reliable techniques for artificial reproduction applicable for a broad range of environmental conditions is required to maintain viable tench culture. Eliminating the use of neuroactive drugs such as dopamine antagonists, is indisputably critical for the safety of the consumers. Therefore, the current study was undertaken to evaluate the potency of three commonly used hormone therapies in artificial reproduction of tench under three thermal regimes.

Hormone treatments based on GnRHa with or without a dopamine antagonist and CPE induced high ovulation rates (≥ 75%) in tench under optimal temperature conditions. The similar potency of the combination and GnRHa alone is a peculiarity of tench in comparison with other cyprinids, and is especially interesting considering the fact that dopaminergic inhibition of LH release in tench has been demonstrated in our previous study (Podhorec et al., 2011b). The negative regulation of dopamine on LH secretion and ovulation increases in intensity along with the degree of reproductive readiness (Peter et al., 1986) but drops in response to optimal internal and external cues at the end of gametogenesis (Dufour et al., 2010). One of the questions addressed in the current study was whether suboptimal water temperature, could enhance dopaminergic inhibition in tench. The results indicate that suboptimal temperature ranges did not increase DA inhibition of LH release and ovulation and did not decrease the pituitary sensitivity to sGnRHa stimulation when compared to fish at optimal temperature. Both the combined treatment and sGnRHa alone showed a pre-ovulatory LH surge with a high ovulation rate (75–100%). However, the administration of a DA inhibitor together with GnRHa significantly modified the LH profile compared to GnRHa alone, irrespective of temperature regime. This is consistent with our earlier report (Podhorec et al., 2011b) that showed slow and gradual increase LH level after GnRHa treatment in contrast to the fast LH surge following stimulation by the combined treatment. As expected in any exothermic organism such as fish the LH kinetic in tench is dependent on the temperature, slowest in fish reared at 18 °C and fastest in fish reared at 26 °C. Gradual LH release and a higher metabolic rate explains the detection of peak LH values in sGnRHa group kept at 26 °C in contrast to lower rate of LH increase in sGnRHa injected groups kept at 18 and 22 °C. However, based on elevation of LH concentration recorded at first and second sampling times as well as on high ovulation success, we suggest that pre-ovulatory LH peaks (> 100 ng mL^{-1}) were also reached for the sGnRHa groups kept at 18 °C and 22 °C. Maximum LH concentrations recorded for sGnRHa group at 26 °C were similar to LH values induced by the combined treatment.

Such situation as seen in tench is in contrast, to the situation in other fish. An adverse effect of unfavourable water temperature on the hypothalamo-pituitary axis via inhibition of LH release by dopamine, has been demonstrated in grass carp (*Ctenopharyngodon idella*) (Glasser et al., 2004) and arctic char (*Salvelinus alpinus*) (Gillet and Breton, 2009). Water temperatures above the natural spawning range have been shown to suppress the LH releasing effect of GnRHa, whereas concomitant administration of GnRHa and dopamine antagonist facilitated ovulation (Glasser et al., 2004; Gillet and Breton, 2009).

The effectiveness of one dose of CPE demonstrated by Kouril (1998) to stimulate oocyte maturation and ovulation in tench, was under suboptimal temperature conditions, despite induction of extremely high exogenous LH concentrations in plasma, lowest of all tested hormone treatments. One of possible explanation can be reduced sensitivity of gonads to exogenous LH (CPE) under unfavourable temperature conditions compared to endogenous LH secreted after stimulation by GnRHa or the combined treatment. Blockade of ovulation under non-optimal temperature conditions may also be due to lack of the sufficient level of 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) or its cognate receptor

in the oocyte. In any case, the use of CPE as a spawning inducing agent is currently of a low priority due to the hazard of transmitting pathogns from donor fish. Current European veterinary legislative issues lead us to a conclusion of unsuitability of using CPE in hormone therapies in tench. As could be expected, the latency period recorded in the present work was found to be negatively correlated with water temperature irrespective of the hormone treatment. This is in line with previous work done by Kouril (1998). In addition, it should be noted that treatment with CPE by which the LH administered acts directly on the ovary resulted in shorter latency than with the administration of GnRH which requires an additional activity at the level of the pituitary.

Based on the results obtained in this *in vivo* study, we can recommend administration of GnRHa without a DA antagonist as a reliable and efficient ovulation inducing therapy under optimal as well as suboptimal temperature conditions.

ACKNOWLEDGEMENTS

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CHAPTER 6

GENERAL DISCUSSION • ENGLISH SUMMARY • CZECH SUMMARY • ACKNOWLEDGEMENTS • LIST OF PUBLICATIONS • TRAINING AND SUPERVISION PLAN DURING STUDY • CURRICULUM VITAE

GENERAL DISCUSSION

The tench has been cultured extensively in ponds in Europe since the middle ages as so-called secondary or by-fish species (Steffens, 1995). The nutritional value of tench meat, together with consumer interest in local foods produced by traditional methods has increased interest in tench culture (Fernandez San Juan, 1995; Wang et al., 2006). High demand for tench fry as one of the most limiting factor for the development and expansion of tench aquaculture has driven research aimed at managing all aspects of their reproduction artificially. Tench females do not ovulate spontaneously under captive conditions of fish farms and required a proper stimulation (Kouril et al., 2008). In Czechoslovak fisheries, the first experiments with the induced ovulation and spawning in fish and specifically tench, using hypothalamic neuropeptide GnRHa were performed by Dr. Kouril and co-workers. Their results demonstrated high responsiveness of tench to a GnRHa treatment (Kouril et al., 1986), were an important stimulus for further development of methods using GnRHa to induce ovulation in several other fish species (Barth et al., 1997). However one important feature of neuroendocrine regulation of LH secretion in tench remained unresolved, the ability of GnRHa alone to induce pre-ovulatory LH surge and ovulation without dopamine antagonist, contrary to what has been reported for all other cyprinids (Peter et al., 1986). The presented work was devoted to clarify basal and applied aspects of using hypothalamic factors for hormonal induction of ovulation in tench.

The dose-response trial of the most commonly used mGnRH analogue ([D-Ala^6, Pro^9, NEt]-mGnRH) demonstrated that low doses of mGnRHa are capable of inducing the prerequisite LH surge and ovulation in a substantial number of tench in accord with our earlier results (Kouril et al., 1986). The stimulation potential of mGnRHa was sufficient to produce results with no statistical differences among concentrations ranging from 1 to 20 μg kg^{-1}. However, when regression analyses were employed, a positive correlation of LH concentration with spawning rate was observed to be associated with higher doses of mGnRHa. This implies that dosages in the range of 10–20 μg kg^{-1} of mGnRHa (ovulation rate ≥ 80%) can be considered reliable and effective, and that higher doses may represent needless overdosing. The potency of mGnRHa alone in inducing spermiation in tench was shown by Caille et al. (2006) when 20 μg kg^{-1} significantly increased milt production. The sensitivity of tench to low mGnRHa concentrations in stimulating LH release from the pituitary and inducing ovulation is similar to that of several fresh and marine members of Perciformes, e.g. silver perch (*Bidyanus bidyanus*), (Levavi-Sivan et al., 2004); sea bream (*Sparus aurata*), (Zohar, 1989); sea bass (*Dicentrarchus labrax*), (Prat et al., 2001); in which a low dose of GnRHa stimulates ovulation and which do not demonstrate dopaminergic control of LH release (Berlinsky et al., 1996; Levavi-Sivan et al., 2004; Prat et al., 2001). In atlantic croaker (*Micropogonias undulatus*) the addition of a DA antagonist to hormonal treatment suppresses the effects of GnRHa on LH release while DA agonists potentiate it.

The ovulation and spermiation in tench after receiving a low dose of GnRHa (≤ 1 μg kg^{-1}) is exceptional within the Cyprinidae. In cyprinids GnRHa alone generally does not induce an ovulatory response (Arabaci and Sari, 2004), with the exception of a minimal ovulation rate when administered at high doses (Heyrati et al., 2007; Mikolajczyk et al., 2003). The minimal effective dose of superactive GnRHa stimulating 40 to 60% of common carp (*Cyprinus carpio*) has been shown to be 40–80 μg kg^{-1} (Mikolajczyk et al., 2004) which is several fold higher than the dose of GnRHa effective in stimulating ovulation in a similar percentage of tench. In goldfish (*Carassius auratus*), and nase (*Chondrostoma nasus*), an even less effective influence of GnRHa alone on LH profile and ovulation has been reported (Chang and Peter, 1983; Szabo et al., 2002). The failure of low doses of GnRHa to induce ovulation in cyprinids has been explained by dopamine inhibition (Peter et al., 1986), which prevents the LH surge prerequisite for ovulation. To date there has been no investigation of dopamine inhibition in tench in spite of the apparent inhibitory tone in other cyprinid species. *In vivo* experiment was carried

out to clarify whether LH release, and thus ovulation, in tench is under inhibitory dopaminergic control. The combined treatment composed of [D-Ala^6, Pro^9, NEt]-mGnRH and the DA D2-receptor antagonist metoclopramide triggered an almost immediate LH release peak with high LH concentrations throughout the experimental period inducing a high ovulation rate. This clear confirmation of DA inhibition in tench was surprising; especially in the light of GnRHa effectiveness in ovulation (60–70%) and the gradually elevated LH profile with values peaking close to ovulation. These data are consistent with our earlier results when as little as 1 μg kg^{-1} of mGnRHa induced gradual elevation of LH with 63% of females ovulating (Kouril et al., 1986). Few studies have compared effects of combined versus GnRHa alone treatments in tench, although Pinillos et al. (2002) reported an enhanced effect of GnRHa alone on secretion of 17,20β-dihydroxy-4-pregnen-3-one, 17,20α-dihydroxy-4-pregnen-3-one, and testosterone and ovulation success compared to a combined treatment. Although in presented study approximately 70% of females ovulated after GnRHa treatment (10 μg kg^{-1}) which is the mean ovulation rate obtained after combined treatment by Kujawa et al. (2010), our and other studies have more commonly reported ovulation rates of 80% or more with GnRHa treatment (Kouril et al., 1986; Kouril et al., 2008). The relatively lower LH mean concentrations in compare with our previous study (Podhorec et al., 2011) were detected in the current trial, although LH concentrations in some ovulated females exceeded detection limits of the assay (> 100 ng mL^{-1}).

Hormonal induction of ovulation in tench is well-established in aquaculture production using a low dose of GnRHa alone (Linhart and Billard, 1995). However, no evaluation has yet been conducted to assess whether suboptimal temperatures reduce the effectiveness of GnRHa alone in inducing ovulation in tench and require the addition of a dopamine inhibitor to increase the potency of hormone therapy. A study was undertaken to evaluate the potency of three commonly used hormone therapies in artificial reproduction of tench under three thermal regimes. Hormone treatments based on GnRHa with or without a dopamine antagonist and CPE induced high ovulation rates (≥ 75%) in tench under optimal temperature conditions. The similar potency of the combination and GnRHa alone is a peculiarity of tench in comparison with other cyprinids, and is especially interesting considering the fact that dopaminergic inhibition of LH release in tench has been demonstrated in the previous study. The negative regulation of dopamine on LH secretion and ovulation increases in intensity along with the degree of reproductive readiness (Peter et al., 1986) but drops in response to optimal internal and external cues at the end of gametogenesis (Dufour et al., 2010). One of the questions addressed in the current study was whether suboptimal water temperature, could enhance dopaminergic inhibition in tench. The results indicate that suboptimal temperature ranges did not increase DA inhibition of LH release and ovulation and did not decrease the pituitary sensitivity to sGnRHa stimulation when compared to fish at optimal temperature. Both the combined treatment and sGnRHa alone showed a pre-ovulatory LH surge with a high ovulation rate (75–100%). As expected in any exothermic organism such as fish the LH kinetic in tench is dependent on the temperature, slowest in fish reared at 18 °C and fastest in fish reared at 26 °C. Gradual LH release and a higher metabolic rate explains the detection of peak LH values in sGnRHa group kept at 26 °C in contrast to lower rate of LH increase in sGnRHa injected groups kept at 18 and 22 °C. However, based on elevation of LH concentration recorded at first and second sampling times as well as on high ovulation success, we suggest that pre-ovulatory LH peaks (> 100 ng mL^{-1}) were also reached for the sGnRHa groups kept at 18 °C and 22 °C. Maximum LH concentrations recorded for sGnRHa group at 26 °C were similar to LH values induced by the combined treatment.

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spawning range have been shown to suppress the LH releasing effect of GnRHa, whereas concomitant administration of GnRHa and dopamine antagonist facilitated ovulation (Glasser et al., 2004; Gillet and Breton, 2009).

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In all conducted trials the administration of a DA inhibitor together with GnRHa significantly modified the LH profile compared to GnRHa alone although both associated with high ovulation rates. In general the injection of GnRHa led to slow and gradual increase LH level in contrast to the fast LH surge following stimulation by the combined treatment. Peter et al. (1986) suggested that the ovulatory response in goldfish (*Carassius auratus*) is dependent on both the magnitude of the LH concentration and the rate of increase in circulating LH levels. Immediate LH surge after the combined treatment was more effective in inducing ovulation than GnRHa-stimulated gradual increase despite comparable LH concentrations (Chang and Peter, 1983; Sokolowska et al., 1984). In tench we cannot confirm this even under unfavourable temperature conditions, as an application of GnRHa alone induced completion of germinal vesicle migration and ovulation in the majority of females after a gradual increase of LH levels. Similar to our results continuously elevated LH levels and high ovulation rates induced by a hormonal treatment have been detected in common carp (Drori et al., 1994; Mikolajczyk et al., 2003; Mikolajczyk et al., 2004) and grass carp (Glasser et al., 2004).

The most widely used synthetic GnRHa in tench artificial reproduction were tested for induction of pre-ovulatory LH surge and ovulation. In goldfish sGnRH is considered to be the main hypophysiotropic form based on its high abundance in the pituitary (Steven et al., 2003). However no difference in the effectiveness of [D-Arg^6, Pro^9, NEt]-sGnRH compared to [D-Ala^6, Pro^9, NEt]-mGnRH or [D-Leu^6, Pro^9, NEt]-mGnRH were detected. Superiority of [D-Ala^6, Pro^9, NEt]-mGnRH previously shown in sea bass (*Dicentrarchu labrax*) (Prat et al., 2001) was not confirmed for the tench, although it induced the maximum LH peak in the trial. All tested GnRHa induced similar LH concentrations and ovulation success and can be equally recommended for hormonal therapies in tench.

The great diversity of the family Cyprinidae has attracted scientific attention and resulted in many comprehensive phylogenetic studies (Briolay et al., 1998; Hanfling et al., 2000; Saitoh et al., 2011). However, despite considerable effort using morphological characters and molecular methods, the systematic position of the genus Tinca is still unclear (He et al., 2008). Chen and Mayden (2009) proposed that Tinca is a member of the terminal clade of cyprinids, the monophyly of which is highly supported. In conclusion, it seems that tench may be representative of ancient cyprinids with several primitive family features conserved including neuroendocrine regulation of LH.

This work represents the first report of dopaminergic control of LH release in tench, which showed

no difference in ovulation rates after administration of GnRHa alone and in combination with DA inhibitor. Significant differences were determined between LH profiles after GnRHa alone or GnRHa with DA inhibitor treatment. High effectiveness of low doses of mGnRHa to induce ovulation in tench was demonstrated with no need to apply higher doses than 20 μg kg^{-1} of mGnRHa or addition of any neuroactive drug. Combined treatment as suggested by Kujawa et al. (2010) for successful induction of ovulation in tench is not necessary. Based on the results obtained in this study, we can recommend administration of GnRHa (10–20 μg kg^{-1}) without a DA antagonist as a reliable and efficient ovulation inducing therapy under optimal as well as suboptimal temperature conditions.

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ENGLISH SUMMARY

Artificial reproduction of tench (*Tinca tinca* L.), with an emphasis placed on hormonal induction of ovulation

Peter Podhorec

The aquaculture of tench relies mainly on fry supplied by artificial reproduction. Fish hatcheries do not always provide optimal conditions for tench reproduction, a situation that, together with other stress factors, may result in reproductive dysfunction at the stage of final oocyte maturation and ovulation. It is generally accepted that this is due to a deficiency in luteinizing hormone surge from the pituitary that stimulates the synthesis of the maturation inducing steroid and final oocyte maturation. Methods used to overcome this dysfunction are either based on application of carp pituitary extract or gonadotrophin-releasing hormone analogue (GnRHa) with or without dopamine inhibitor (DI). Although cyprinids are considered typical of a fish group with decisively demonstrated DA inhibition of LH release and ovulation, it appears that this does not apply to tench. For successful hormonal induction of FOM and ovulation in female tench, a low dose of GnRHa is sufficient, contrary to what has been reported for all other cyprinids. The objective of the presented thesis is to clarify basal and applied aspects of using hypothalamic factors for hormonal induction of ovulation in tench.

A broad range of effective GnRHa doses is used in aquaculture of tench however, the specification of a minimal effective GnRHa dose based on LH profile and ovulation success is still missing. Hormonal therapy applied to excess can result in needlessly increased production costs and have detrimental impacts on broodstock fecundity and quality of eggs. An experiment was design to test the dose-response of the most commonly used GnRH analogue, [D-Ala^6, Pro^9, NEthylamide]-mGnRH, to stimulate a pre-ovulatory LH surge with subsequent ovulation in tench. The stimulation potential of mGnRHa was sufficient to produce results with no statistical differences among concentrations ranging from 1 to 20 μg kg^{-1}. However, when regression analyses were employed, a positive correlation of LH concentration with spawning rate was observed to be associated with higher doses of mGnRHa. This implies that dosages in the range of 10–20 μg kg^{-1} of mGnRHa (ovulation rate ≥ 80%) can be considered reliable and effective, and that higher doses may represent needless overdosing.

To evaluate whether LH release and ovulation in tench is under inhibitory dopaminergic control different treatments with or without DA D2-receptor antagonist metoclopramide were administered. The combined and GnRHa alone treatments were both associated with high LH concentrations and ovulation rates but GnRHa treatment led to a slow and gradual LH release, while a fast LH surge was seen following the combined treatment. The addition of DI did not influence the ovulation rate but significantly modified LH pre-ovulatory profile in tench.

Dopaminergic inhibition is not constant over the reproductive period in cyprinids. Increases in intensity along with the degree of reproductive readiness but drops in response to optimal internal and external cues at the end of gametogenesis. The questions addressed in this study was whether suboptimal water temperature, could enhance dopaminergic inhibition in tench like in other cyprinids. The results indicate that suboptimal temperature ranges did not increase DA inhibition of LH release and ovulation and did not decrease the pituitary sensitivity to sGnRHa stimulation when compared to fish at optimal temperature. Both the combined treatment and sGnRHa alone showed a pre-ovulatory LH surge with a high ovulation rate (75–100%), irrespective of thermal regime.

The current study represents the first report of dopaminergic control of LH release in tench which showed no difference in ovulation rates after administration of GnRHa alone and in combination with a DA. We demonstrated high effectiveness of low doses of mGnRHa (≤ 20 μg kg^{-1}) to induce ovulation in tench with no need of dopamine inhibitors under optimal as well as suboptimal thermal conditions.

CZECH SUMMARY

Umělá reprodukce lína obecného (*Tinca tinca* L.), s důrazem kladeným na hormonální indukci ovulace

Peter Podhorec

Rybniční produkce lína obecného (*Tinca tinca*) je v současné době plně závislá na produkci násadového materiálu vyprodukovaného pomocí umělého výtěru. Metodika umělého výtěru lína je založená na hormonální indukci ovulace a spermiace, buď s pomocí aplikace extraktu kapří hypofýzy, nebo syntetického analogu spouštěcího hormonu gonadotropinu (GnRHa). Kaprovité druhy ryb se zpravidla vyznačují velmi silnou dopaminovou inhibicí sekrece luteinizačního hormonu (LH), která zabraňuje úspěšné stimulaci ovulace po samotném podání GnRHa bez dopaminního inhibitoru (DI). Jednou ze zvláštností lína je schopnost dosáhnout ovulace po aplikaci nízké dávky GnRHa, což je výjimečný a doposud nepopsaný jev u jiných druhů ryb čeledi Cyprinidae. Cílem předložené disertační práce byla optimalizace hormonální indukce ovulace při zaměření na hypothalamické faktory GnRH a dopamin.

Výše účinné dávky GnRHa je jedním z nejvýznamnějších faktorů ovlivňujících úspěšnost hormonální indukce ovulace u lína. Nadměrně vysoká dávka GnRHa zbytečně zatěžuje rybí organismus a může mít negativní dopad na pohlavní produkty, zatímco nízká dávka nevyvolává žádanou odezvu v podobě sekrece LH a následné ovulace. Kvůli nalezení optimální dávky GnRHa bylo testováno pět různých výší dávky (1; 2,5; 5; 10 a 20 μg kg^{-1}) nejčastěji využívaného GnRHa ([D-Ala^6, Pro^9, NEthylamide]-mGnRH). Všechny testované dávky GnRHa významně zvýšily sekreci LH a stimulovaly ovulaci u většiny (≥ 60%) ošetřených jikernaček lína. Analýza získaných dat pomocí nelineární regrese zvýraznila pozitivní korelaci mezi vzrůstající dávkou mGnRHa a % ovulujících jikernaček. Jako optimální pro indukci ovulace byla u lína stanovena dávka mGnRHa v rozpětí 10–20 μg kg^{-1}.

Vysoká účinnost samostatně aplikovaného GnRHa u lína je v kontrastu se situací u ostatních kaprovitých druhů ryb, vyznačující se silnou dopaminovou inhibicí sekrece LH s nutností kombinované aplikace GnRHa a DI. Byl navržen experimentální desain, v rámci kterého byly jikernačky lína hormonálně ošetřeny, jednak samotným GnRHa, jednak kombinací GnRHa a DI. Samostatně podaný GnRHa, stejně jako kombinované ošetření, indukovalo výrazné zvýšení hladiny LH s následnou ovulací většiny ošetřených jikernaček. Zajímavým zjištěním byla výrazná modifikace profilu LH podaným DI. Samostatně aplikovaný GnRHa indukoval pozvolný vzestup hladiny LH s dosažením maximálních koncentrací v době krátce před ovulací, ve srovnání s téměř okamžitým dosažením maximálních hodnot LH po aplikaci GnRHa spolu s DI. Navzdory stejné účinnosti při indukci ovulace GnRHa, jako i GnRHa spolu s DI, byla na základě různého profilu LH identifikovaná dopaminová inhibice LH sekrece u lína.

Dopaminová inhibice u kaprovitých ryb není stejně silná v průběhu celého roku. Stupňuje se s postupující připraveností ryb k výtěru, i vlivem nevhodných vnějších podmínek ve výtěrovém období. Pro optimalizaci hormonálního ošetření v suboptimálních teplotních podmínkách, resp. ověření nutnosti ztlumení dopaminové inhibice, byly aplikovány tři druhy hormonálních preparátů při třech teplotních režimech. Ve srovnání s výsledky zjištěnými u jiných kaprovitých druhů ryb, kde kombinované podání GnRHa s DI zvyšuje % ovulujících jikernaček, nebyl u lína zjištěn žádný pokles účinnosti samostatně podaného GnRHa (10 μg kg^{-1}) stimulovat ovulaci. Navzdory námi prokázané dopaminové inhibice jsme u lína nezaznamenali potřebu současné aplikace GnRHa s DI v suboptimálních teplotních podmínkách.

Dosažené výsledky představují významný posun v pohledu na neuroendokrinní regulaci sekrece LH u lína, stejně jako na praktické využití hypothalamických faktorů při řízené reprodukci lína. Vůbec

poprvé se podařilo prokázat dopaminní inhibici sekrece LH u lína, u kaprovitého druhu ovulujícího po aplikaci samotného GnRHa. Bližší specifikace účinné dávky mGnRHa, stejně jako i nalezení optimálního způsobu hormonálního ošetření jikernaček lína v suboptimálních teplotních podmínkách, představuje významný přínos pro rybářskou praxi.

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LIST OF PUBLICATIONS

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Podhorec, P., Socha, M., Sokołowska-Mikolajczyk, M., Policar, T., Svinger, V.W., Kouril, J., 2011. The effects of water temperature and various hormonal treatments on luteinizing hormone release and ovulation in tench (*Tinca tinca*). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology. (submitted)

Podhorec, P., Socha, M., Sokołowska-Mikolajczyk, M., Policar, T., Svinger, V.W., Drozd, B., Kouril, J., 2011. Determination of dopamine control of luteinizing hormone release in tench (*Tinca tinca*). General and Comparative Endocrinology. (submitted)

Podhorec, P., Socha, M., Sokołowska-Mikolajczyk, M., Drozd, B., Policar, T., Stejskal, V., Kouril, J., 2011. Effective dose of mGnRHa for induction of ovulation in tench (*Tinca tinca* L.). Aquaculture 319, 184–187.

Policar, T., **Podhorec, P.,** Stejskal, V., Kouril, J., Alavi, S.M.H., 2011. Growth and survival rates, puberty and fecundity in captive common barbel (*Barbus barbus* L.) under controlled conditions. Czech Journal of Animal Science. (in press)

Policar, T., **Podhorec, P.,** Stejskal, V., Hamáčková, J., Alavi, S.M.H., 2010. Fertilization and hatching rates and larval performance in captive common barbell (*Barbus barbus* L.) throughout the spawning season. Journal of Applied Ichthyology 26, 812–815.

Podhorec, P., Kouril, J., 2009. Induction of final oocyte maturation in Cyprinidae fish by hypothalamic factors: a review. Veterinarni Medicina 54, 97–110.

ABSTRACTS AND CONFERENCE PROCEEDINGS

Podhorec, P., Socha, M., Sokołowska-Mikolajczyk, M., Policar, T., Svinger, V.W., Kouril, J., 2011. The effects of water temperature and various hormonal treatments on luteinizing hormone release and ovulation in tench (*Tinca tinca*). Diversification in Inland Finfish Aquaculture, Písek, Czech Republic, May 16–18, 2011, 55 p.

Podhorec, P., Kouril, J., 2008. Endocrine regulation and artificial stimulation of ovulation in tench (*Tinca tinca* L.), review. Vth International Workshop on Biology and Culture of the Tench (*Tinca tinca* L.), Ceresole d´Alba, Italy, September 29 – October 3, 2008, 39 p.

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