LH secretion and 17β-estradiol concentration in the blood plasma and hypothalamus of goldfish (*Carassius auratus gibelio* B.) and common carp (*Cyprinus carpio* L.) treated with fadrozole (aromatase inhibitor) and GnRH analogues

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ABSTRACT: Fadrozole – a non-steroidal aromatase inhibitor was applied to common carp and goldfish to examine its ability to potentiate the stimulatory action of GnRH analogues on LH secretion *in vivo*. The goal of the project was to find a substitute for antidopaminergic drugs used with GnRH analogues in fisheries practice to stimulate ovulation in fish bred in captivity. The first trials on goldfish showed a moderate ability of fadrozole to potentiate salmon GnRH analogue stimulation, weaker than that obtained with pimozide (dopamine antagonist). No ovulation in fadrozole-treated fish was observed. Several experiments performed in two consecutive reproductive seasons (different treatment regimes and doses of fadrozole) neither improved nor confirmed the results obtained in the first year. The analysis of 17β-estradiol levels in the blood plasma and in hypothalami showed no changes in the concentration of this steroid in fadrozole-treated fish in comparison with the controls. This shows that fadrozole is not able to replace the antidopaminergic drugs used in fisheries practice and cannot be considered as a potential ovulation inducer in cyprinids.

Keywords: aromatase inhibitor; LH; estradiol; goldfish; carp; ovulation

Production of cyprinid fish is the most important domain in world freshwater aquaculture with production centres located in Southeast Asia and Europe. Successful development of this branch of aquaculture was possible thanks to the cognition of physiological processes responsible for sexual maturation and reproduction and, as a consequence, development of the efficient and simple techniques for inducing maturation, spermiation and ovulation. Starting from the late eighties the most effective and widespread technique was the use of superactive synthetic GnRH analogues. However, in cyprinids, because of the strong dopamine inhibitory tonus upon LH secretion (Chang and Peter, 1983; Chang et al., 1984), it was necessary to combine the treatment with GnRH analogue with dopamine antagonist (Yaron, 1995; Peter and Yu, 1997; Zohar and Mylonas, 2001). Unfortunately, the presence of antidopaminergic drugs in the formulations used for induction of ovulation is a major obstacle for its common use in the countries of European Union. Antidopaminergic drugs like pimozide (synthesised by Janssen and Allewijn, 1968) are well-known neuroleptic compounds with extremely long half-life, exhibiting a wide range of physiological effects. This arouses serious problems for carp farmers and commercial hatcheries in which the products containing antidopaminergic drugs were used for

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years. It was recently discovered that the high level of dopamine, affecting LH secretion, is determined by the high levels of circulating estradiol (Linard et al., 1995; Saligaut et al., 1998). In the last stages of ovarian maturity, just before ovulation, the drop of dopamine production follows a rapid drop of estradiol levels. Thanks to this phenomenon the potentiation of GnRH action and the appearance of LH ovulatory surge and ovulation may occur (Fitzpatrick et al., 1986; Saligaut et al., 1999). The aim of this study was to provoke the LH ovulatory wave in carp and goldfish by the use of an aromatase inhibitor – fadrozole. Aromatase is a key enzyme converting testosterone into estradiol in gonads and in the brain. The blockade of this enzyme action provokes the diminution of estradiol production. Lower estradiol levels can in consequence decrease the production of dopamine – the main LH release inhibitory factor. Thus the application of GnRH analogues could evoke the LH ovulatory wave and stimulate ovulation in the examined fish.

MATERIAL AND METHODS

Nine independent *in vivo* experiments were performed in 3 consecutive years 2003–2005 in the Department of Ichthyobiology and Fisheries (DIF) at the University of Agriculture in Cracow and at Fisheries Research Station in Zator, Poland. The experiments were conducted in May and June – the natural reproductive season for carp and goldfish in Poland.

Animals

Goldfish (*Carassius auratus gibelio* B., mean body weight 123 ± 25 g) were purchased on Zator Fish Farm. They were 3-years-old sexually mature females. After netting from an outdoor pond they were transported to the laboratory, and stocked in 3–4 flow-through plastic basins (800 l in vol.) supplied with river water. During that time the fish were fed frozen *Chironomus* larvae (at a ratio of 0.5% of total body weight). Two to three days before the experiment the appropriate amount of females (10 fish per group) were netted, weighed, tagged and placed in aquaria (350 l in vol.) filled with recirculated river water (one group per aquarium). Common carp (*Cyprinus carpio* L.) originated from the Fisheries Station of the DIF. They were 4-years-old sexually mature females (mean body weight 2.35 ± 0.64 kg). After netting from an outdoor earthen pond they were transported to the laboratory, weighed, tagged and placed in concrete flow-through basins (1 000 l in vol.) supplied with deep well water. In the case of both species the water temperature in aquaria or basins was gradually increased up to 21°C and kept constant during the experiments. The fish were not fed during the experiments and were exposed to a simulated natural photoperiod. Before each manipulation the fish were anaesthetised with ethylene glycol monophenyl ether (0.4 ml/l). The last experiment (no. 9) was performed in the commercial hatchery of the Zator Fish Farm. Thirty individually tagged carp females (6–8 years old) weighing from 6.5 to 12.5 kg were placed in steel basins (750 l in vol., 5 fishes per basin) supported with a recycled water system. They were manipulated as described above.

Drugs

The non-steroidal aromatase inhibitor fadrozole (CGS 16949A) was a generous gift of Novartis Pharma AG (Basel, Switzerland). The injection solutions with appropriate concentrations of fadrozole were freshly prepared in a buffered saline solution (0.8% NaCl) prior to each intraperitoneal injection. Salmon GnRH analogue (Des-Gly^{10}, D-Arg^{6}, Pro^{9}-NHEt^{9}) salmon GnRH (sGnRHa) was purchased from Bachem Biochimie SARL (Voisins-le-Bretonneux, France). Gonazon^{TM} containing (D-Nal(2)^{6}, aza-Gly^{10})-LH-releasing-hormone (azagly-nafarelin) was a generous gift from Intervet International B.V. (Boxmeer, The Netherlands). Gonazon is the first and the only officially approved inducer of ovulation in the EU and Norway (Haffray et al., 2005). It was dissolved to a working concentration (64 μg/ml) in the original vehicle supplied by Intervet International B.V. Pimozide (PIM), a dopamine D2 receptor antagonist, was purchased from Sigma-Aldrich (Poznan, Poland). It was dissolved in acidified propylene glycol (POCh Gliwice, Poland) at a concentration of 10 mg/ml. The intraperitoneal injection (i.p.) volume for all the drugs used in the experiments was 0.5 ml/kg. Injections were performed with a 1-ml syringe attached to 0.8 × 40 mm (for carp) or 0.4 × 13 mm (for goldfish) needle. Blood samples (100–150 μl for goldfish or 500–800 μl for carp) were taken from the caudal

vasculature with heparinized 1-ml syringes. After centrifugation the resulting plasma was stored at –20°C until analysis.

Experimental protocols

Experiments performed in 2003

Experiments 1, 2, and 3 were conducted on goldfish females in May and June 2003. In each experiment sGnRHa was injected intraperitoneally at a dose of 10 μg/kg (time 0) and there were 3 control groups: saline, sGnRHa, and sGnRHa + PIM (5 mg/kg) treated fish. Each group consisted of 10 fishes.

Exp. 1. Three experimental groups which received sGnRHa (time 0) were pretreated (single injection) with different doses of fadrozole (5, 50 or 500 μg/kg) at 72 hours before the injection of sGnRHa. Blood samples were taken at –72, 0, 6, 12 and 24 h after sGnRHa injection.

Exp. 2. Three experimental groups which received sGnRHa were injected with fadrozole at a dose of 50 μg/kg (dose per one injection) at different times before sGnRHa application. The first group received 2 injections of fadrozole (24 h before and at the time of sGnRHa application). The second group received three injections (at –48, –24 and at time 0). The third group received four injections of fadrozole (at –72, –48, –24 and at time 0). The same protocol for blood sampling was applied as in exp. 1.

Exp. 3. Two experimental groups received four injections of fadrozole at a dose of 1 or 10 mg/kg (dose of single injection) given at 24-h intervals before the application of sGnRHa. Blood samples were taken at 0, 12 and 24 h after sGnRHa injection.

Experiments performed in 2004

Experiments 4, 5, 6 were performed on goldfish females (10 fishes per group) in May and June 2004. They were identical (repetition). In each experiment there were 3 control groups: saline, sGnRHa alone and sGnRHa + PIM treated fish. Two doses of sGnRHa (10 and 50 μg/kg) were applied at time "0". Experimental groups received four injections of 50 μg/kg fadrozole in 24-h intervals before sGnRHa application.

Experiments performed in 2005

Experiments 7, 8, 9 were performed on mature common carp females in May and June 2005. The design of experiments, experimental groups and sampling protocol are presented below.

Exp. 7 consisted of 5 groups of carp (10 fishes per group):

Group 1. Control – intraperitoneal injection of sGnRHa (10 μg/kg) at time 0 h.

Group 2. Fadrozole 4 × 50 μg/kg (–72, –48, –24 and 0 h) + sGnRHa at time 0 h.

Group 3. Fadrozole 4 × 100 μg/kg (–72, –48, –24 and 0 h) + sGnRHa at time 0 h.

Group 4. Fadrozole 4 × 200 μg/kg (–72, –48, –24 and 0 h) + sGnRHa at time 0 h.

Group 5. sGnRHa + PIM (5mg/kg) at time 0 h.

In experiment 8, three groups of fish were used (10 fishes per group).

Group 1. Control, intraperitoneal injection of sGnRHa (10 μg/kg) at time 0 h.

Group 2. Fadrozole 4 × 50 μg/kg (–72, –48, –24 and 0 h) + sGnRHa at time 0 h.

Group 3. Fadrozole 7 × 50 μg/kg (–144 h till 0 h in 24-h intervals) + sGnRHa at time 0 h.

The last experiment (no. 9) was performed in a commercial hatchery of Zator Fish Farm and consisted of 3 groups (8 fishes per group):

Group 1. Control, intraperitoneal injection of Gonazon™ at time 0 h.

Group 2. Fadrozole 4 × 50 μg/kg (–72, –48, –24 and 0 h) + Gonazon™ at time 0 h.

Group 3. Gonazon™ + PIM (5 mg/kg) as positive control.

Blood samples in experiments 7–9 were taken at 0, 12 and 24 h after sGnRHa or Gonazon™ injection.

Hormone level analysis

Blood plasma samples were assayed for LH and 17β-estradiol by ELISA. The analysis of LH was done according to the protocol developed by Kah et al. (1989). The measurement of estradiol in blood plasma was performed after extraction in the mixture of cyclohexane and ethyl acetate (1:1) according to the method described by Szczerbik et al. (2007). In order to analyse estradiol levels in the hypothalami (experiments 3, 5, 6) whole brains were sampled from fish after decapitation. Each brain was placed on an ice-cold plateau and with the use of Weckert's scissors the hypothalamus was cut off the remaining parts of the brain. Cuts were done behind the telencephalon and behind the lobi inferiores of the ventral brain. The optic

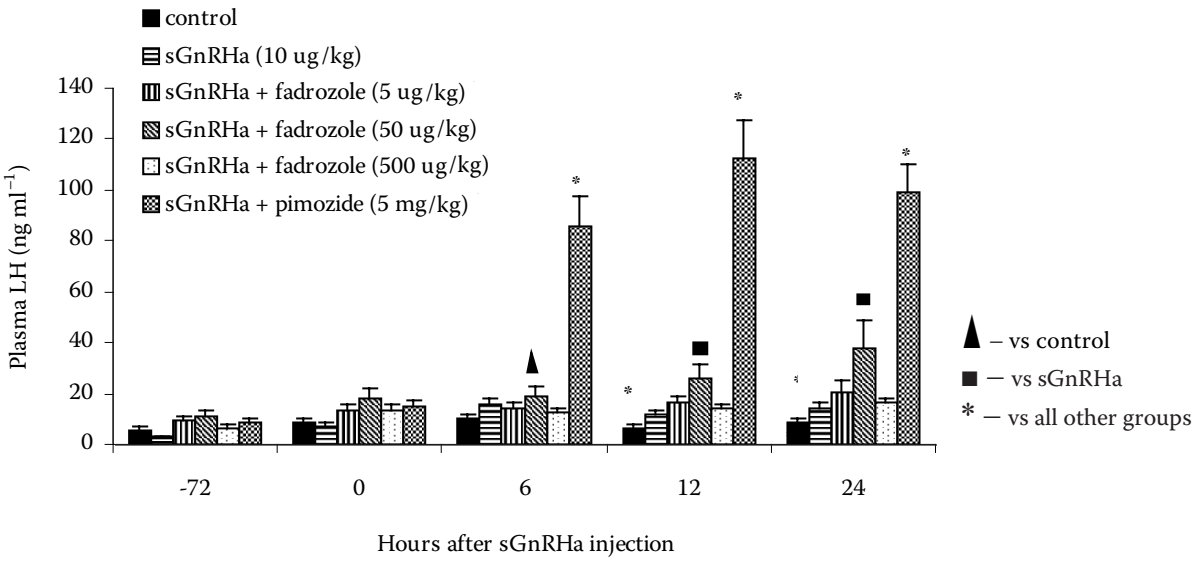

Figure 1. The effects of different doses of fadrozole (5, 50 or 500 μg/kg) applied in the form of single injection at 72 h before the injection of sGnRHa on plasma LH levels in female goldfish. Bars represent mean LH levels ± SE

tectum of the mesencephalon was also removed. The hypothalami were frozen immediately at –20°C until analysis. After thawing the brain tissue was homogenised in PBST buffer and subjected to the extraction as described above.

Statistics

The significance of differences in plasma LH and E2 concentrations between groups was calculated using nonparametric Mann-Whitney *U*-Test at the $P \leq 0.05$ level of significance. The significance of differences in ovulation rates between groups was calculated using *Z*-test at the $P \leq 0.05$ level of significance.

RESULTS

Experiments on goldfish in 2003

Exp. 1. Single injection of fadrozole at a dose of 50 μg/kg at time –72 h had a slight but significantly stimulatory effect on spontaneous LH secretion (18.3 ng/ml) in comparison with the control group (8.8 ng/ml) as measured at time 0 h (Figure 1). Other doses of fadrozole (5 or 500 μg/kg) had no effects on LH secretion. The injection of sGnRHa at time 0 h evoked a moderate (14–16 ng/ml) but significant increase in LH secretion when compared to the control group (6–9.5 ng/ml) at all post-injection sampling times. In the group treated with 50 μg/kg of fadrozole + sGnRHa there were significantly higher LH levels (26.5 to 38 ng/ml) in 12 and 24 hours after injection than in fish treated with sGnRHa only. The other doses of fadrozole had no effect on sGnRHa action. The highest LH levels (ranging from 85 to 112 ng/ml) were measured in the group to which the classical treatment (sGnRHa + pimozide) was applied. These levels were significantly higher than in all other groups at all post-injection times (Figure 1). Only in this group 80% of females ovulated. The analysis of 17β-estradiol concentrations in blood plasma showed no differences between treatment groups (data not shown).

Exp. 2. In this experiment only one dose of fadrozole (50 μg/kg) was used but at different treatment times and periods. The analysis of plasma LH concentrations showed that the single injection of fadrozole at time –72 h + sGnRH at time 0 h, similarly like in exp. 1, significantly increased LH secretion (19 ng/ml) in comparison with sGnRHa alone (12–13 ng/ml) in 12 and 24 hours post injection (Figure 2). Four injections of fadrozole (starting at –72 h) had a more pronounced effect on sGnRHa action. LH levels in this group reached half (48 ng/ml) levels in the sGnRHa + pimozide treated group (99.8 ng/ml). Triple application of fadrozole (started at –48 h) had no significant influence on sGnRHa stimulated LH secretion. There was no effect of fadrozole on spontaneous secretion of LH, either. The highest LH levels as well as the ovulation of 80% of females were

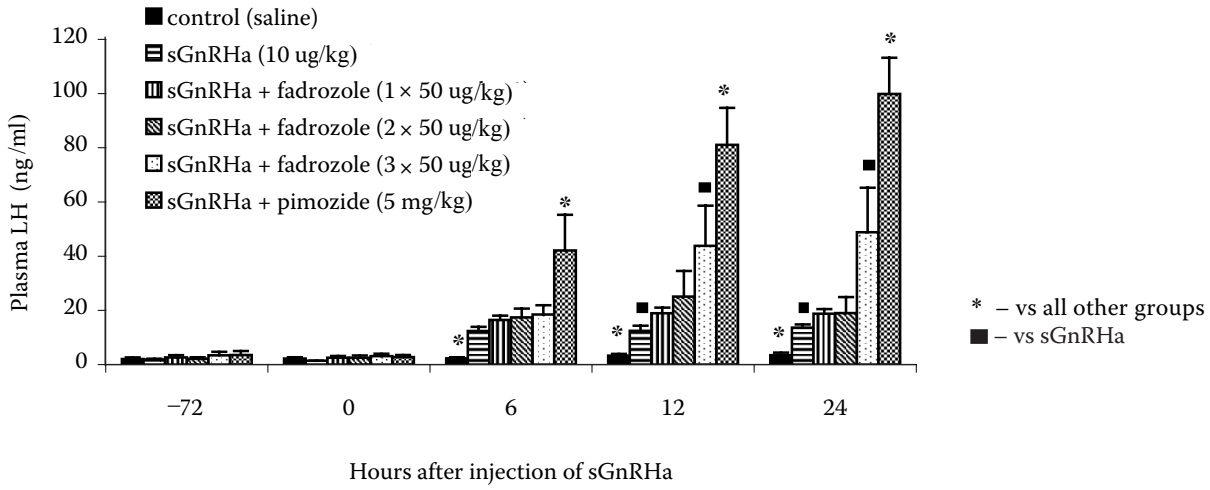

Figure 2. The effects of single, double or triple injection of fadrozole at a dose of 50 μg/kg given in 24-h intervals on sGnRHa stimulated LH secretion in female goldfish. Bars represent mean LH levels ± SE

observed in the group treated with sGnRHa + PIM. There were no significant differences in plasma 17β-estradiol levels between treatment groups (data not shown).

Exp. 3. The application of 4 injections of fadrozole at a dose of 1 mg/kg (starting from –72 h) significantly increased sGnRHa stimulated LH secretion (22.5 and 35.5 ng/ml) in comparison with the group treated with sGnRHa only (6.1 and 7.5 ng/ml) in 12 and 24 h after sGnRHa injection, respectively (Figure 3). There was also a slight stimulatory effect of this dose on spontaneous LH secretion (time 0). Fadrozole at a dose of 10 mg/kg had no effect on sGnRHa action. The highest LH level (84.2 ng/ml) as well as the highest percentage of ovulating fish (70%) were detected in the sGnRHa + pimozide treated group. There were no significant differences in concentrations of 17β-estradiol in the blood plasma and hypothalami between treatment groups (data not shown).

Experiments on goldfish in 2004

Experiments 4, 5 and 6 were identical. Each next one was performed as soon as the results from a preceding experiment were known. Surprisingly,

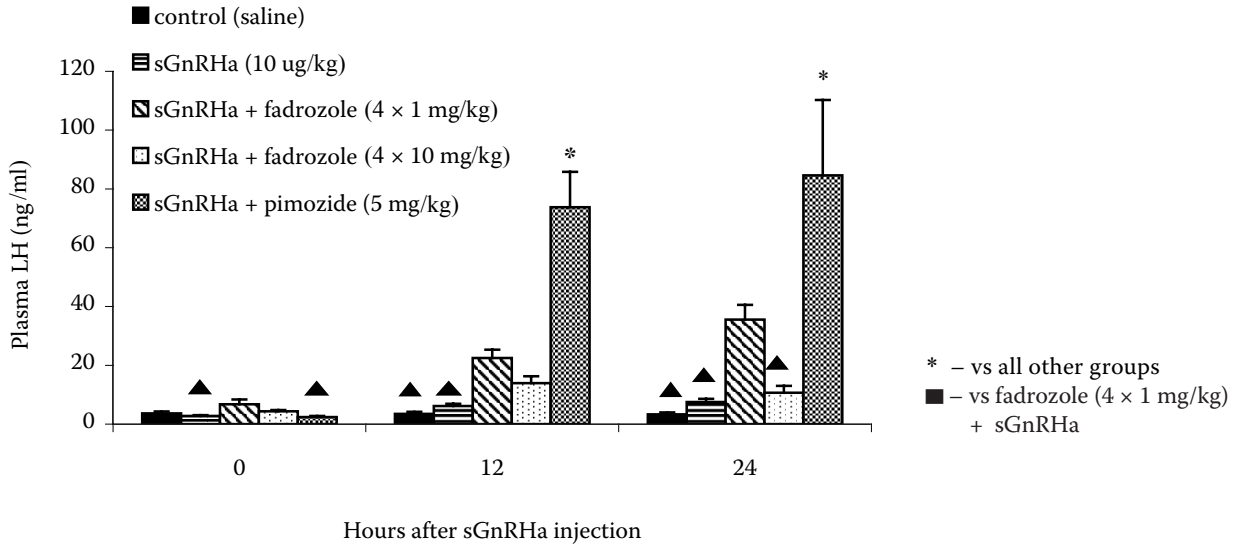

Figure 3. The effects of four injections of different doses of fadrozole (1 or 10 mg/kg) applied in 24-h intervals starting at 72 hours before sGnRHa application on plasma LH secretion in female goldfish. Bars represent mean LH levels ± SE

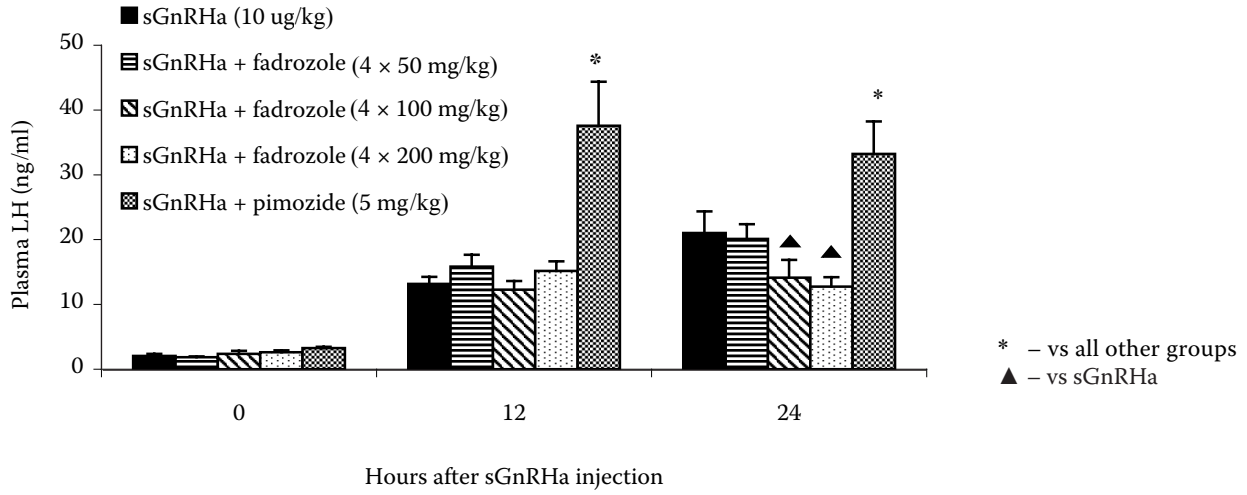

Figure 4. The effect of four injections of different doses of fadrozole (50, 100 and 200 μg/kg) applied in 24-h intervals starting at 72 hours before sGnRHa application on plasma LH secretion in female carp. Bars represent mean LH levels ± SE

in all three experiments there was no effect of fadrozole (4 × 50 μg/kg) either on spontaneous or on sGnRHa stimulated LH secretion. Thus it was impossible to repeat and confirm the results from 2003. There were no differences in 17β-estradiol levels in the blood plasma and hypothalami between the respective groups, either (data not shown).

Experiments on common carp in 2005

Exp. 7. Fadrozole treatment, regardless of the dose applied (4 × 50, 100 or 200 μg/kg), had no effect on spontaneous LH secretion in common carp (Figure 4). In 12 h after injection there were no differences in LH secretion between the sGnRHa treated group and that exposed to fadrozole treatment + sGnRHa. In 24 h after sGnRHa injection the LH levels in groups treated with 100 or 200 μg/kg of fadrozole were significantly lower (14.1 and 12.7 ng/ml, respectively) when compared with the control group treated with sGnRHa alone (21.0 ng/ml). Fadrozole at a dose of 50 μg/kg had no effect on sGnRHa stimulated LH secretion. The highest average level of LH (37.6 ng/ml) and ovulation rate (50% of females) were detected in the group treated with sGnRHa + PIM.

In exp. 8 despite the prolonged (up to 6 days before sGnRHa injection) exposition to fadrozole (50 μg/kg per injection) there were no changes

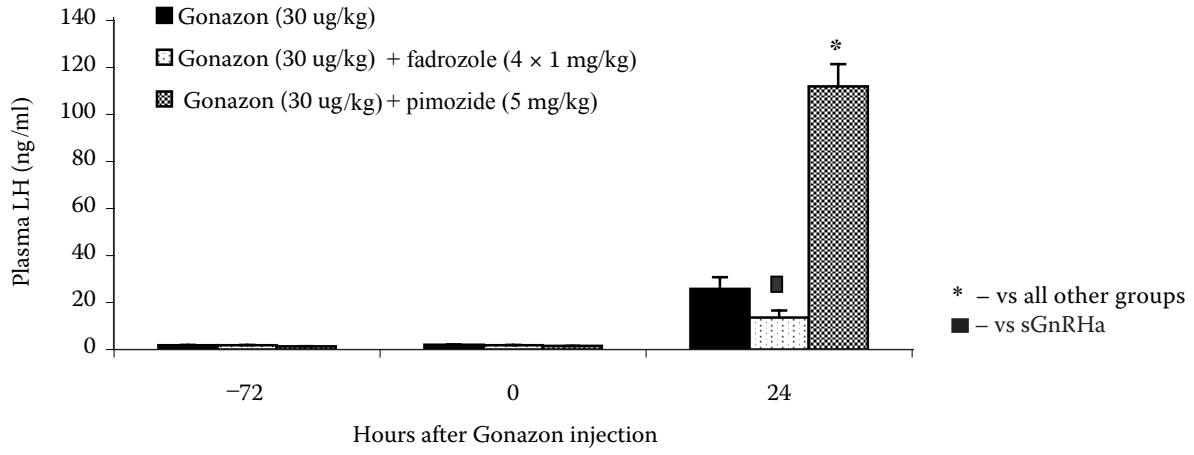

Figure 5. The effect of four injections of fadrozole at a dose of 1 mg/kg applied in 24-h intervals starting at 72 hours before gonazon application on LH secretion in female spawners in the commercial hatchery conditions. Bars represent mean LH levels ± SE

either in spontaneous or in sGnRHa stimulated LH secretion. There were no significant changes in 17β-estradiol levels in the blood plasma between treatment groups, either (data not shown).

In the last experiment (no. 9) performed on common carp broodstock in the commercial hatchery, instead of sGnRHa a commercially available product GONAZON^{TM} was used. In contrast to all other investigations done so far (except exp. 7), in this experiment the fadrozole treatment (4 × 1 mg/kg) evoked a significant inhibition of gonazon stimulated LH secretion (13.6 ng/ml) in comparison with the control group treated with gonazon alone (25.8 ng/ml) in 24 hours after gonazon injection (Figure 5). There were no differences in spontaneous LH secretion and plasma 17β-estradiol levels between treatment groups (data not shown).

DISCUSSION

Cytochrome P450 aromatases A and B are the key enzymes in steroidogenesis in fish enabling the conversion of C19 androgens into C18 estrogens (for review see Pellegrini et al., 2005). However, a dominant role is played by aromatase B (Chiang et al., 2001; Valle et al., 2002). Its sites of action are mainly gonads and brain but its presence has also been detected in the pituitary, adipose tissue and liver of fish (Callard et al., 1978; Andersson et al., 1988). It was also shown that the activity of brain aromatase in fish is about 100 to 1 000 times higher than in mammals and birds (Borg et al., 1989; Gonzales and Piferrer, 2003). Estrogens can be produced in the brain of fish of both sexes (Afonso et al., 2000). Since there is no aromatase in the testes of males (Callard et al., 1978; Depeche and Sire, 1982), brain is the main source of estrogens in the blood circulation in males (Timmers, 1988).

There are some data indicating the use of aromatase inhibitors in the modulation of reproductive cycle. Afonso et al. (1999, 2000) showed the acceleration of maturation and spawning in Coho salmon with the use of fadrozole. In black porgy aromatase inhibitors increased LH secretion (Lee et al., 2001) but it also blocked the natural sex change (Lee et al., 2002). On the contrary, in tilapia it was possible to achieve sex reversal after treatment with aromatase inhibitors (Afonso et al., 2001). The activity of brain aromatase changes significantly along the reproductive cycle, being associated with changes in the metabolism of brain catecholamines. It was shown that the application of estradiol to fish evoked an increased activity of pituitary neurones producing dopamine (Linard et al., 1995, 1996; Saligaut et al., 1998). Moreover, there is immunohistochemical evidence that the dopamine containing neurons reaching the pituitary gland possess estrogenic receptors (Linard et al., 1996). In the last stages of ovarian maturation, just before ovulation, the rapid estradiol level drop is followed by the diminution of dopamine production. Thanks to this phenomenon there is a potentiation of GnRH action and appearance of LH ovulatory surge and ovulation as observed in coho salmon (Fitzpatrick et al., 1986) or rainbow trout (Saligaut et al., 1999).

Data presented in this paper, obtained in 2003 on goldfish (exp. 1, 2, 3), support and confirm the results obtained by others authors on different fish species. The application of fadrozole at different doses (50 μg/kg or 1 mg/kg) three days before the injection of sGnRHa significantly increased LH secretion stimulated by sGnRHa (Figure 1). The effect was more pronounced if fadrozole treatment was applied every 24 h in comparison with single administration (Fi-gure 2). However, the stimulatory action of fadrozole was much weaker than that of dopamine antagonist – pimozide (PIM). LH levels in PIM + sGnRHa treated groups reached easily 100 ng/ml, while in fadrozole + sGnRHa treated fish the LH levels were twice or three times lower. Only in PIM + sGnRHa groups ovulation was observed. This moderate stimulatory effect of fadrozole was obtained without visible changes in plasma 17β-estradiol. Unfortunately, in these experiments there was no measurement of 17β-estradiol in the brains of fish, which could have helped to explain this phenomenon.

Based on the results of the first year of the project a new set of experiments was planned and performed on goldfish in 2004. Surprisingly, it was impossible to repeat and confirm the results of the preceding experiments. In four consecutive experiments there was no stimulation of either spontaneous or sGnRHa stimulated LH release. There were no significant changes in a 17β-estradiol concentration in the plasma and hypothalami. Because the main goal of the project was to change and/or modify the procedure of induced ovulation in common carp, it was decided to repeat the experiments on this species. In experiment 8 there were no effects of fadrozole (50 μg/kg) administered for 3 or 7 days on spontaneous and sGnRHa stimulated LH secretion. There were no changes in the plasma 17β-estradiol concentration, either. Surprisingly, in two other experiments

(no. 7 and 9) a significant inhibition of the stimulatory action of sGnRHa and gonazon by 100 and 200 μg/kg (Figure 4) and 50 μg/kg of fadrozole (Figure 5) was observed. This phenomenon was associated with the absence of changes in 17β-estradiol concentrations in the blood plasma and hypothalami between the respective groups. All these observations draw a confusing picture of the role and the effects of aromatase inhibitors in the processes involved in the regulation of LH secretion in cyprinids. It seems that the effects of fadrozole are highly dependent on the actual hormonal status of fish, state of gonadal development and origin of fish. At the moment, it is impossible to explain why, in some cases, fadrozole stimulated LH secretion (despite of the absence of changes in 17β-estradiol concentration) while in other experiments despite of changes observed in 17β-estradiol levels, there was no influence on LH secretion. In our opinion the only explanation of the observed phenomenon is a too short time of the exposition of fish to fadrozole. In most cases this time was limited to 3 days before sGnRHa or gonazon injection, while other authors applied much longer (several weeks or months) periods of aromatase inhibitor treatment (Anatonopoulou et al., 1995; Afonso et al., 1999, 2000; Lee et al., 2001). Such a protocol would be difficult to accept by carp farmers and in carp hatcheries. The routine protocol of ovulation induction in carp involves a very short time (2 to 3 days) of the presence of broodstock in the hatchery. A longer exposition of fish to the hostile environment of hatchery and to a constant spawning-inducing temperature causes poor spawning results (Epler et al., 1989) and significantly increases the costs (water, energy, personnel work etc.).

In conclusion, it has to be stated that because of striking differences between the results obtained so far the treatment with an aromatase inhibitor – fadrozole in cyprinids cannot be recommended as a potential tool for spawning induction.

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