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Peter Podhorec, Magdalena Socha, Imen Ben Ammar, Mirosława Sokolowska-Mikolajczyk, Elzbieta Brzuska, et al.. The effects of GnRHa with and without dopamine antagonist on reproductive hormone levels and ovum viability in tench *Tinca tinca*. *Aquaculture*, 2016, 465, pp.158-163. 10.1016/j.aquaculture.2016.09.012 . hal-02994636

HAL Id: hal-02994636

<https://hal.univ-lorraine.fr/hal-02994636>

Submitted on 29 Aug 2023

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The effects of GnRHa with and without dopamine antagonist on reproductive hormone levels and ovum viability in tench *Tinca tinca*

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Published in Aquaculture. Available online:
<http://dx.doi.org/10.1016/j.aquaculture.2016.09.012>

Keywords

Tench, Aquaculture, Reproduction, GnRHa, Metoclopramide.

Abstract

The study evaluated the impact of mGnRHa ([D-Tle6, Pro9, NEt]-mGnRH), with or without metoclopramide, on release of luteinizing hormone (LH) and sex steroids and on viability of ova in tench *Tinca tinca*. Four experimental groups were subjected to the following treatments given as intraperitoneal injection: 0.9% NaCl (C); 25 1g kg⁻¹ mGnRHa (Gn); 251g kg⁻¹ mGnRHa with 20 mg kg⁻¹ metoclopramide (GnM); and or 20 mg kg⁻¹ metoclopramide (M). GnM treatment induced LH surge reaching maximum concentrations 6 h post-treatment followed by a slight but significant decline ($P < 0.05$). Gn group showed gradually increasing LH values ($P < 0.05$). No significant differences were found between GnM and Gn treated groups in 17 β -estradiol (E2) or testosterone blood level. Significant decrease from baseline ($P < 0.05$) was found in E2 values at the end of trial period in both Gn and GnM. Testosterone values increased with Gn and the GnM, peaking 12 h post-treatment and dropping to baseline levels at 24 h. GnM was associated with significant increase of 17 α , 20 β -dihydroxy-4-pregnen-3-one (17,20 β -P) values at 6 h ($P < 0.05$), with its concentration peaking at 12 h similar to that in Gn. Both treatments induced ovulation in a high proportion of females, while M and C group showed no ovulation. No significant differences were found in ovulation rate, latency, ovum weight, or relative fecundity. Nevertheless, significant differences ($P < 0.05$) between Gn and GnM treatment were observed in fertility (77% vs. 54%) and hatching (40% vs. 23%). GnM stimulated rapid LH release with a potentiating effect on 17,20 β -P secretion, possibly the source of the adverse effects on ovum viability. We describe for the first time the negative impact of metoclopramide combined with mGnRHa hormone treatment on ovum quality in tench.

Statement of relevance: Improvement of fish artificial reproduction.

1. Introduction

Cyprinid rearing is the dominant segment of freshwater aquaculture worldwide. Tench (*Tinca tinca*) is an important cultured cyprinid highly rated for flesh quality and relatively undemanding rearing conditions. Its intensive culture is dependent on supply of fry from natural reproduction in spawning ponds (Morawska, 1984) or, preferably, through artificial reproduction of captured broodstock (Kujawa et al., 2011).

The hypothalamo-pituitary-gonad axis plays vital roles in fish reproduction -among other processes it controls gonadotropin and steroid hormone production (Somoza et al., 2002). Gonadotropin-releasing hormone (GnRH) synthesized and secreted from neurons of the hypothalamus is the main stimulator of luteinizing hormone (LH) synthesis and secretion from the pituitary (Zohar et al., 2010). Secreted LH induces synthesis and release of the 17 α , 20 β -dihydroxy-4-pregnen-3-one (17,20 β -P) from the ovary with subsequent final oocyte maturation (FOM) and ovulation (Nagahama and Yamashita, 2008). The hypothalamic neurotransmitter dopamine (DA) inhibits GnRH stimulation (Peter et al., 1986). Dopaminergic inhibition of basal and GnRH-induced LH release is especially prominent in cyprinids, peaking at the end of vitellogenesis (Peter and Yu, 1997).

Tench females do not undergo final oocyte maturation and ovulation in captivity (Kouril et al., 2008). Artificial conditions in

fish hatcheries result in reduction or inhibition of LH secretion that regulates final stages of gametogenesis (Zohar and Mylonas, 2001). For cyprinids, a treatment combining synthetic GnRH analogue (GnRHa) with DA antagonists generally leads to a LH surge and ovulation (Podhorec and Kouril, 2009). However, in tench despite confirmed DA inhibition of LH secretion (Podhorec et al., 2012a), FOM and ovulation can be achieved with administration of GnRHa without removing of dopaminergic inhibition by DA antagonists (Kouril et al., 1986; Podhorec et al., 2011). The combined and GnRHa-alone treatments are both associated with high ovulation rates, but GnRHa alone leads to a more gradual LH release, while an acute LH surge is observed following the combined treatment (Podhorec et al., 2011, 2012a, 2012b). This LH release pattern which characterizes the combined treatment raises questions about the possible impact of LH release profile on subsequent FOM and ovulatory processes which may, consequently, affect gamete quality. To the best of our knowledge no study has addressed so far on this issue in tench or other Cypriniformes.

The objectives of the present in vivo study were to assess the impact of GnRH analogue treatment, with or without DA inhibitor, on LH and sex steroid level, as well as on ovulation. Hormone changes were linked with the impact of used treatments on the production of viable ova.

2. Material and Methods

2.1. Broodstock and treatments

Sexually mature female tench of 1053 \pm 46 g (mean \pm SEM) body weight (BW) were harvested from 1.5 ha ponds 1.5 m deep in southern Poland in spring when daily mean water temperature had reached 18– 20 °C. Fish were transferred to the research facility of the Institute of Ichthyobiology and Aquaculture in Gołysz, Poland. Preselection of females was based on external characteristic -distended and soft abdomen, which, during the natural spawning period, constitutes a reliable method of determining spawning readiness and by the evaluation of oocyte stage development – eccentric position of germinal vesicle (Podhorec et al., 2011). Selected fish were randomly divided into four experimental groups (n = 10) with each group placed in a 3000 L flow-through tank supplied with pre-warmed pond water. After two days of acclimatization and gradual temperature increase, the water conditions were as follows: temperature 24.0 \pm 0.3 °C, natural photoperiod (16 h:8 h light:dark), and dissolved oxygen 7.0 \pm 0.6 mg L⁻¹. After a two-day acclimatization period, fish received a single intraperitoneal injection of either 0.9% NaCl, Braun Melsungen AG, Germany (C); 25 Eig kg⁻¹ [D-Tle6, Pro9, NET]-mGnRH, NORDIC Pharma s.r.o., Czech Republic, (Gn); 25 Eig kg⁻¹ mGnRHa combined with 20 mg kg⁻¹ metoclopramide, Sigma–Aldrich, USA, (GnM); or 20 mg kg⁻¹ metoclopramide (M), (Drori et al., 1994). To increase the quantity and quality of sperm used for fertilization, males were treated with a single intraperitoneal injection of 25 Eig kg⁻¹ mGnRHa alone. Prior to manipulation, fish were anesthetized in a solution of Propiscin (Inland Fisheries Institute, Poland) at 0.5 mL L⁻¹.

A heparinized 21-gauge needle with 1 mL syringe was used to collect serial blood samples (1000 EIL) by caudal venipuncture prior to injection (0 h) and 6,12, and 24 h post-injection (PI). Blood samples were centrifuged at 4000 \times g for 10 min at 8 °C, and plasma was stored at –80 °C until analysis.

Females were checked for ovulation every 2 h as from 24 h post injection (PI) until 36 h PI. Fish releasing ova in response to gentle abdominal pressure were immediately stripped into a dry bowl, ova were weighed to the nearest gram and fertilized with heterosperm collected from at least 4 males into an immobilizing solution Kurokura 180 prepared according to Rodina et al. (2004). After elimination of stickiness by enzyme alcalase (Sigma–Aldrich, USA) (Linhart et al., 2003), a sample of ova from each female were incubated with milt (0.5 mL) in a Petri dish in water at 23.2 \pm 0.5 °C, in triplicate. Optimal conditions for incubation were maintained by an 80% water change every 12 h. Fertilization and hatching rate were determined under a stereoscope at 24 and 72 h PI, respectively.

The following parameters were recorded: ovulation rate (number of ovulated females within 36 h), latent period (time from injection to ovulation), relative fecundity index ([weight of stripped ova/pre- stripping BW] \times 100), fertility index ([fertilized ova/total number of ova] \times 100), and hatching rate ([hatched ova/fertilized ova] \times 100).

The experiment was conducted in accordance to the principles of the Ethical Committee for the Protection of Research

Animals at the University of South Bohemia and the Polish Academy of Sciences.

CV intra-assay varied from 2.3 to 3.0% and CV inter-assays from 2.4 to 4.7%.

2.2. Hormone 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 a parallel displacement curve of serially diluted tench plasma with the standard common carp curve. Sensitivity of the performed assay was at the range 0.6–200 ng mL⁻¹ with the intra- and inter-assay coefficients of variance (CV) at 5% and 9% for low and high levels, respectively.

Plasma 17 α , 20 β -dihydroxy-4-pregnen-3-one was extracted with dichloromethane and quantified according to the method described by Szczerbik et al. (2008). CV intra-assay was 7.9% and CV inter-assays was 8.7%.

17 β -Estradiol (E2, ng mL⁻¹) was assayed in 50 μ L of blood plasma using the DIALsource E2 ELISA kit (DIA-source, KAP0621). When necessary, plasma samples were diluted 1:3–1:5. The detection limit of the assay was 5 \pm 2 pg mL⁻¹,

Testosterone (T, ng mL⁻¹) was assayed in 25 μ L of blood plasma using the DIALsource Testosterone ELISA Kit (DIALsource, KAPD1559). When necessary, plasma samples were diluted 1:2 to 1:4. The detection limit of the assay was 0.083 ng mL⁻¹, CV intra-assay varied from 1.5 to 9.5%, and CV inter-assays varied from 7.6 to 13.9%.

2.3. Statistical analysis

Statistical analyses were performed using the STATISTICA software (StatSoft Inc., Tulsa, OK, USA). Data are presented as a mean \pm standard error of the mean (SEM). Normality and homogeneity of variances were tested by the Kolmogorov–Smirnov and Bartlett methods, in order to comply with the prerequisites of analysis of variance (ANOVA). When necessary, data were log-transformed. Within-group differences in LH and sex steroid levels were analyzed by a repeated measures ANOVA, and between-group differences with one-way ANOVA at each sampling time. To compare individual differences between groups, Tukey's HSD test and Fisher's LSD test were applied. Ovulation rates were compared with χ^2 analysis. Fertility and hatching data after arcsine transformation, latent period, and fecundity data were compared by Student's t-test. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Luteinizing hormone (LH)

All experimental treatments elicited significantly higher LH plasma concentrations than found in controls at one or more sampling times ($P < 0.05$). Significant differences among all groups were found at 6 h PI, with the highest mean LH levels observed with GnM treatment ($P < 0.05$). At 12 h, GnM group reached significantly ($P < 0.05$) higher levels than Gn group and both groups differ from M and C group ($P < 0.05$). At 24 h, similar LH levels were observed in Gn and GnM group, with both significantly higher ($P < 0.05$) than the other groups (Fig. 1).

The GnM treatment induced an early LH surge, reaching maximum concentrations at 6 h PI, followed by a slight but significant decline from the peak ($P < 0.05$). In contrast, the Gn treatment resulted in gradually increasing LH values, peaking at 24 h ($P < 0.05$). The administration of metoclopramide evoked a modest increase in circulating LH levels, reaching significantly higher levels than C group only at 6 h PI ($P < 0.05$) and returning to the baseline levels after 24 h (Fig. 1).

3.2. 17 β -estradiol

No differences among groups were found in E2 level, with the exception of M and Gn at 24 h PI ($P < 0.05$), with higher levels in the M group. Levels of E2 significantly declined ($P < 0.05$) over the experimental period in the Gn, GnM and C groups (Fig. 2).

3.3. Testosterone

The GnM and Gn treatments led to significantly higher T concentrations at 12 h PI ($P < 0.05$) than in baseline measures and control. At 12 h PI, a significant difference was found between the Gn and M groups, but not between GnM and M group. At 24 h, a significant decline compared to 12 h in T values was observed in the Gn group. Fluctuations of T levels were observed only in the C group, with a slight decline at 6 and 12 h from pre-treatment levels (Fig. 3).

3.4. 17 α , 20 β -dihydroxy-4-pregnen-3-one

The GnM was associated with significantly greater 17,20 β -P values 6 h PI than in other groups ($P < 0.05$). At 12 h, a surge in 17,20 β -P was observed in plasma concentrations in the GnM and Gn groups, but not in the M and C groups ($P < 0.05$). No difference in 17,20 β -P levels was found among the GnM treatment and Gn groups at 12 h. A significant reduction in 17,20 β -P values was observed at 24 h compared to the previous sampling points in the GnM and Gn groups ($P < 0.05$), slightly more pronounced in the GnM treatment ($P < 0.05$). Non-significant fluctuations in 17,20 β -P level throughout the trial were found in the M group compared to C. Nevertheless, significant induction of 17,20 β -P output by M was observed when comparing levels at 12 h with the baseline (Fig. 4).

3.5. Reproductive performance

A high proportion of females receiving GnM and Gn treatment ovulated, while no ovulation was seen in M and C groups. No significant differences were found between GnM and Gn group in ovulation rate, latency period and relative fecundity.

Differences were detected between GnM and Gn groups in fertility index and hatching rate, with the Gn group performing significantly better ($P < 0.05$) (Table 1).

4. Discussion

In this study, we showed for the first time the negative impact of metoclopramide added to mGnRHa treatment on the egg quality of tench: GnM treatment, in contrast to Gn, stimulated rapid LH release with a potentiating effect on $17,20\beta$ -P secretion, linked with adverse effects on ova viability.

The ovulation-inducing effect of Gn observed in the current study is the confirmation of previous studies of tench (Podhorec et al., 2011, 2012a, 2012b). This is atypical for Cyprinidae, a group with fish species that clearly demonstrate DA regulation of LH secretion (Podhorec et al., 2012a). The ability of GnRHa alone to induce ovulation in tench is similar to several marine species that lack dopaminergic regulation of LH release (Barbaro et al., 1997; Copeland and Thomas, 1989; Prat et al., 2001). However, it should be noted that in these species the effects of DA antagonist addition to GnRHa treatment are more suppressive than inductive with respect to LH release and ovulation. In tench, DA inhibition of pre-ovulatory LH release appears to be just a modest regulator of extended LH release rather than a robust inhibitor as in other cyprinids. Consistent with this statement, our current and earlier results (Podhorec et al., 2011, 2012a, 2012b) showed a significantly different effect of GnM and Gn on LH level dynamics. GnM stimulated a precocious and persistent LH surge, in contrast to the gradual rise in LH induced by Gn treatment alone. Billard et al. (1984) suggested that high pre-ovulatory LH levels induced by DA inhibitor plus GnRHa could have a deleterious effect on ova quality in rainbow trout. Similar negative impact of DA inhibitor with GnRHa on ova survival has been observed in arctic charr, *Salvelinus alpinus* (Gillet et al., 1996), emphasizing negative correlation between LH levels or kinetics of LH release and ova survival. It is assumed that in the present study, GnM treatment suppressed DA influence on pre-ovulatory LH release, leading to an early massive LH surge. Luteinizing hormone levels at 6 h PI were twice as high as those with Gn and were later associated with a significant decrease in fertility and hatching rates in comparison to Gn. Assessment of whether the deleterious impact on ova results from excessively high LH levels or from a direct negative effect of metoclopramide is difficult, due to the inability of metoclopramide alone to induce any ovulation. However, in crucian carp (*Carassius carassius*), metoclopramide alone produced a similar or higher ovulation and hatching rates than when combined with mGnRHa (Cejko and Kucharczyk, 2015). Higher survival rates of arctic charr ova after treatment with DA inhibitor alone compared to GnRHa alone (Gillet et al., 1996) imply that metoclopramide per se has no harmful effect on arctic charr ova. In the current study no negative effect was observed on ovulation rate or relative fecundity in either GnM or the Gn group. In certain fish species, overstimulation by hormone treatment has been shown to result in reduction of fecundity and ova quality (Barbaro et al., 1997; Ibarra-Castro and Duncan, 2007; Taranger et al., 1992). In the meagre *Argyrosomus regius* at higher than the optimal GnRHa doses, latency time was shorter and oocyte maturation was accelerated resulting in lower ova quality and relative fecundity (Fernandez-Palacios et al., 2014). In tench we did not observe differences in latency time among treatments even when the precocious rise of

$17,20\beta$ -P in GnM group suggests acceleration of FOM resulting in lower reproductive performances. Fertility indices and hatching rates following Gn treatment found in the present study were similar to, or lower than, values reported in tench by other authors (Linhart et al., 2006; Rodina et al., 2004; Rodriguez et al., 2008), a phenomenon that could be due to different quality of the broodstock or a different protocol of ova stickiness elimination. In contrast to our results, there are reports on good reproductive performance in tench following treatment with GnRHa plus DA inhibitor (Kujawa et al., 2011; Targonska et al., 2012), but without comparisons to a parallel treatment with GnRHa alone it is difficult to assess this results.

No differences were found between GnM and Gn treatment on E2 and T level. Secretion patterns for these steroids correspond to the expected shift in the biosynthetic pathway from the production of mainly C19 and C18 to C21 steroids (Nagahama, 1994). Significant decrease in E2 values from baseline at the end of the trial period was observed for both GnM and Gn treatments. A similar E2 level pattern was found in common carp *Cyprinus carpio* (Levavi - Zermosky and Yaron, 1986), reflecting a shift in the steroidogenic enzymes from the prevalence of P450 aromatase to 20β hydroxysteroid dehydrogenase, resulting in the production of the maturation inducing steroid, 17α , 20β -dihydroxy-4-pregnen-3-one ($17,20\beta$ -P), (Nagahama and Yamashita, 2008). No decrease in E2 values was detected in the M group, suggesting that metoclopramide alone does not induce an LH surge sufficient to stimulate any effect by inhibition of aromatase activity as probably occurred following GnRHa treatment. In GnM and Gn treatments, T values increased, peaking 12 h PI and dropping to the baseline levels at 24 h PI. A similar T secretion pattern after Gn treatment was reported in a trial with more frequent blood sampling (Pinillos et al., 2002). The T pattern changes were probably due to a shift in the steroidogenic pathway leading to $17,20\beta$ -P production from precursor 17α -hydroxyprogesterone. This is in line with the peak of $17,20\beta$ -P, 6 h later than the peak of T (Pinillos et al., 2002). 17α , 20β -dihydroxy-4-pregnen-3-one is one of the two C21 steroids detected in tench plasma around the time of ovulation (Pinillos et al., 2002) and is reported to be released into the water at a high rate following GnRHa treatment (Antonopoulou et al., 2011). The potential of $17,20\beta$ -P to induce final oocyte maturation was demonstrated in several fish species (Canario and Scott, 1988; Goetz, 1983; Yamauchi et al., 1984). In tench females treated with $17,20\beta$ -P, 40–60% underwent ovulation (Epler et al., 1993). Both Gn and GnM treatments stimulated a surge of $17,20\beta$ -P detected at 12 h PI. Pinillos et al. (2002) reported a peak of $17,20\beta$ -P occurring 6 h later, with subsequent decline seen at 24 h. The rapid LH surge observed shortly after GnM was reflected in earlier onset of $17,20\beta$ -P increase 6 h PI, which was not seen with Gn treated fish. Interestingly $17,20\beta$ -P production by the oocytes reaches the same level in both Gn and GnM groups at 12 h PI despite significantly different LH levels. The pathway to oocyte maturation via a maturation promoting factor and that leading to ovulation are clearly distinct, although both oocyte maturation and ovulation are promoted by the $17,20\beta$ -P (Nagahama, 1994). Overstimulation of $17,20\beta$ -P production by artificially induced LH surge could result in slight asynchrony between the

process of meiotic maturation and the process of ovulation, leading to decreased viability of oocytes, as suggested by Mylonas et al. (1992).

The stimulation of final oocyte maturation and ovulation by the GnM treatment did not lead to reduction in ovulation rate or quantity of obtained ova, but significantly decreased fertility and hatchability of the ova. We conclude that induction of a gradual LH release by Gn is the more physiologically appropriate treatment for stimulation of FOM and ovulation in tench.

Combined treatment with a synthetic analogue of GnRH and a dopamine inhibitor has been effective in several fish species (Heyrati et al., 2007; Peter et al., 1988; Podhorec and Kouril, 2009) as a tool for overcoming reproductive dysfunction and obtaining good quality eggs in captivity. Commercial

preparations (e.g. Ovaprim, Western Chemical Inc., USA; Ovopel, Interfish Kft., Hungary) enable widespread use of the combined treatment throughout the freshwater aquaculture industry (Brzuska, 2014; Brzuska and Adamek, 2008; Nowosad et al., 2014; Targonska et al., 2014; Viveiros et al., 2015), yet there is a dearth of information on possible deleterious effects of combined treatment on fish gametes of species displaying some degree of dopamine inhibition of LH secretion. Our study revealed that in tench, combined treatment is unnecessary and may significantly decrease viability of stripped eggs. These results are of significance for aquaculture, especially among cyprinids in which combined treatment is widely used as universal therapy for induction of ovulation (Yaron et al., 2009). We strongly recommend caution in using the combined treatment even in species displaying some degree of dopamine inhibition and suggest its use only in those species for which it is necessary.

Table 1. Effect of 0.9% NaCl, mGnRHa, mGnRHa with metoclopramide and metoclopramide on tench males (*Tinca tinca*) reproductive parameters.

Treatment	Body weight (g)	Ovulation rate	Latency period (h)	Fecundity index (%)	Fertility index (%)	Hatching rate (%)
0.9% NaCl	1102 ± 83	0/10 ^a				
mGnRHa (25 µg kg ⁻¹)	944 ± 85	8/10 ^b	26.2 ± 0.6 ^a	4.47 ± 1.11 ^a	77.2 ± 2.5 ^a	40.0 ± 3.2 ^a
mGnRHa (25 µg kg ⁻¹) + metoclopramide (20 mg kg ⁻¹)	1165 ± 96	9/10 ^b	25.1 ± 0.6 ^a	4.25 ± 0.61 ^a	53.7 ± 6.9 ^b	22.5 ± 2.6 ^b
Metoclopramide (20 mg kg ⁻¹)	1000 ± 98	0/10 ^a				

Data are expressed as the mean ± SEM. Different superscripts denote statistical differences between groups (P < 0.05).

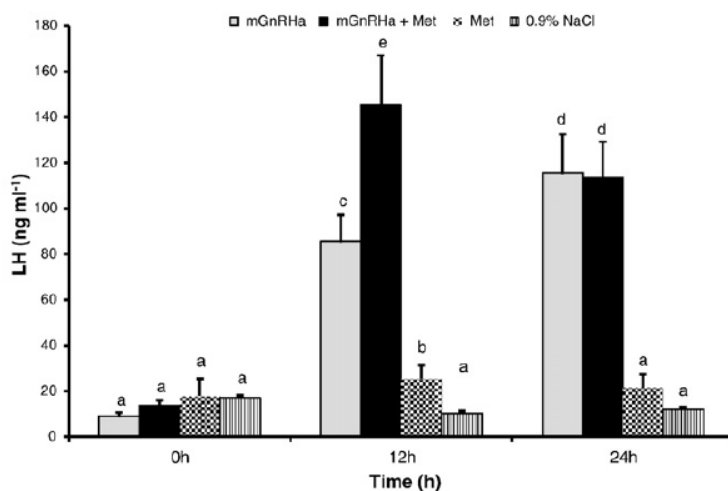


Figure 1.

Effects of 0.9% NaCl, mGnRHa, mGnRHa with metoclopramide, and metoclopramide on luteinizing hormone (LH) release in male tench *Tinca tinca*. Data are expressed as the mean ± SEM. Different superscript letters indicate significant differences (P < 0.05).

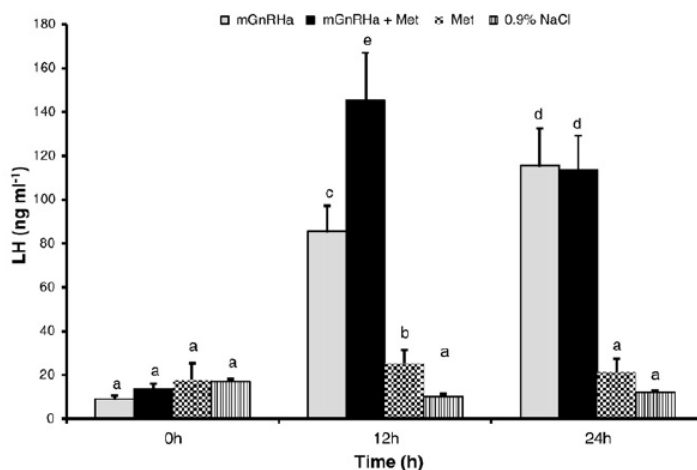


Figure 2.

Effects of 0.9% NaCl, mGnRHa, mGnRHa with metoclopramide and metoclopramide on testosterone release in male tench *Tinca tinca*. Data are expressed as the mean ± SEM. Different superscript letters indicate significant differences (P < 0.05).

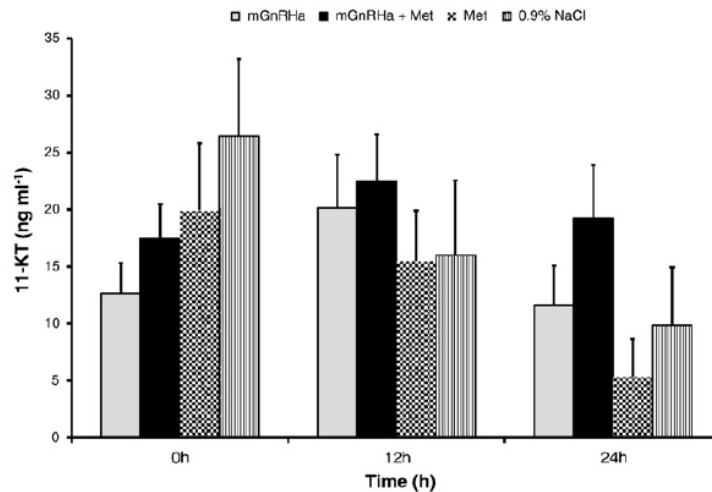


Figure 3.

Effect of 0.9% NaCl, mGnRH α , mGnRH α with metoclopramide and metoclopramide on 11-ketotestosterone release in male tench *Tinca tinca*. Data are expressed as the mean \pm SEM. Different superscript letters indicate significant differences ($P < 0.05$).

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6. Acknowledgements

We would like to express our deep gratitude to the head of the Institute of Ichthyobiology and Aquaculture of the Polish Academy of Sciences in Gofysz, Dr. I. Irnazarow, for enabling us to conduct the present study. We would also like to thank everyone involved in conducting controlled reproduction (Mgr K. Szambelan, Mgr D. Sitko, Mgr R. Ronchetti, U. Czudek, R. Wrzecionko). The study was financially supported

by the Grantová Agentura české Republiky (GP13-39438P), Ministry of Education, Youth, and Sports of the Czech Republic - projects CENAKVA (no. CZ.1.05/2.1.00/01.0024), CENAKVA II (no. LO1205 under the NPU I program), and Národní Agentura pro Zemědělský Výzkum (QJ1510117).