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Efficacy of poly (lactic-co-glycolic acid) microparticles as a gonadotropin-releasing hormone analogue delivery system to stimulate ovulation of peled *Coregonus peled*

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Abstract: The aim of the study was to determine the efficacy of poly (lactic-co-glycolic acid) microparticles as a carrier of gonadotropin-releasing hormone analogues (GnRHa) for induction of ovulation in peled *Coregonus peled*. Female peled were injected intraperitoneally with 1) saline solution only (control), 2) mammalian GnRHa at 25 µg/kg body weight, 3) GnRHa in 753-type microparticles at 50 µg/kg, or 4) GnRHa in 653-type microparticles at 50 µg/kg. Blood plasma samples were taken on days 0, 4, 8, and 12 post-injection. All hormone treatments induced synchronous ovulation and higher cumulative ovulation compared to controls. Hormone treatments did not affect relative fecundity or the percentage of eyed eggs. Testosterone level decreased toward the onset of ovulation. On day eight of the trial, the testosterone level was significantly lower in hormone-treated groups compared to the control group. The level of 17β-oestradiol showed a decreasing trend post-injection, with the lowest observed level on day eight. Our results demonstrate that ovulation can be induced in the peled by the sustained – release of GnRHa in poly (lactic-co-glycolic acid) microparticles, but the treatment does not improve reproductive performance.

Keywords: induction of ovulation; GnRHa; salmonids; sustained drug release; steroid feedback

Peled *Coregonus peled* is native to Russia and has been introduced into areas including Belarus, Germany, Finland, Latvia, Estonia, and Czechia (Gordeeva et al. 2008). It has been successfully adapted to intensive aquaculture (Savini et al. 2010; Stejskal et al. 2018; Matousek et al. 2020). Peled matures at two years, with spawning typically occurring in winter. Broodstock must be regularly checked for ovulation during the reproductive season since spontaneous spawning can extend over several weeks. Temperature below 2 °C may stimulate mass ovulation (Hochman 1987).

Acceleration of sexual maturation and synchronisation of spawning offers the potential to increase the economic efficiency of gamete collection (Olito et al. 2001). Synthetic analogues of gonadotropin-re-

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leasing hormone (GnRHa) can be used to stimulate and synchronise ovulation in salmonids (Mikolajczyk et al. 2008; Noori et al. 2010; Anderson et al. 2017). The prolonged luteinising hormone surge that is required for ovulation and spawning can be provided by multiple injections or by sustained drug release systems (Zohar and Mylonas 2001). Long-acting preparations show advantages over conventional administration, providing convenience, controlled release of the agent, low toxicity, and possibly greater effectiveness (Lin et al. 2014).

Administration in microparticles can optimise the effects of pharmaco-therapies. As a drug delivery system, microparticles offer protection of the active agent against enzymatic degradation, controlled drug release ranging from a few hours to several weeks, and easy administration not available with the standard long-acting preparations often used in aquaculture, such as implants (Siepmann and Siepmann 2006). Another advantage over implants is the simplicity of different release profiles that can be achieved by adjusting polymer selection, particle size and drug loading, and most importantly, the multiply character, which allows more homogenous distribution in the physiological environment (Vyslouzil et al. 2013). A wide range of biodegradable polymers has been used to formulate microparticles (da Silva and Pinto 2019; Shah et al. 2019; Strobel et al. 2020). Poly (lactic-co-glycolic acid) (PLGA) shows excellent biocompatibility and biodegradability in the body (Makadia and Siegel 2011), and its use in microparticles is a promising approach to induction of gametogenesis, showing high potential as a vehicle for long-acting pharmaceutical preparation in fish reproduction (Matejkova and Podhorec 2019).

The objectives of the study were to determine the efficacy of sustained GnRHa release from two types of PLGA microparticles in inducing ovulation in the peled, as well as its impact on egg quality and steroid feedback.

MATERIAL AND METHODS

The experiment was carried out in mid-December 2018 at a commercial fish farm in South Bohemia. On December 4, 2018, 250 sexually mature 3-year-old peled were obtained (Mydlovary, Czechia) from Kinski JSC Fish Farm (Zdar nad Sazavou, Czechia). Fish were sorted by sex and placed in separate flow-through tanks (four tanks for females, one for males) supplied with water from a local river and held for acclimatisation for seven days under ambient water temperature and photoperiod. Fish were not fed during the study. Water temperature decreased continuously from 7.2 °C at the beginning of the experiment to 3.5 °C during the incubation of eggs (Figures 1 and 2).

On December 11, females were randomly divided into four groups of 50 fish. Fish were anaesthetised in a clove oil bath (0.03 ml/l), and the preparations were injected intraperitoneally using a 2 ml syringe equipped with a 0.33 × 12 mm needle. The experimental groups were: CON: saline solution only (Braun Melsugen AG, Melsungen, Germany), a single injection of 0.9% NaCl at dose 1 ml/kg body weight; mGnRHa: single injection of Supergestran® (Nordic Pharma, Jesenice, Czech Republic) at 25 µg/kg body weight [mammalian GnRHa (D-Tle^6, Pro^9, NEt-mGnRHa)]; M753: 10-day release microparticles with encapsulated GnRHa [(D-Ala^6, des-Gly^{10}) GnRH-ethylamide] formulated with

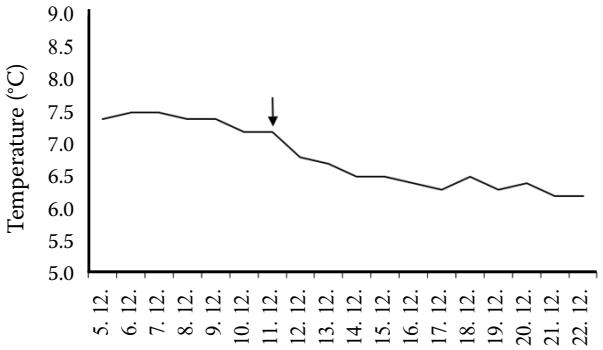

Figure 1. Water temperature in the fish tanks during the trial during December 2018

Arrow indicates the date of hormone injection

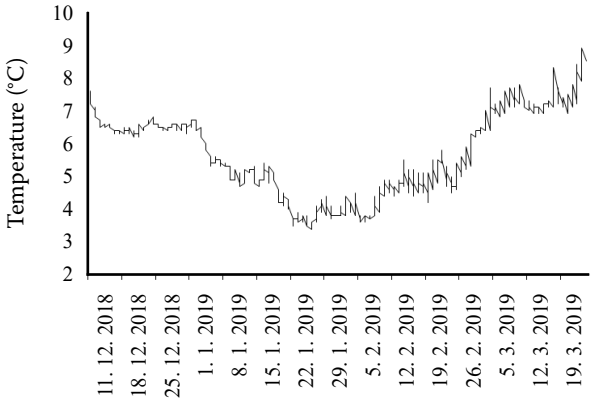

Figure 2. Water temperature during egg incubation of *Coregonus peled*

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Resomer RG 753H (Evonik, Darmstadt, Germany) at 50 µg/kg GnRHa with 5 µg/kg released per day; M653: 10-day release microparticles with encapsulated GnRHa, formulated with Resomer RG 653H (Evonik, Darmstadt, Germany), at 50 µg/kg GnRHa with 5 µg/kg released per day.

Resomer RG 753H that was used for the formation of PLGA applied in group M753 contains 10% more polylactic acid than Resomer RG 653H used in group M653, which decreases its hydrophilicity, suggesting that its use will provide slower onset of hormone release and a more prolonged effect.

After injection, fish were checked for ovulation at four-day intervals. Ovulation was considered to have occurred if eggs were released by a gentle massage of the abdomen. Ovulated eggs were stripped into separate dishes for each female, and a small sieve and filter paper were used to remove the ovarian fluid. Eggs from each female were weighed to 0.01 g (PCB 1000-2; Kern & Sohn GmbH, Balingen, Germany). A sample of unfertilised eggs was used to calculate the absolute and relative fecundity.

Sperm from 50 males that spontaneously released sperm upon the gentle massage of the abdomen was separately collected at the time of fertilisation into a 2 ml syringe. Eggs from each female were separately fertilised with an equal volume of sperm from three males. Sperm was mixed with the eggs, hatchery water was added, and the mixture was gently stirred for 1 h using a horizontal shaker (Duomax 1030; Heidolph Instruments GmbH, Schwabach, Germany). Three samples from each ovulating female, each containing ~150 eggs, were separately incubated in small plexiglass incubators (1.7 l), equipped with dirigible inflow (Kallert 2009) with regulated water flow. The mean pH was 7.62 ± 0.05, and oxygen saturation was maintained at 10.4 ± 1.06 mg/l. Water temperature was measured hourly by an automatic datalogger (EL-USB-1-RCG; Lascar Electronics, Whiteparish, UK). Dead eggs were counted and removed using a 3 ml plastic pipette. On February 5, the percent of eyed eggs in the total number of eggs in the incubator was calculated.

Microparticle preparation

The standard double water-in-oil-in-water ($w_1/o/w_2$) principle was used to prepare both microparticle treatments. Alarelin acetate (APExBIO, Houston, TX, USA) (10 mg) was dissolved in 1.5 g of 9.1% gelatine solution ($water_1$ phase) at 50 °C. Two types of Resomer with carboxyl groups on the polymer ends and different ratios of glycolic acid to lactic acid (65 : 35 for RG 653H and 75 : 25 for RG 753H) were used to investigate the optimal carrier properties for GnRHa encapsulation. Resomer was chosen since it has been found useful in various products (Schwach et al. 2003; Duvvuri et al. 2005)

For type 653 microparticles, 800 mg of Resomer RG 653H (0.32–0.44 dl/g) (Evonik, Darmstadt, Germany) was used, and 800 mg Resomer RG 753H (0.32–0.44 dl/g) (Evonik, Darmstadt, Germany) was used for type 753. Resomer was dissolved in 5 ml dichloromethane) (Penta, Prague, Czech Republic), pre-mixed on a vortex for 30 s, and homogenized (Ultra-Turrax T25; Ika Werke, Staufen Im Bresgau, Germany). The resulting w_1/o emulsion was pre-mixed for the 60 s on a homogeniser with 12 g of 1% poly (vinyl alcohol) solution at 50 °C (PVA, Mw 31 000–50 000; 98–99% hydrolysed; Sigma Aldrich, St. Louis, MO, USA) to create a concentrated double emulsion $w_1/o/w_2$. The concentrated emulsion was immediately poured into 200 g of 0.1% PVA/2.0% NaCl. The resultant $w_1/o/w_2$ emulsion was stirred for 2 h to completely evaporate dichloromethane. The particles were collected via centrifugation, resuspended in purified water, and lyophilised. Four runs were prepared from each Resomer.

Blood sampling and steroid assay

Blood samples were taken prior to the injection of the preparations (day 0), and on days 4, 8, and 12 post-injection. Ten females from each group were sampled at each sampling point and each female was sampled only once. Blood samples (1 ml) were taken by caudal venipuncture using heparinised syringes (5 ml) and 0.7 × 30 mm needles. Plasma was obtained by 10 min centrifugation at 5 000 RPM, 4 °C (5427R; Eppendorf AG, Hamburg, Germany) and stored at –80 °C until analysis. The level of sex steroids in the blood plasma were measured twice in each sample.

The concentration of testosterone (T) (KAPD-1559) and17β-oestradiol (E2) (KAP0621) were analysed by enzyme-linked immunosorbent assay using commercially available kits (DIAsource,

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Ottignies-Louvain-la-Neuve, Belgium) according to the manufacturer's instructions. The samples were checked for dilution accuracy and diluted (1 : 2) with Calibrator 0, if necessary. The absorbance was read at 450 nm with a microplate plate reader (PlateReader AF2200; Eppendorf AG, Hamburg, Germany). The sensitivity of the assays was 0.083 ng/ml for T and 5 pg/ml for E2. Intra-assay coefficients of variation for all assays were less than 9%, and inter-assay coefficients of variation were less than 8%.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analysis was conducted with Statistica v12 Cz (StatSoft, Tulsa, OK, USA). Normality and homogeneity (Cochran C., Hartley, Bartlett) of data were tested and significant differences were analysed by one way ANOVA (Tukey or HSD test). For all tests, the level of significance was set at $P < 0.05$.

RESULTS

All treatments were associated with significantly greater synchronisation of ovulation than seen in the control group, with no significant differences found among the treated groups. First ovulation occurred four days post-injection in all hormone-treated groups, while first ovulation in the control group was recorded on day 12 of the experiment. On day 12, the percentage of ovulated females was 82.5% in mGnRHa, 70% in M753, and 90% in M653 (Figure 3). The latency period was significantly shorter in mGnRHa (8.4 ± 0.6 days) than in control (12 days), with no significant differences in latency found among the treated groups.

No significant differences were observed among groups in terms of relative and absolute fecundity. Mean relative fecundity was 19 087 ± 1 136 eggs/kg fish body weight (Figure 4).

The level of T decreased towards ovulation. The decrease was slighter in the controls compared to treatment groups. On day eight, the T level was significantly lower in treatment groups compared to controls, with no significant differences among treatments. At the end of the trial, plasma T levels were similar among groups, with no significant differences on day 12 (Figure 5).

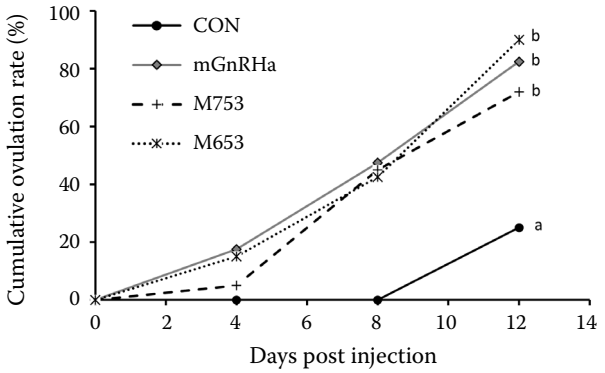

Figure 3. Cumulative ovulation rate of *Coregonus peled*
CON = saline solution only (control); M653 = gonadotropin-releasing hormone analogue (GnRHa) in 653-type microparticles at 50 µg/kg; M753 = GnRHa in 753-type microparticles at 50 µg/kg; mGnRHa = mammalian GnRHa at 25 µg/kg body weight
[a,b]Groups with different letters are significantly different ($P < 0.05$)

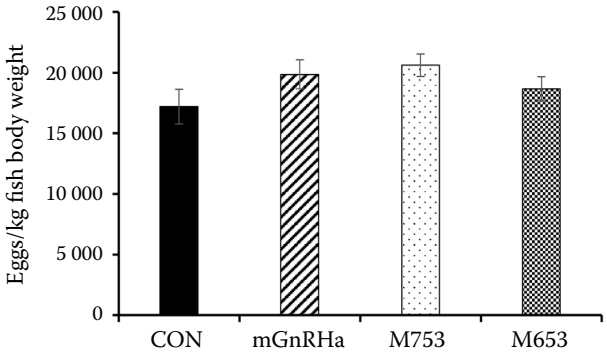

Figure 4. Relative fecundity in *Coregonus peled* in different treatments
CON = saline solution only (control); M653 = gonadotropin-releasing hormone analogue (GnRHa) in 653-type microparticles at 50 µg/kg; M753 = GnRHa in 753-type microparticles at 50 µg/kg; mGnRHa = mammalian GnRHa at 25 µg/kg body weight

The E2 level showed a decreasing trend with the minimum level in hormone-treated groups on day eight and, in the control group, on day 12. The M753 group showed significantly lower E2 concentration on day eight compared to controls and mGnRHa. At the end of the trial, both microparticle-treated groups showed an increasing trend in E2 compared to controls (Figure 6).

There was no significant difference in the percentage of eyed eggs in samples from tested groups: CON 50.6 ± 1.6%; mGnRHa, 49.9 ± 1.3%; M753, 52.6 ± 1.2%; and M653, 54.1 ± 1.1%.

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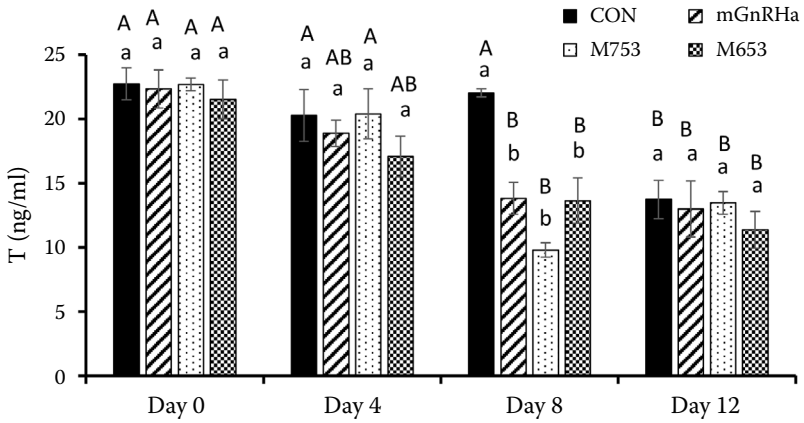

Figure 5. Changes in plasma testosterone (T) in *Coregonus peled*

CON = saline solution only (control); M653 = gonadotropin-releasing hormone analogue (GnRHa) in 653-type microparticles at 50 µg/kg; M753 = GnRHa in 753-type microparticles at 50 µg/kg; mGnRHa = mammalian GnRHa at 25 µg/kg body weight

[A,B]Different letters indicate significant differences within an experimental group over the course of the experiment (one way ANOVA)

[a,b]Different letters indicate significant differences among groups on a sampling day (one way ANOVA)

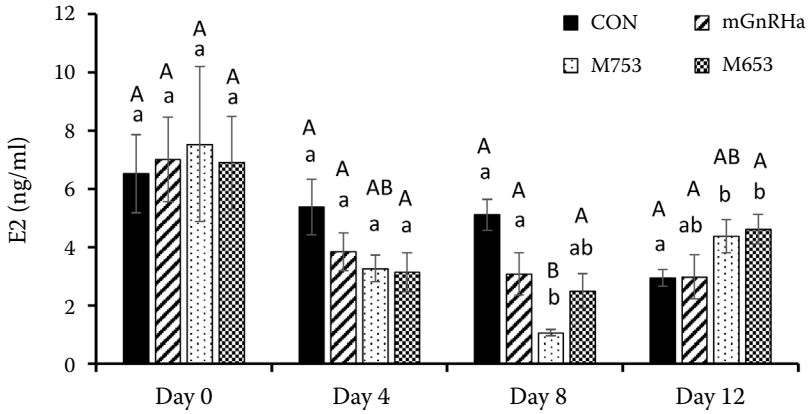

Figure 6. Changes in plasma 17β-oestradiol (E2) in *Coregonus peled*

CON = saline solution only (control); M653 = gonadotropin-releasing hormone analogue (GnRHa) in 653-type microparticles at 50 µg/kg; M753 = GnRHa in 753-type microparticles at 50 µg/kg; mGnRHa = mammalian GnRHa at 25 µg/kg body weight

[A,B]Different letters indicate significant differences within an experimental group over the course of the experiment (one way ANOVA)

[a,b]Different letters indicate significant differences among groups on a sampling day (one way ANOVA)

DISCUSSION

Induction and synchronisation of ovulation in peled by single injection or prolonged-release GnRHa was significantly increased compared to non-hormone-treated controls. Similar results have been reported for other salmonids (Olito et al. 2001; Noori et al. 2010; Svinger et al. 2013). We found no difference in results of prolonged-release and acute treatment in peled, although a positive effect of prolonged released preparations compared to the acute administration has been frequently reported. Sustained-release GnRHa with Freund's incomplete adjuvant at 25–50 µg/kg in rainbow trout *Oncorhynchus mykiss* resulted in significantly higher ovulation compared to the acute hormone treatment (Arabaci et al. 2004; Vazirzadeh et al. 2008). In the same species, 100% ovulation was obtained with sustained-release GnRHa (12.5–50.0 µg/kg) encapsulated in a polyglycolic-

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polylactic matrix, results not reached with a single injection of GnRHa at 20 µg/kg (Breton et al. 1990). In the present study, a single injection of mGnRHa at 25 µg/kg was effective at induction and synchronisation of ovulation, in accordance with other studies in other salmonids (Mikolajczyk et al. 2007; 2008).

The hormone treatment in our experiment did not affect the relative fecundity or the proportion of eyed eggs. No effect of hormone treatment on the volume of eggs was observed in Atlantic salmon *Salmo salar* (Anderson et al. 2017). In some species, ovulation induced by GnRHa has been associated with a negative effect on egg quality (Bobe and Labbe 2010). GnRHa implants were associated with reduced egg buoyancy, fertilisation, number of viable eggs, and smaller oil globules (Agulleiro et al. 2006; Bonnet et al. 2007; Garber et al. 2009). On the other hand, long-lasting GnRHa implants in greater amberjack *Seriola dumerili* led to higher fecundity compared to spontaneous spawning and to single hormonal injections (Jerez et al. 2018). A positive effect of prolonged GnRHa release from implants was also observed in Atlantic salmon males in which total expressible milt increased, while sperm density and motility was unchanged (Goren 1995).

In our experiment, the percentage of eyed eggs was similar in all tested groups. A lack of effect of hormone treatment on egg quality was also reported by other authors (Billard et al. 1984; Dabrowski et al. 1994; Park et al. 2007; Zarski et al. 2019). In contrast, a lower percentage of eyed eggs was observed in brown trout *Salmo trutta* treated with a double injection of GnRHa at 10 µg/kg (Mylonas et al. 1992). Reduced number of offspring was reported in chinook salmon *Oncorhynchus tshawytscha* treated with GnRHa at 25 µg/kg compared untreated controls (Olito et al. 2001).

In the present study, the level of E2 and T showed a similar pattern in all groups, with levels decreasing near ovulation. However, T tended to decrease later than E2 in all groups. The trend was also observed in Atlantic salmon (Dickhoff et al. 1989; Taranger et al. 1998) and European grayling *Thymallus thymallus* (Szmyt et al. 2021). Similar results were observed in wild brown trout, in which the highest concentration of plasma E2 occurred 30 days prior to ovulation, while, in cultured brown trout, the peak level was 14 days before ovulation (Norberg et al. 1989).

CONCLUSIONS

Sustained delivery system and a single GnRHa injection can be successful in induction and synchronisation of ovulation in peled if administered near the natural spawning period. Poly (lactic-co-glycolic acid microparticle treatment does not increase fecundity or percentage of eyed eggs. Further investigation is needed to determine the effect of microparticles on the brood fish and the survival of the fry.

Conflict of interest

The authors declare no conflict of interest.

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