Induction and advancement of ovulation in wild Arctic grayling (*Thymallus arcticus arcticus*) using D-Tle^6,Pro^9,NEt-mGnRHa Lecirelin

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ABSTRACT: The effect of single and double injections of D-Tle^6,Pro^9,NEt-mGnRHa (Supergestran®) on advancement and induction of ovulation in Arctic grayling was assessed. Sexually mature wild Arctic grayling females (most 2–4 years old) were caught in the Yenisey River at the beginning of May 2010. After a 4-day acclimatization, the females were randomly divided into four groups and intramuscularly injected as follows: group A, control group, treated with physiological saline only; group B, treated with a single injection (SI) of Supergestran® at 25 μg/kg body weight (BW); group C, injected twice (DI) with 25 μg/kg BW 3 days apart; group D, injected twice with 10 μg/kg BW 3 days apart. After stripping, the pseudo-gonadosomatic index was calculated, and an eggs sample from each female was fertilized. Only fish in the groups treated with DI protocols ovulated. No differences between the two groups were found in the timing of ovulation, ovulation rate, or mean time to ovulation. No females in either group A or B ovulated, since the experiment had to be prematurely terminated due to technical problems at the field hatchery. The DI of 10 μg/kg proved sufficient to induce and advance ovulation in Arctic grayling. Hormone treatments seem to be a promising tool to obtain viable eggs of Arctic grayling in a short time window and thereby to ensure satisfactory numbers of fry for restocking programs.

Keywords: salmonids; reproduction; single injection; double injection; GnRHa

The Siberian Arctic grayling (*Thymallus a. arcticus*) is one of the most abundant fish species in the upper Yenisey River in central Siberia. Recently, there have been some efforts to develop controlled artificial reproduction of this species, since its natural wild stocks are in decline through poaching and overfishing, intrusion of man-made structures, and environmental pollution (Vincent, 1962; Kaya, 1992; Barndt and Kaya, 2000). However, because of the unfavourable and extreme variation in climatic conditions, it is difficult to develop adequate hatcheries in most of the Siberian territories. Rudimentary temporary buildings, often with inexperienced staff, are insufficient to ensure the welfare of the wild Arctic graylings, which are mainly captured at spawning grounds in floating nets and frequently injured in the process. Following capture, graylings are usually transported to hatcheries in simple iron-plate containers without aeration or oxygenation with the aim to allow complete sexual maturation and apply manual stripping. Since wild salmonid, thymalid, and coregonid stocks are highly sensitive to stress caused by capture, handling, and environmental

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conditions in captivity, the procedures result in high prespawning mortality (Turunen et al., 1994; Mikolajczyk et al., 2005, 2008).

Synthetic analogues of hypothalamic neuropeptide gonadotropin hormone-releasing hormone (GnRHa) have been widely used in salmonids as effective tools to induce/advance or synchronize ovulation (Breton et al., 1990; Taranger et al., 1992; Haraldsson et al., 1993; Jansen, 1993; Arabaci et al., 2004; Park et al., 2007; Vazirzadeh et al., 2008; Noori et al., 2010). Gonadotropin hormone releasing hormone acts on the pituitary gland to stimulate secretion of luteinizing hormone (LH). Luteinizing hormone initiates ovarian production of maturation inducing factors (MIS), e.g. 17α,20β-DP, and maturation promoting factors (MPF) followed by ovulation (Nagahama and Yamashita, 2008).

The dosage of GnRHa effective in inducing ovulation in salmonids varies from 1 μg/kg body weight (Taranger et al., 1992) to 50 μg/kg BW (Arabaci et al., 2004) given either as a single injection (SI) or as a double injection (DI) spaced some days apart. The response of salmonid females to the hormone treatment protocol and dosage depends strongly on the environmental conditions that influence endocrine stage and oocyte maturation stage at the time of the hormone treatment (Gillet et al., 1996; Vikingstad et al., 2008). In some cases, SI at a very low dose is sufficient to induce ovulation (Fitzpatrick et al., 1984; Mylonas et al., 1992; Taranger et al., 1992). In other cases SI induces ovulation in only a small percentage (Arabaci et al., 2004; Mikolajczyk et al., 2005; Noori et al., 2010) of the treated broodstock. In contrast, a DI protocol at doses of 5–25 μg/kg BW provides reliably strong induction and advancement if administered 3–4 weeks prior to natural ovulation time (Mylonas et al., 1992; Slater et al., 1995; Svinger et al., 2010). However, injecting broodstock twice increases handling, stress, and risk of injury. To avoid this, GnRHa sustained release preparations have been developed (for review see Mylonas and Zohar, 2001). In contrast to an acute SI, these ensure a progressive and prolonged increase in plasma LH levels, which is more suitable with respect to the duration of gametogenesis under gonadotropin control in salmonids (Breton et al., 1983, 1990; Zohar, 1988). Unfortunately, the high cost of these preparations and the limitation of their use in small and sensitive broodstock such as Arctic grayling (200–400 g) necessitate the use of single or double injections of GnRHa dissolved either in physiological saline or contained in commercial preparations.

In this study, we evaluated the efficacy of SI at one dosage and DI at various dosages of mammalian D-Tle^6,Pro^9,NEt-mGnRHa (Lecirelin) contained in the commercial preparation Supergestran® in inducing and advancing ovulation in wild Arctic grayling. Although there have been previously some experiments with GnRHa conducted in the subfamily *Thymallidae* in European grayling (*Thymallus thymallus*) (Kouřil et al. 1987; Mikolajczyk et al., 2008), this is the first report of the use of GnRHa in Arctic grayling.

MATERIAL AND METHODS

Broodstock

Broodstock samples of Arctic grayling were captured in the first half of May 2010 from the Yenisey River, Siberia, Russian Federation. Swimming snares were used to capture sexually ripe individuals of both sexes (45 males and 74 females) on migration routes to the spawning grounds. The broodstock (most 2–4 years old, mean weight 253 ± 43 g) were transported to a field hatchery supplied with water from the Yenisey River in the village of Kononovo (Krasnoyarsk region: 56°N, 93°E). The fish were acclimatized for 4 days in 4 open square fiberglass flow-through tanks (1.2 m^3, 0.33 l/s) under natural photoperiod conditions. Average water temperature during acclimatization was 3.1°C. Out of the 74 females, 8 died during the first 24 h acclimatization due to injuries and stress during capture and transport. Of the remaining females, individuals with a swollen urogenital papilla were selected and kept at the facility for the experiment. On the second day of acclimatization, 30% of the surface of the rearing tanks was covered with dark tarpaulins to provide shelter.

Experimental design

During the acclimatization period, females were randomly divided into four tanks, each containing 13 individuals. However, due to mortalities on day 4 of acclimatization, the number of fish in the experimental groups was reduced to 8 or 9 as follows: group A (control), treated with physiological saline only (9 individuals); group B, SI of 25 μg/kg

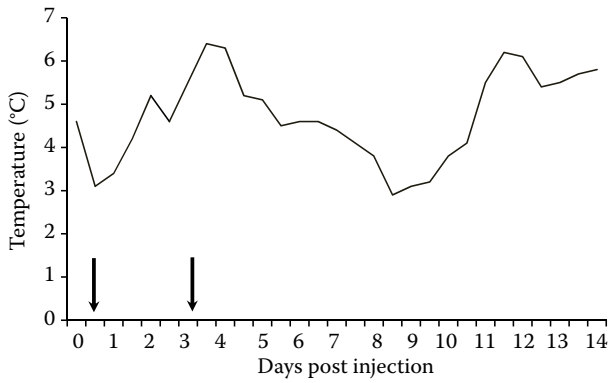

Figure 1. Temperature fluctuation during the experiment. Arrows indicate days of hormone administration

BW (8 individuals); group C, DI of 25 µg/kg BW given 3 days apart (8 individuals); group D, DI of 10 µg/kg BW given 3 days apart (8 individuals).

Water temperature fluctuations from the first injection to the termination of the experiment are given in Figure 1. Oxygen content during this period was 11.6 ± 0.8 mg/l.

Hormone preparation and application

The commercial preparation Supergestran® (Nordic Pharma, Jesenice, Czech Republic) contains 25 µg/ml of D-Tle^6,Pro^9,NEt-mGnRHa in 2 ml ampoules. Physiological saline (0.09%) was used when appropriate to dilute the compound to the dosage of the peptide administered in this experiment. This preparation was chosen for ease of use in the field and its stability in adverse environments. Before hormone administration, females were anesthetized (clove oil, 0.03 ml/l) and weighed to the nearest 1 g. The hormone preparation was injected intraperitoneally using 1 ml insulin syringes.

After the second injection in groups C and D, all groups were checked for ovulation every second day. Females were considered to have ovulated if eggs were released with gentle manual pressure on the abdomen. The latency period was calculated as the average number of days between the first injection and ovulation. Pseudo-gonadosomatic index (pGSI = weight of stripped eggs/body weight of female before stripping) was calculated for each stripped female. Fertilization rate was calculated as the percentage of non-developing white eggs in a sample of 100 eggs taken from each female, which were fertilized with 1 ml of a mixture of milt from 3–4 males. After the stripped females had recovered, they were released into the river. Some females with low pGSI were killed and dissected immediately after stripping to assess the stage of oocytes remaining in ovaries. The experiment was terminated on the 28th of May because of technical problems at the field facility leading to the death of 5 females in the control group.

Statistical analysis

Ovulation progress differences were assessed using survival analysis (Z-test) and χ^2 test. Differences in fertilization rate and pGSI were tested by *t*-test after arcsin data transformation. Nonparametric Mann-Whitney test was used to compare mean time to ovulation. A significance level (α) of 0.05 was applied to all tests except where indicated. Data are presented as means ± SEM.

RESULTS

First ovulations in both DI groups occurred 9 days after the first injection, when 50% of females in these groups ovulated (Figure 2). The next ovulations occurred on day 11, with the ovulation rate reaching 87.5% and 75% in groups C and D, respectively. The final ovulated female was recorded in group D on day 13, and an ovulation rate of 87.5% was reached in this group (Figure 2). Seven out of the 8 females in group C were stripped over the course of 3 days. No ovulations were recorded

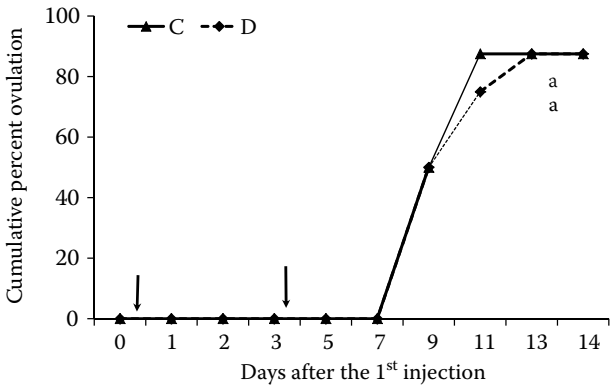

Figure 2. Ovulation progress in groups treated with double injection (DI) (group C – 2 × 25 µg/kg, group D – 2 × 10 µg/kg). Arrows indicate days of hormone administration. Because no ovulation was recorded either in group A or B, these are not included in the figure

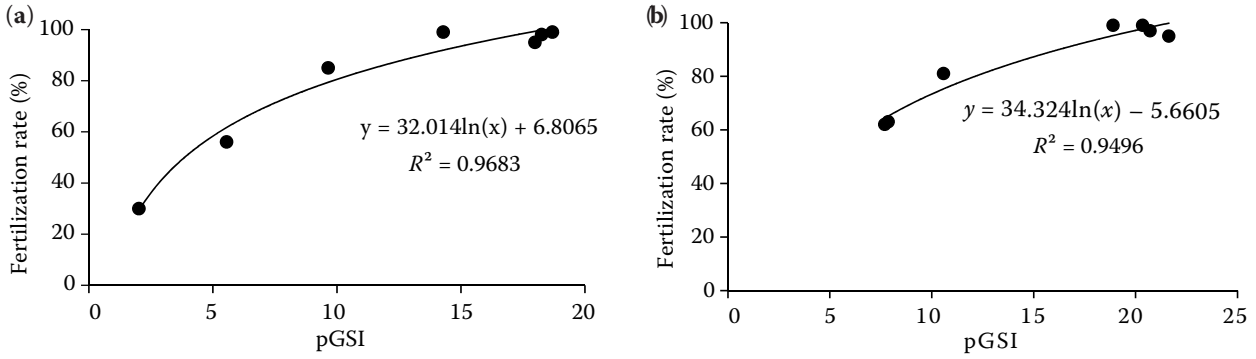

Figure 3. Relationship between pGSI level and fertilization rate in group C (**a**) and group D (**b**) ($P < 0.01$)

in the SI group or the control group during the experimental period. There were no significant differences found in the timing of ovulation between groups C and D. The latency period did not differ significantly and was 9.9 ± 1.0 and 10 ± 1.5 days for groups C and D, respectively.

Mean pGSI levels reached 12.3 ± 6.2 and 15.4 ± 5.9% in groups C and D, respectively, with no significant differences. No significant differences between groups C and D were found in fertilization rate, which was 80 ± 25% and 85 ± 15.4%. However, there was a significant positive correlation observed ($P < 0.01$, $R^2 = 0.97$ and $R^2 = 0.95$ for groups C and D, respectively) between pGSI and fertilization rate in both groups, suggesting that females with higher pGSI had higher fertilization rates (Figure 3). Females showing pGSI from 2 to 8% reached fertilization rates of 30 to 63%, whereas females with pGSI of 9.6 to 20.3% exhibited fertilization rates of 85 to 99% (Figure 3).

DISCUSSION

Natural spawning time of Arctic grayling in the Yenisey river occurs when water temperature increases to 7–8°C (V.I. Zadelenov, Scientific Research Institute of Ecology of Fishery Reservoirs, Krasnoyarsk, 2010, personal communication). Barndt and Kaya (2000) observed spawning of North American Arctic grayling (*Thymallus arcticus*) at temperatures of 9.8–10.5°C. In our experimental conditions, the hormone injections were administered at lower temperatures, from 3 to 4.6°C. There is strong evidence that elevated temperature can modulate the effects of endocrine rhythms in salmonids (Gillet et al., 1996; Pankhurst and Thomas, 1998; Taranger et al., 2003; King and Pankhurst, 2004a, b; Gillet and Breton, 2009; Gillet et al., 2011). These effects also have the capacity to influence the efficacy of hormone treatment. It is not known whether lower than optimum temperatures may have the same delaying effect on steroidogenic and ovulatory responses as do more elevated temperatures (Pankhurst and Tomas, 1998). Crim et al. (1983) treated female rainbow trout (*Oncorhynchus mykiss*) with a pelleted long-lasting GnRHa preparation at 25 µg/kg concluding that pituitary stimulation occurred well in advance of ovulation, despite temperatures around 2°C. However, sustained release hormone preparations are known to have stronger capacities to counteract suboptimal environmental conditions in salmonids and to be much more reliable in eliciting advanced ovulation than is a single acute injection (Breton et al., 1990; Arabaci et al., 2004; Vazirzadeh et al., 2008).

Both DI dosages of Supergestran® were highly effective in induction and advancement of ovulation in Arctic grayling, and 7 out of 8 females were stripped in both groups. In contrast, SI of this preparation did not induce ovulation, and no ovulation was recorded in the control group. This is in contrary to studies performed in European grayling by Kouřil et al. (1987), who report that SI of D-Ala^6,Pro^9,NEt-GnRHa at 10–40 µg/kg BW induced ovulation in 76–90% of the treated broodstock. Similar results were achieved by Mikolajczyk et al. (2008) using the preparation Gonazon™ containing the synthetic analogue azaglynafarelin, with SI of dosages of 16–48 µg/kg inducing ovulation with up to 100% efficacy. In both these studies, however, some females in control groups ovulated at the same time as those in experimental groups, which suggests that the hormone was administered shortly before, or concurrent with,

the beginning of natural spawning time, when the broodstock has the highest physiological sensitivity to the treatment (Taranger and Hansen, 1993; Vikingstad et al., 2008). This can also explain the shorter latency periods in studies by Kouřil et al. (1987) and Mikolajczyk et al. (2008) in European grayling, which were approximately 5 days shorter than in Arctic grayling.

Mainly due to high mortality, no natural ovulation was recorded in the control and SI groups in the present study; thus it was not possible to estimate advance of ovulation time in the DI injected Arctic grayling. However, several females captured two weeks after the beginning of the experiment ovulated naturally during the second week in June. This suggests that ovulation was advanced by approximately 1 month in the DI groups, and that the lack of response to the SI of D-Tle^{6},Pro^{9},NEt-mGnRHa Lecirelin at 25 μg/kg may have been due to its inability to counteract suboptimal environmental conditions and/or insufficient physiological readiness of females.

Hormone treatments and their influence on egg quality is still a question. Results of previous investigations in salmonids are inconsistent. Some authors have reported that hormone treatment affects egg quality (Fitzpatrick et al., 1984; Mylonas et al., 1992; Taranger et al., 1992; Noori et al., 2010). In other studies no such effect was found (Slater et al., 1995; Arabaci et al., 2004; Park et al., 2007; Vazirzadeh et al., 2008). These discrepancies may reflect a large number of variables such as hormone dose levels (Olito et al., 2001), physiological stage of females (Billard et al., 1984; Gilet et al., 1996), asynchrony between processes of meiotic maturation and ovulation (Mylonas et al., 1992), methods used for fertilization, age of broodstock (Kallert, 2009), and ovarian plasma properties (Lahnsteiner et al., 1999; Dietrich et al., 2007; Wojtczak et al., 2007).

In our experiment, fertilization rate was the highest in the females with the higher pGSI level. Further, females with higher pGSI were stripped later (on days 11 and 13). This suggests that some females with low pGSI were possibly not completely ripe at the time of stripping, and only a small proportion of eggs stripped were fertilizable. This was confirmed by dissection of these females, which showed large numbers of oocytes remaining in the ovaries. Other treated females reached fertilization rates of 85–99%, a satisfactory level for Arctic grayling fry production and comparable to fertilization rates in European grayling treated with Gonazon™ containing D-Nal(2)^{6}aza-Gly^{10}-GnRHa Azaglynafarelin (Mikolajczyk et al., 2008).

We conclude that Supergestran® is an effective tool for induction and advancement of ovulation in wild Arctic grayling if administered in DI at 10 μg/kg BW. This treatment made it possible to collect fertilizable eggs prior to an increase in mortality in the sensitive wild broodstock. Our results augment available data on artificial reproduction of this Siberian species. However, the field conditions available for this experiment were not sufficient to develop optimized guidelines for hormone treatment without thorough research into the influence of such hormone manipulation on the sex product quality, which can be provided only with appropriate laboratory facilities.

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