Fakulta rybářství
a ochrany vod
Faculty of Fisheries
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Jihočeská univerzita
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University of South Bohemia
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Optimization of hormone-induced ovulation in economically important fish species

Optimalizace metod hormonální indukce ovulace u hospodářsky významných druhů ryb

Viktor W. Švinger

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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1.1. HORMONE TREATMENTS FOR CONTROL OF OVULATION

Many fish species held in aquaculture conditions exhibit varying degrees of reproductive dysfunction. Dysfunction has been categorized into three levels, based on severity, by Mylonas and Zohar (2001). The most severe form is characterized by a failure to undergo vitellogenenis, for example, in eels (*Anguilla japonica, Anguilla anguilla*) (Ohta et al., 1997), Mediterranean amberjack (*Seriola dumerilii*, Carangidae) (Garcia et al., 2000), and the Mekong river giant catfish (*Pangasius gigas*, Pangasiidae) (Donaldson, 1996). The mid-range and most common reproductive dysfunction at central European latitudes is observed in Cyprinidae (carp), Siluridae (catfish), and pike (Esocidae). In fish exhibiting this degree of dysfunction vitellogenesis proceeds normally, but the developing oocytes fail to mature with the onset of the spawning season, requiring hormone treatment to induce oocyte maturation to obtain fertilizable eggs. The Salmonidae are among typical representatives exhibiting the lowest level of dysfunction. Salmonids reared in captivity undergo vitellogenesis and oocytes reach maturation (Bromage and Cumaranatunga 1988), but fish fail to spawn and must be stripped manually. Spontaneous spawning in females also rarely occurs in captivity in brook trout (*Salvelinus fontinalis*) (personal observation) and brown trout (*Salmo trutta fario*) (Ronny Seyfried, personal communication). The spawning season in salmonid females, for example, in Arctic charr (*Salvelinus alpinus*), rainbow trout (*Oncorhynchus mykiss*), and brown trout, is not synchronized and can extend over more than 3 months (Breton et al., 1990). To ensure fertilization success, broodstock must therefore be checked for ovulation at least weekly to prevent the over-ripening of eggs prior to stripping (Leitritz, 1969; Sakai et al., 1975; Bry, 1981; Springate et al., 1984, 1985; Bromage et al., 1992a). Hormone treatment is used for control of temporal aspects of the final stages of the reproductive cycle, synchronization of oocyte maturation and ovulation, and for limiting the duration of the spawning season (Figure 1).

Disproportionate decline in egg production due to high pre-spawning broodstock mortality or stress may occur in Pacific salmon (*Oncorhynchus* sp.) (Hunter et al., 1978) and some physically fragile species such as brook trout, European grayling (*Thymallus thymallus*), Arctic grayling (*Thymallus arcticus*), huchen (*Hucho hucho*), and coregonids (Coregonidae sp.). In these species hormone treatments can be used to advance ovulation to approximately 30 days earlier than natural spawning (Jungwirt et al., 1979; Mikolajczyk et al., 2008; Švinger et al., 2013a).

Hormone treatment is not essential for salmonids to undergo ovulation. Hormonal synchronization or acceleration of ovulation is used to provide flexibility of hatchery management during stripping. Employment of these methods must be linked to existing conditions and requirements of the aquaculture protocol as well as to species.

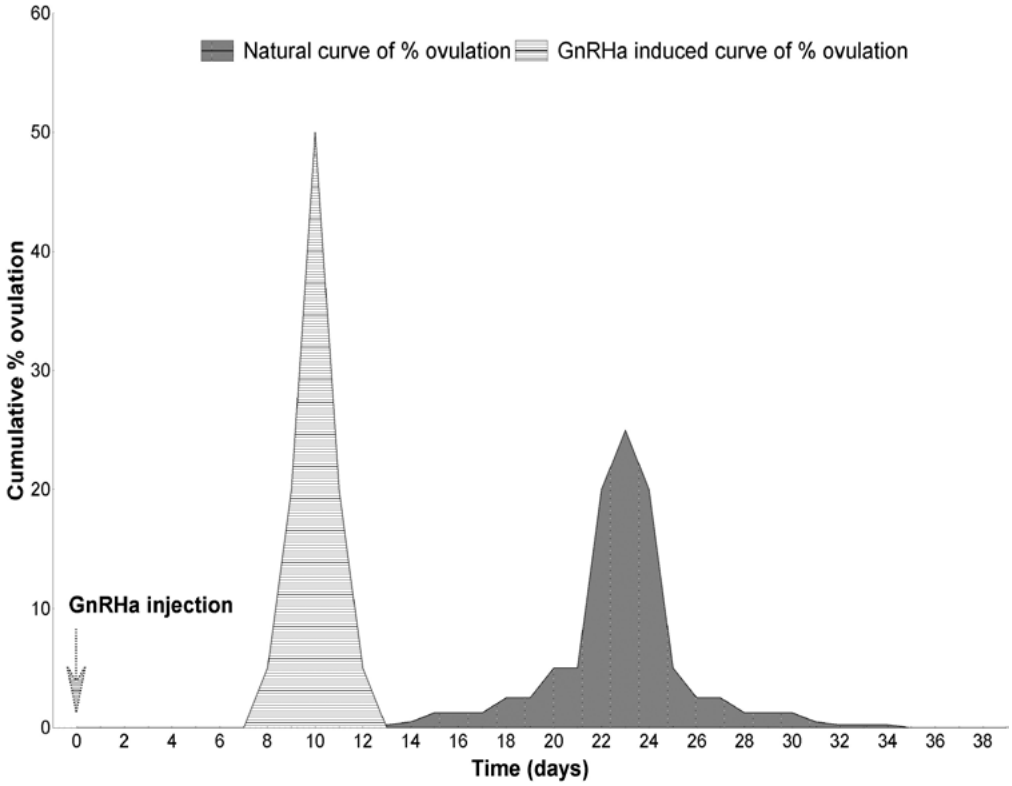

Figure 1. Effect of the GnRHa treatment on the spawning duration in salmonids (blue). Horizontally hatched area represents % of ovulation in GnRHa treated salmonid broodstock. Shaded area demonstrates % of ovulation in naturally ovulating "standardized" salmonid broodstock.

1.2. HISTORY OF HORMONE TREATMENTS IN SALMONIDS

Records of hormone treatments in salmonids date from the 1930s, when ovulation was successfully induced using carp pituitary gonadotropin hormone (hypophysation) in rainbow trout (von Ihering, 1937; Hasler et al., 1939). Later attempts were carried out in the USA and Japan using gonadotropic substances in blueback salmon (*Oncorhynchus nerka*) (Burrows et al., 1952; Palmer et al., 1954) and rainbow trout (Migita et al., 1952). During spawning migration Pacific salmon have traditionally been captured in rivers and transported to hatcheries to be artificially stripped at sexual maturity. Any extension of this period would be accompanied by increased mortality of broodstock resulting in reduced egg production (Hunter et al., 1978). Dried chinook (*Oncorhynchus tshawytscha*) and chum salmon (*Oncorhynchus keta*) pituitary was used successfully to accelerate spawning, whereas attempts to use horse pituitary extract failed (Burrows et al., 1952; Palmer et al., 1954). In the late 1970s, carp pituitary injection was used in huchen with good results (Jungwirt et al., 1979), and although hypophysation is basically an outmoded method, it is still in use for artificial reproduction of huchen under conditions that do not allow holding

of broodstock to sexual maturity (Aleksandar Bajić, 2012, personal communication, II International Hucho Symposium, September 19–22, Łopuszna, Poland). In the early 1970s, methods allowing isolation and purification of Chinook salmon gonadotropin were developed (Donaldson et al., 1972). Administration of purified gonadotropin represented the most advanced hypophysation method. Purified salmon gonadotropin was widely used from the mid-1970s to the early 1980s in Canada for acceleration and synchronization of ovulation in chinook salmon (Hunter et al., 1978) and coho salmon (*Oncorhynchus kisutch*) (Jalabert et al., 1978; Donaldson et al., 1981a; Hunter et al., 1981). The end of the 1970s introduced the era of synthetic gonadotropin releasing hormone analogues (GnRHa), which underwent substantial development during the subsequent 30 years of practical use. Initially GnRHas were used in combination with purified salmon gonadotropin (Donaldson et al., 1981b; Sower et al., 1984) (Table 1). Fitzpatrick et al. (1984) demonstrated high potency of GnRHa alone in coho salmon administered in single and multiple acute injections. Around the same time, Crim et al. (1983a,b) and Crim et al. (1984) reported enhanced effects of GnRHa treatment using cholesterol pellet implants to ensure prolonged GnRHa release. This represents the beginning of GnRHa treatment separation into acute injections and sustained release treatments. In the mid-1980s dopaminergic inhibitors were first employed in rainbow trout and brown trout (Billard et al., 1984). The work of Billard et al. (1984) dealing with dopaminergic inhibition in salmonids can be considered pioneering. Since the 1980s, various GnRHas and GnRHa delivery methods have been applied in most commercially important salmonid species (Table 1). In 2005, Gonazon, the first drug containing GnRHa officially approved by the European Union, was introduced and tested, primarily in Arctic charr, rainbow trout, Atlantic salmon, European grayling, and European whitefish (Haffray et al., 2005; Mikolajczyk et al., 2005; Mikolajczyk et al., 2008). In the USA and Canada, the commercial preparations Ovaplant, OvaRH, and Ovaprim were developed, intitially for salmonid reproduction, and are available in Europe and used in other species. The GnRHa-containing drug Supergestran was developed in the Czech Republic independent of the EU, and is now fully approved for use in a wide variety of fish species, including some salmonids, by veterinary prescription. Recent decades therefore have seen the development of a market for GnRHa preparations, allowing users to select preparations tailored to their specific needs.

1.3. MAJOR AIMS OF THE DISSERTATION

In the Czech Republic, GnRHas were formerly applied in salmonids only rarely in European grayling (Kouřil et al., 1987; Randák et al., 2002). Until recently there was a lack of information with respect to their use in other salmonid species such as brook charr (Chapter 2), northern whitefish (*Coregonus peled*) (Chapter 3), native populations of brown trout (Švinger and Kouřil, 2012b), and endangered Arctic grayling (Chapter 4). The main goal of this work was to develop and refine procedures using synthetic GnRHas in these economically important and endangered salmonids. Since GnRHa treatments presented in this work were used for the first time in these species, special attention was paid to optimization of GnRHa dose, GnRHa delivery method, post-treatment survival of broodstock, and effects of GnRHa treatment on egg quality.

1.4. BRAIN-PITUITARY-GONAD AXIS IN SALMONIDS

The reproductive cycle of vertebrates is controlled by the hormone brain-pituitary-gonad axis (BPG axis). It is well established that the native hypothalamic neurotransmitter gonadotropin releasing hormone (GnRH) plays a central role in the neural control of vertebrate reproduction via the BPG axis. GnRH is a neurodecapeptide secreted by neurons that, in teleosts, directly innervate the pituitary gland (Amano et al., 2010). Two or three native species-specific molecular forms of GnRH are present in fish brain (Oka, 1997; Okubo and Nagahama, 2008). While fish such as flounder (Pleuronectidae) and percids (Perciformes) possess three native forms, only two have been identified in the brain of salmonids: the chicken **c**GnRH-2 and the salmon **s**GnRH (Okuzawa et al., 1990; Okubo and Nagahama, 2008). Okuzawa et al. (1990) measured sGnRH and cGnRH-II content in rainbow trout, and brain distribution of these forms was studied in two Pacific salmon species, *Oncorhynchus nerka* and *Oncorhynchus masou* (Amano et al., 1992, 1997; Amano, 2010). In all cases, sGnRH was detected in the olfactory bulb, ventral telencephalon, preoptic area, and pituitary, whereas cGnRH-II was localized in the midbrain tegmentum, but not in the pituitary. Potential to stimulate secretion of gonadotropin hormones was demonstrated by both molecular forms, probably due to similar amino acid sequence (Amano et al., 2010). High amounts of sGnRH in salmonid pituitary suggest that this form acts to stimulate synthesis and secretion of luteinizing hormone (LH), earlier known as GTH-II (Breton et al., 1998), and to some extent follicle stimulating hormone (FSH, GTH-I) (Ando et al., 2004; Amano et al., 2010; Zohar et al., 2010). During vitellogenesis in the prespawning period, sGnRH stimulates FSH secretion but not LH. Conversely, during the spawning season, sGnRH is an effective stimulant of LH secretion but not of FSH (Ando et al., 2004).

Follicle stimulating hormone plays a dominant role during the follicular growth of oocytes during vitellogenesis (Kawauchi et al., 1989; Swanson et al., 1989; Swanson, 1991; Prat et al., 1996; Nagahama and Yamashita, 2008). Follicle stimulating hormone acts directly on the follicular layer of oocyte *theca folliculi* to produce testosterone. Testosterone is aromatized on 17β-estradiol (E_2) by enzyme P450 aromatase ($P450_{arom}$) produced by the granulose layer of follicular cells (Pankhurst and Carragher, 1991; Kagawa et al., 1982; Nagahama, 1994). E_2 stimulates hepatic synthesis of yolk protein vitellogenin, which is transported by the bloodstream to be sequestered in the growing oocytes (Specker and Sullivan, 1994).

A surge of LH in blood plasma occurs at the beginning of spawning. Luteinizing hormone stimulates ovarian secretion of maturation-inducing steroid 17α20β-dihydroxy-4-pregnen-3-one (DHP) (Young et al., 1983) produced by the follicular layer of granulose cells through 20β-hydroxysteroiddehydrogenase-mediated conversion of thecal 17α-hydroxyprogesterone (17P) (Young et al., 1986; Nagahama, 1994; Nagahama et al., 1994; Nagahama, 2008). Maturation-inducing steroid triggers maturation promoting factor, the final inducer of the onset of oocyte maturation (FOM) (Nagahama and Yamashita, 2008). Oocyte maturation is defined by resumption and completion of the first meiotic division with subsequent progression to methaphase II (Nagahama, 1994). Typical migration of the germinal vesicle to the animal pole indicates the onset of FOM, followed by germinal vesicle breakdown, chromosome condensation, formation of the division spindle, and extrusion of the first polar body, characterizing completion of the first meiotic division (Nagahama, 1994; Kotani and Yamashita, 2002).

To be fertilizable, oocytes have to be released from follicular cells in the process of ovulation (Nagahama and Yamashita, 2008; Goetz and Garczynski, 1997). Ovulation is composed of follicular separation, detachment of microvillar connections between the follicular wall and oocytes, follicle rupture, opening of the follicular wall, and expulsion of the oocyte (Goetz and Garczynski, 1997). An essential role in ovulation is played by hydrolytic enzymes (proteases) present in the follicles of vertebrates (Ogiwara et al., 2005). Arachidonic acid and its metabolites including prostaglandins $PGF_{2\alpha}$ and PGE_2 (eicosanoids), vasoactive peptides, catecholamines, and inhibitors of proteases are also involved in ovulation in fish (Goetz et al., 1982; Bradley and Goetz, 1994; Goetz and Garczynski, 1997; Patiño et al., 2003). Besides initiating oocyte maturation, DHP is supposed to be a key hormone for triggering ovulation through binding to nuclear progesterone receptors (Nagahama and Yamashita, 2008).

Application of synthetic GnRH analogues induces ovulation via the above-mentioned BPG axis. Basically, GnRHa acts at the pituitary level of the BPG axis and fundamentally differs from previously used methods (hypophysation) that directly stimulate ovarian DHP production and thus act at the ovarian level.

1.5. REGULATORY FACTORS AND MECHANISMS OF THE SALMONID REPRODUCTION CYCLE

1.5.1. Photoperiod

Salmonids attain sexual maturity annually, with the spawning date determined by photoperiod changes inducing pineal melatonin secretion (Porter et al., 1999; 2001). Sexual maturity is reached approximately 5–8 months after the longest days of the year (21 June). The photoperiod is the most important factor controlling the reproductive cycle of salmonids (Bromage and Duston, 1986; Bromage et al., 1992a; Gillet, 1994; Siikavuopio et al., 2009) and is key to their commercial production (Peterson and Hormon, 2005; Nordgarden et al., 2005). Various techniques of photostimulation are currently used to advance or delay the spawning date by several months (Macquarrie et al., 1979; Bromage et al., 1992a; Taranger et al., 2010), to accelerate parr-smolt transformation (Stefansson et al., 1991; Björnsson et al., 1989), to prevent grilsing in Atlantic salmon (Porter et al., 1999), to enable crossbreeding between species by synchronizing their spawning date (Peter Aschenbrenner, personal communication), and to stimulate growth of selected age groups (Saunders et al., 1985; Kråkenes et al., 1991; Endal et al., 2000; Taylor et al., 2006). Continuous light and high temperature can trigger sexual maturation during smoltification (Taranger et al., 1999; Fjelldal et al., 2011).

A detailed discussion of photoperiod function is beyond the scope of this thesis, and this chapter primarily serves to define photoperiod as a major regulating factor in salmonid reproduction and to suggest the potential for utilizing photostimulation to shift spawning time with subsequent induction of ovulation with GnRHa (Taranger et al., 2003; Bonnet et al., 2007; King and Pankhurst, 2007). Delaying or advancing the spawning date through photoperiod alterations may mean that maturation and ovulation take place at water temperatures that differ from those of the natural spawning period (Taranger et al., 2003). Thus, the primary regulatory factor (photoperiod) may become out of phase with the chief modulation factor (water temperature).

1.5.2. Temperature

While photoperiod is considered the chief regulatory factor of the reproductive cycle in salmonids (*Zeitgeber* of internal rhythms of the organism) (Bromage et al., 2001), temperature has a major modulating role (Pankhurst and King, 2010) but an imperceptible effect on the timing of spawning (Hokanson et al., 1973; King et al., 2003). When conducting GnRHa treatment in salmonids, water temperature has fundamental significance for efficacy of the treatment as well as the resulting egg quality. The reproductive cycle has two main phases: the proliferation, growth, and differentiation of gametes (vitellogenesis, spermatogenesis), and the period of oocyte or sperm maturation and preparation for their release (FOM and spermiation) (Mylonas and Zohar, 2001). Each fish species is characterized by its physiologically optimal thermal range for the course of endocrine processes responsible for the control of each phase. If the temperature exceeds the optimum range, particularly in cold water fish species, disruption or cessation of these processes occurs, in the best case resulting in the deterioration of the quality of sex products or, at worst, total failure of vitellogenesis or oocyte maturation. When rearing salmonid broodstock, a basic rule is that a certain number of degree days per annum cannot be exceeded without risk of over-ripening and inferior quality of sex products (Mayer, 2001). Reproductive endocrinology of salmonids was the subject of research in the 1980s and 1990s, predominantly in rainbow trout (Campbell et al., 1980; Scott et al., 1980; Bromage et al., 1982; Scott and Sumpter, 1983; Schulz, 1984; Springate et al., 1985; Fostier and Jalabert, 1986; Bromage and Cumaranatunga, 1988; Tyler et al., 1990), charr (Goetz et al., 1987; Mayer et al., 1992; Foster et al., 1993; Frantzen et al., 1997), Atlantic salmon (Crim et al., 1986; Andersen et al., 1991; Cotter et al., 2000), and Pacific salmon (Sower and Schreck, 1982; Dye et al., 1986; Slater et al., 1994). However, the effect of elevated temperature on circulation of reproductive hormones and its implications for aquaculture have been studied only during the past decade (Pankhurst and King, 2010).

Vitellogenesis, a phase of rapid ovarian growth characterized by hepatic production and ovarian absorption of yolk protein precursor vitellogenin (Specker and Sullivan, 1994; King and Pankhurst, 2003), extends over several months of the female reproductive cycle. During vitellogenesis oocytes multiply in size, and vitellogenin content may reach more than 90% of the total oocyte volume (Tyler, 1991; Tyler et al., 2000). Since the spawning season of salmonids occurs mainly in autumn and winter (Scott, 1990), vitellogenesis takes place primarily during summer and early autumn (Bromage and Cumaranatunga, 1988; Tyler et al., 1990; Estay et al., 1998; King and Pankhurst, 2003) within a period when natural temperatures tend to increase to physiological threshold levels (King et al., 2003). Sexually maturing Atlantic salmon females kept for 3 months from an early stage of vitellogenesis at temperatures of 16, 18, and 22 °C exhibited symptoms of impaired vitellogenesis compared to fish kept at 14 °C (King and Pankhurst, 2000; King et al., 2003). Impaired vitellogenesis was characterized by reduced levels of E_2 and its precursor, testosterone (T), as well as vitellogenin. Physical alterations in ovulated oocytes were observed at 22 °C, including reduced egg size and failure of zonagenesis (Hyllner and Haux, 1995) accompanied by deformations of micropyle (King et al., 2003). The assumption of King et al. (2003) that these changes are elicited via reduction in the cytochrome $P450_{arom}$ activity responsible for conversion of T to E_2 was later confirmed by Watts et al. (2004). Identical results to those in Atlantic salmon were obtained in rainbow trout at 21 °C (Pankhurst et al., 1996), several weeks prior to spawning during the late vitellogenic stage in Arctic charr at 8–11 °C

(Gillet et al., 1991), and in lake trout at 10–12 °C (*Salvelinus namaycush*) (Švinger and Kallert, unpublished data). According to results of Australian studies, repeat spawning Atlantic salmon are more resistant to thermal impairment of reproduction than maiden individuals (Pankhurst et al., 2011).

In salmonids, elevated temperatures are also known to inhibit maturation (Gillet et al., 1991; Taranger and Hansen, 1993; Gillet et al., 1996, 2011; Gillet and Breton, 2009,; Pankhurst et al., 1996; Pankhurst and Thomas, 1998; Taranger et al., 2003; King and Pankhurst, 2004ab; Vikingstad et al., 2008). The onset of FOM and ovulation was delayed in Atlantic salmon maintained at 11 °C compared to those at 6 °C (King and Pankhurst, 2004a). A temperature of 6 °C shows a positive effect on synchronization of ovulation. Temperatures of 14–16 °C modulated profiles of E_2 and T levels and inhibited the pre-ovulatory surge of maturational steroid DHP with simultaneous inhibition of ovulation (King and Pankhurst, 2000; Taranger et al., 2003). Rainbow trout oocytes maintained at 18 °C *in vitro* exhibited high $P450_{arom}$ activity (suggesting a potential for stimulation of E_2 production) along with low 20β-HSD activity mediating production of DHP (Pankhurst and Thomas, 1998) (see section 1.4). Further, GnRHa treatment is not effective in salmonid females if carried out at elevated temperatures: 18 °C in rainbow trout, 14–16 °C in Atlantic salmon, 10 °C in Arctic charr (Pankhurst and Thomas, 1998; King and Pankhurst, 2004a; Gillet and Breton, 2009). The lack of effectiveness probably results from reduced sensitivity of pituitary to GnRHa treatment, which impedes hypophyseal LH secretion and subsequent stimulation of ovarian DHP. King and Pankhurst (2004a) suggest that reduced ovarian DHP production may be due to lack of a substrate (17P) and its 20β-HSD-mediated conversion to DHP, since 20β-HSD activity was still present at 16 °C in Atlantic salmon. That would mean disruption of enzyme processes preceding production of 17P (conversion of cholesterol to 17P via pregnenolone and progesterone) (Nagahama, 1997), which are also regulated by the LH surge (King and Pankhurst, 2004a).

Elevated temperatures during the final stages of oogenesis can result in total collapse of BPG axis pathways via reduced sensitivity of pituitary to GnRH (Taranger et al., 2003; Vikingstad et al., 2008; Gillet and Breton, 2009); inhibition of LH secretion (Gillet et al., 1996; Gillet and Breton, 2009); reduced enzyme activity, probably at the 20β-HSD level (Pankhurst and Thomas, 1998) and impaired synthesis of its substrate 17P (King and Pankhurst, 2004a); and low follicular feedback to LH stimulation due to lack of follicular LH receptors and/or their reduced affinity to LH (Gillet et al., 2011), which impedes DHP production with subsequent delay or cessation of FOM and ovulation (King and Pankhurst, 2004a). These changes are not irreversible; resumption of normally functioning BPG axis occurs when fish are moved back into cold water (Gillet et al., 1991; Gillet and Breton, 2009, Gillet et al., 2011; King and Pankhurst, 2004b).

1.5.3. Temperature and dopaminergic inhibition in salmonids

LH secretion is subject to a stimulatory effect of hypothalamic GnRH and an inhibitory effect of gonadotropin release-inhibiting factor (GRIF), the dopamine (Pasqualini et al., 2004). The intensity of dopaminergic inhibition is species specific. Strong dopaminergic inhibition is generally present in the *Cyprinidae*, whereas in gilthead sea bream (*Sparus aurata*) and Atlantic croaker (*Micropogonias undulatus*), for example, no inhibitory action of dopamine has been observed (Zohar et al., 1995; Copeland and Thomas, 1989). The inhibitory role of LH secretion in salmonids is

controversial and was traditionally considered of little importance (Van der Kraak et al., 1986; Dufour et al., 2005). However, several studies have strongly suggested that dopaminergic inhibition in salmonids exists (Billard et al., 1984; Van der Kraak et al., 1986; Gillet et al., 1996; Park et al., 2007), since administration of a dopamine inhibitor induced ovulation. Existence of dopaminergic inhibition was definitively demonstrated by French scientists Gillet and Breton (2009) in Arctic charr at an elevated temperature (10 °C) during final stages of oogenesis.

This underscores temperature as the primary modulating factor of the reproductive cycle of salmonids via the neuroendocrine system. Dopaminergic inhibition of hypothalamic GnRHa secretion and pituitary LH secretion is probably an adaptive control mechanism preventing release of sex products into conditions inadequate for fertilization and embryo development. Arctic charr is the most northerly distributed teleost species, usually living at water temperatures ranging from 0–10 °C (Johnson, 1980), and, for production of high quality eggs, water temperatures below 7 °C prior to the spawning season are critical (Johnston, 2002). It is therefore presumed that dopaminergic inhibition of LH secretion is potentiated in other members of the Salmonidae by higher temperatures than is the case for Arctic charr.

Negative effects of elevated temperatures during the ovulatory period can be counteracted by dopamine inhibitors (pimozide, domperidone, metoclopramide) (Gillet et al., 1996; Park et al., 2007), techniques ensuring sustained GnRHa release (Taranger et al., 2003; Vikingstad et al., 2008), and repeated acute GnRHa treatment (Pankhurst and Thomas, 1998). However, use of dopamine inhibitors is problematic, since no limits for residues of dopamine inhibitors in foods have been established according to Directive 2004/28/EC of the European Parliament and of the Council.

1.6. ACUTE VS. SUSTAINED GNRHA TREATMENTS

Acute GnRHa treatment refers to administration of GnRHa diluted in 0.7–0.9% physiological saline (NaCl). A single acute injection of GnRHa is generally insufficient to produce a high ovulation rate in salmonids (Mylonas et al., 1992; Taranger et al., 1992; Švinger et al., 2010), because the elicited LH surge is rapid and short-lived (Breton et al., 1990), lasting 2–4 days depending on water temperature, species, and the GnRHa molecule (Cook and Peter, 1980; Breton et al., 1990; Gillet and Breton, 2009; Mylonas and Zohar, 2001). Therefore injection must be repeated, usually after 2 or 3 days (double acute injection). Although double acute injection is highly effective, it necessitates increased handling of the broodstock. To counteract this, sustained release GnRHa delivery systems have been developed to ensure an LH stimulating effect lasting for several days or weeks (for review see Mylonas and Zohar, 2001). Unfortunately, the high cost and complex manufacture requiring technical expertise and specialized equipment, makes them unfeasible for on-site preparation.

Adjuvants are used to initiate and augment the inflammatory reaction necessary for induction of optimal innate and adaptive immune responses to vaccines, as well as to ensure long lasting immunity (Safari et al., 2011). Adjuvants can also increase the potency of antivenins, allowing a lower dose (Pratanaphon et al., 1997) and reducing costs (Singh and O'Hagan, 1999). Freund's incomplete adjuvant (FIA) has been used as a GnRHa carrier in an attempt to develop an anti-maturation vaccine in rainbow trout (Riley and Secombes, 1993). For synchronizing ovulation in salmonids, GnRHa-

FIA combinations have shown an effect similar to that of commercial sustained release formulae (Arabaci et al., 2004; Vazirzadeh et al., 2008; Park et al., 2007). FIA is a viscous liquid containing paraffin oil (continuous phase) along with mannide mono-oleate as a surfactant. Mixing FIA with GnRHa dissolved in physiological saline creates a water-in-oil (W/O) emulsion with GnRHa trapped in the dispersed phase, stabilized with the surfactant (emulsifier) present at the interface of the two phases (Guy, 2007). After administration, FIA-emulsified GnRHa is slowly released, ensuring a long-lasting LH stimulatory effect. Using this method is inexpensive and practicable without extensive the need for technical expertise (see Švinger and Kouřil, 2012b). In addition, it enables the use of GnRHa sustained release in small-sized broodstock.

1.7. EFFECTIVENESS (SUPERACTIVITY) OF GNRHA MOLECULES IN SALMONIDS

The effectiveness of distinct GnRHa molecules can differ considerably (Nagahama et al., 1995). Effectiveness of a GnRHa molecule is measured by its potency in stimulating pituitary LH secretion. Native GnRH molecules generally have much lower potency than their synthetic superactive analogues (Peter et al., 1985; Van der Kraak, 1987; Crim et al., 1988; Weil et al., 1992; Forniés et al., 2003). Goren et al. (1990) and Zohar et al. (1990a) demonstrated *in vitro* that two main proteolytic enzymes, Tyr^5–Gly^6 endopeptidase a Pro^9–Gly^{10}–NH_2 peptidase, are responsible for degradation of native GnRH molecules in liver, kidney, and pituitary. Substitution of the D amino acid at position 6 and modification of the carboxy terminus from glycine-amide to ethyl-amide results in formation of a GnRH analogue highly resistant to enzyme degradation, contributing considerably to its superactivity (Zohar et al., 1990b). Nevertheless, superactivity as the major source of the resistance to endo-enzymatic processes has not been shown in rainbow trout (Weil et al., 1992). More likely, modification of native peptides enhances affinity to hypophyseal GnRH receptors, leading to higher biological GnRHa activity as observed in goldfish (*Carassius auratus*) (Habibi et al., 1989), African catfish (*Clarias gariepinus*) (De Leeuw, 1988), and winter flounder (*Pseudopleuronectes americanus*) (Crim et al., 1988).

A wide range of distinct GnRHa molecules have been used in salmonid species (Table 1). In rainbow trout the analogue of native salmon GnRH D-Arg^6,Pro^9-NHEt-**s**GnRHa was shown to be the most effective stimulant of LH secretion, whereas D-Trp^6,Pro^9NHEt-sGnRHa showed the lowest efficacy (Breton et al., 1990). Similar to Breton et al. (1990), Van der Kraak (1987) demonstrated D-Ala^6 and D-Arg^6 derivates of native salmon GnRH to exhibit the highest efficacy, as well as providing the most prolonged LH stimulation, in coho salmon. As in rainbow trout, D-Arg^6,Pro^9-NHEt-sGnRHa proved to be highly effective in Atlantic salmon, although maximal superactivity was shown with an analogue of the mammalian peptide D-hArg(Et_2)^6,Pro^9NHEt-**m**GnRHa (Crim et al., 1988). In the same study, Crim et al. (1988) reported that D-Arg^6-Pro^9NHEt]-sGnRHa, D-hArg(Et_2)^6,Trp^7,Leu^8-sGnRHa and D-hArg(Et_2)^6,Trp^7,Leu^8,Pro^9-NHEt-sGnRHa stimulated the most prolonged duration of LH secretion.

Based on these results, with the exception of the experiment with Arctic grayling, we used the synthetic analogue of native salmon GnRH D-Arg^6,Pro^9-NHEt-sGnRHa in our trials. This analogue is widely used as a standard active substance in many commercial hormone preparations (e. g. Ovaplant, OvaRH, Dagin, Ovaprim).

Table 1. Effective hormone treatments for induction and synchronization of ovulation in salmonids.

Species	Delivery method	Dose per kg body weight	Hormone treatment	Reference
Coregonus laveratus	ip	16 or 32 µg.kg^{-1}	D-Nal(2)^{6},Pro^{9}-aza-Gly (Gonazon™)*	Mikolajczyk et al. (2005)
Coregonus peled	im	25 + 25 µg.kg^{-1}	D-tertLeu^{6},Pro^{9}-NHEt (Supergestran®)	Švinger et al. (2010)
	ip	25 µg.kg^{-1}	D-Arg^{6},Pro^{9}-NHEt	Švinger and Kouřil (2012a)
	ip-FIA	25 µg.kg^{-1}	D-Arg^{6},Pro^{9}-NHEt	Švinger and Kouřil (2012a)
Hucho hucho	im	0.4 + 3.6 mg.kg^{-1}	cP	Jungwirt (1979)
Oncorhynchus keta	ip	70 µg.kg^{-1}	D-Ala^{6},Pro^{9}-NHEt	Park et al. (2007)
	ip	70 µg.kg^{-1} + 0.7 mg.kg^{-1}	D-Ala^{6},Pro^{9}-NHEt + PIM	Park et al. (2007)
Oncorhynchus kisutsch	ip, im	0.1+ 25 mg.kg^{-1}	SG-G100 + SPE	Hunter et al. (1981)
	ip, im	10 + 50 mg.kg^{-1}	SPE	Hunter et al. (1981)
	ip	100 + 11 µg.kg^{-1}	SG-G100 + D-[Ser(tBu)]^{6},Pro^{9}-NHEt	Donaldson et al. (1981a)
	ip	100 + 200 µg.kg^{-1}	SG-G100 + D-Ala^{6},Pro^{9}-NHEt	Donaldson et al. (1981a)
	ip	0,1 + 10 mg.kg^{-1}	SG-G100 + tamoxifen	Donaldson et al. (1981b)
	ip	0,1 + 0.062 mg.kg^{-1}	SG-G100 + D-Ala^{6},Pro^{9}-NHEt	Sower et al. (1982)
	ip	20 µg.kg^{-1}	D-Ala^{6},Pro^{9}-NHEt (GVBD)	Van Der Kraak et al. (1983)
	ip	100 + 0,5 µg.kg^{-1}	SG-G100 + D-Ala^{6},Pro^{9}-NHEt	Sower et al. (1984)
	ip	0.5 + 0.5 µg.kg^{-1}	D-Ala^{6},Pro^{9}-NHEt *	Fitzpatrick et al. (1984)
	EVAc	25 µg.kg^{-1}	D-Ala^{6},Pro^{9}-NHEt or D-Arg^{6},Pro^{9}-NHEt	Zohar et al. (1990)
	EVAc	33 µg.kg^{-1}	D-Ala^{6},Pro^{9}-NHEt (Reproboost®)	Goren et al. (1995)
Oncorhynchus mykiss	si or epi	25 µg.kg^{-1}	D-Trp^{6},Pro^{9}-NHEt (pelleted)	Crim et al. (1983a)
	ip	100 + 60 µg.kg^{-1}	SG-G100 + D-Ala^{6},Pro^{9}-NHEt	Sower et al. (1984)
	ip	1 µg.kg^{-1}+ 10 mg.kg^{-1}	D-Ala^{6}, Pro^{9}-NHEt +PIM *	Billard et al. (1984)
	im	20 µg.kg^{-1}	D-Arg^{6},Pro^{9}-NHEt	Breton et al. (1990)
	p[LGA]	12,5 µg.kg^{-1}	D-Trp^{6}-GnRH	Breton et al. (1990)

Oncorhynchus mykiss	EM	90 µg.kg^{-1} (MD)	D-Arg^{6},Pro^{9}-NHEt	Breton et al. (1995)
	ip	100 µg.kg^{-1}	D-Ala^{6},Pro^{9}-NHEt	Pankhurst and Thomas (1998)
	FIA	25 µg.kg^{-1}	D-[Ser(tBu)]^{6},Pro^{9}-NHEt	Arabaci et al. (2004)
	ip	32 µg.kg^{-1}	D-Nal(2)^{6},Pro^{9}-aza-Gly (Gonazon™)	Haffray et al. (2005)
	ip-FIA	25 µg.kg^{-1}	D-Arg^{6},Pro^{9}-NHEt	Vazirzadeh et al. (2008)
Oncorhynchus nerka	ip	5 + 5 µg.kg^{-1}	D-Ala^{6},Pro^{9}-NHEt	Slater et al., (1995)
	EVAc	75 µg.kg^{-1}	D-Ala^{6},Pro^{9}-NHEt (SHD)	Sato et al. (1997)
Oncorhynchus tshawytscha	ip	0,1 + 2.5 mg.kg^{-1}	SG-G100+SG-G100	Hunter et al. (1978)
	ip	0,1 + 50 mg.kg^{-1}	SG-G100+SPE	Hunter et al. (1978)
	ChBM	25 µg.kg^{-1}	D-Arg^{6},Pro^{9}-NHEt (Ovaplant®)*	Olito et al. (2001)
	EVAc	75 µg.kg^{-1}	D-Ala^{6},Pro^{9}-NHEt (Reproboost®), RBI	Berejikijan et al. (2003)
Salmo salar	ChBM	125 µg pellets	D-Trp^{6}, Pro^{9}-NHEt	Crim et al. (1983b)
	ChBM	38 µg.kg^{-1}	D-Trp^{6}, Pro^{9}-NHEt	Crim and Glebe (1984)
	SIMP	6.7 mg implants	[D-Nal(2)^{6}]-LHRH	Crim et al. (1986)
	EVAc	25 µg.kg^{-1}	D-Ala^{6},Pro^{9}-NHEt or D-Arg^{6},Pro^{9}-NHEt	Zohar et al. (1990)
	ip	10 µg.kg^{-1}	D-Ala^{6},Pro^{9}-NHEt	Taranger et al. (1992)
	EVAc	35 µg.kg^{-1}	D-Ala^{6},Pro^{9}-NHEt (Reproboost®)	Goren et al. (1995)
	ip or ChBM	25 µg.kg^{-1}	D-Ala^{6},Pro^{9}-NHEt	King and Pankhurst (2004)
	ip	32 µg.kg^{-1}	D-Nal(2)^{6},Pro^{9}-aza-Gly (Gonazon™)	Haffray et al. (2005)
	ip	10 µg.kg^{-1} + 5 mg.kg^{-1} 25% + 75% divided	D-Arg^{6},Pro^{9}-NHEt + DOM (Ovaprim™)	King and Pankhurst (2007)
	BdM,im	50 µg.kg^{-1}	D-Ala^{6},Pro^{9}-NHEt	Vikingstad et al. (2008)
Salmo trutta	SRI	5 µg.kg^{-1} + 10 mg.kg^{-1}	D-Ala^{6},Pro^{9}-NHEt +PIM	Billard et al. (1984)
	ip	10 + 10 µg.kg^{-1}	D-Ala^{6},Pro^{9}-NHEt *	Mylonas et al. (1992)
	EVAc	125 µg.kg^{-1}	D-Ala^{6},Pro^{9}-NHEt	Goren et al. (1995)

Salmo t. caspius	ip	100 + 100 µg.kg^{-1}	D-Ala^{6},Pro^{9}-NHEt *	Noori et al. (2010)
Salmo t. fario	ip, FIA	15-30 µg.kg^{-1}	D-Arg^{6},Pro^{9}-NHEt	Švinger and Kouřil (2012b)
Salmo t. lacustris	ip	25 + 25 µg.kg^{-1}	D-Arg^{6},Pro^{9}-NHEt	Švinger and Kouřil (2012b)
Salvelinus alpinus	ip	2 µg.kg^{-1} (1 mg.kg^{-1}) + 8 µg.kg^{-1} (4 mg.kg^{-1})	D-Arg^{6},Pro^{9}-NHEt + DOM (Ovaprim™)	Jansen (1993)
	im	30 µg.kg^{-1}	D-Ala^{6}, Pro^{9}-NHEt	Haraldsson et al. (1993)
	ip at 5°C	20 µg.kg^{-1} + 5 mg.kg^{-1}	D-Arg^{6},Pro^{9}-NHEt + PIM *	Gillet et al. (1996)
	p[LGA] at 10 °C	20 µg.kg^{-1}	D-Trp^{6}, Pro^{9}-NHEt	Gillet et al. (1996)
	ip at 5°C	20 µg.kg^{-1}	D-Arg^{6},Pro^{9}-NHEt	Gillet et al. (1996)
	ip	32 µg.kg^{-1}	D-Nal(2)^{6},Pro^{9}-aza-Gly (Gonazon™)	Haffray et al. (2005)
	ip	20 µg.kg^{-1}	D-Arg^{6},Pro^{9}-NHEt	Gillet and Breton (2013b)
Salvelinus fontinalis	ip, FIA	25 µg.kg^{-1}	D-Arg^{6},Pro^{9}-NHEt	Švinger et al. (2013)
Salvelinus namaycush	ip	10 µg.kg^{-1}	D-Ala^{6},Pro^{9}-NHEt	Erdahl and McClain (1987)
Thymallus arcticus	ip	10 + 10 µg.kg^{-1}	D-tertLeu^{6},Pro^{9}-NHEt (Supergestran®)	Švinger et al. (2013a)
Thymallus thymallus	im	5 µg.kg^{-1}	D-Ala^{6},Pro^{9}-NHEt	Kouřil et al. (1987)
	ip	40 µg.kg^{-1} + 20 mg.kg^{-1}	D-Ala^{6},Pro^{9}-NHEt (Ovopel)	Szmyt et al. (2007)
	ip	16 µg.kg^{-1}	D-Nal(2)^{6},Pro^{9}-aza-Gly (Gonazon™)	Mikolajczyk et al. (2008)
	ip-FIA	15–20 µg.kg^{-1}	D-Arg^{6},Pro^{9}-NHEt	Švinger and Kouřil (2012b)

PIM – pimozide; **DOM**: domperidone; **MET**: metoclopramide; **cP**: carp pituitary; **SPE**: salmon pituitary extract; **SG-G100**: purified salmon gonadotropin; **tx:** antiestrogen tamoxifen; **GVBD**: injected dose caused germinal vesicle breakdown; **SHD:** shortening of homing duration; **p[LGA]**: polygycolic-polylactic biodegradable matrix; **EVAc**: ethylene vinyl acetate co-polymer; **FIA**: Freund´s incomplete adjutant; **ChBM**: cholesterol-based matrix implant; **RBI**: reproductive behavior improvement; **SIMP**: silastic implant; **BdM**: biodegradable microspheres; **SRI**: silicone rubber implant; **EM**: enteric microcapsules; **MD:** maturation diet; **ip**: intraperitoneal injection; **im**: intramuscular injection; **si**: surgical implant; **epi**: egg pore insert; *: egg quality alteration

1.8. EGG QUALITY FOLLOWING GNRHA TREATMENT

Good quality eggs are characterized by high fertilization, eying, and hatching rates; production of healthy alevins; and fast growing juveniles (Bromage et al., 1992b). Salmonids are a low fecundity species, and egg quality is a crucial consideration in hormone treatment decisions. Sources reporting egg quality data diverge considerably. Fitzpatrick et al. (1984), Mylonas et al. (1992), Taranger et al. (1992), Gillet et al. (1996), Olito et al. (2001), Mikolajczyk et al. (2005), Noori et al. (2010), and Bonnet et al. (2007) reported negative effects on eggs with GnRHa treatment, whereas Hunter et al. (1978), Hunter et al. (1981), Donaldson et al. (1981a), Donaldson et al. (1981b), Billard et al. (1984), Zohar et al. (1990), Slater et al. (1995), Arabaci et al. (2004), Park et al. (2007), and Vazirzadeh et al. (2008) did not observe changes in egg quality. Egg quality was reported to be improved by Sower et al. (1984), Jansen (1993), and Svinger et al. (2013b) (Chapter 2). Several hypotheses exist with respect to factors potentially affecting egg quality. Hyperstimulation of the pituitary by high GnRHa dose (100–150 $\mu g.kg^{-1}$) (Crim and Glebe, 1984; Haraldsson et al., 1993; Taranger et al., 1992; Olito et al., 2001) or its combination with a dopamine inhibitor, may result in secretion of high levels of LH (Billard et al., 1984; Gillet et al., 1996). This hypothesis is indirectly supported by the statement of Mylonas et al. (1992) that GnRHa treatment causes asynchrony between FOM and ovulation, and that the asynchrony can be intensified by high LH levels. However, low quality eggs are also commonly observed in salmonids stripped without hormone treatment. Rainbow trout eggs stripped immediately after ovulation were shown to have lower survival rates than eggs stripped 4 to 10 days post-ovulation (Sakai et al., 1975; Bry, 1981; Springate et al., 1984). Currently, post-ovulatory time required to attain a suitable level of egg ripeness in salmonid females is the only known reliable factor influencing fertilization success (Bromage et al., 1992b). This issue is further discussed in Chapter 5.

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CHAPTER 2

SYNCHRONIZATION OF OVULATION IN BROOK CHAR (*SALVELINUS FONTINALIS*, MITCHILL 1814) USING EMULSIFIED D-Arg^{6}Pro^{9}NEt sGnRHa

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Synchronization of ovulation in brook char (*Salvelinus fontinalis*, Mitchill 1814) using emulsified D-Arg^{6}Pro^{9}NEt sGnRHa

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Abstract Effects of physiological saline (PS)-dissolved or Freund′s incomplete adjuvant (FIA)-emulsified sGnRHa on the induction and advancement of ovulation in brook char were assessed. Two-year-old females were randomly divided into 5 groups. Groups A and B received intraperitoneal injection of FIA-emulsified sGnRHa (sGnRHa-FIA) at dosages of 50 and 25 μg kg^{-1} body weight (BW), respectively. Females in group C were treated with a double injection (DI) of PS-dissolved sGnRHa (sGnRHa-PS) at 25 μg kg^{-1} BW spaced 3 days apart. Fish in group D received a 25 μg kg^{-1} BW single injection (SI) of sGnRHa-PS. Group E was established as a control group. After stripping, ovarian plasma pH level was measured, and an egg sample was taken from each female to record egg weight and diameter and survival to the eyed stage. Females in the GnRHa-treated groups ovulated significantly earlier than did females in the control group ($P < 0.01$). No significant differences were found among GnRHa-treated groups in ovulation dynamics and mean time to ovulation. Ovarian fluid pH was significantly higher in groups A, B, and C compared to control group E ($P < 0.05$). Significantly lower egg weight was found only in group B ($P < 0.01$), although all advanced groups tended to have lower egg weight than the control group. Egg diameters paralleled egg weight. Survival to the eyed stage was significantly higher in GnRHa-advanced groups compared to the control. A negative relationship was found between egg weight end eyed eggs percent ($R^2 = 0.26$). No pre-spawning or post-spawning mortality was observed during a 6-month period. Neither sGnRHa-PS nor sGnRHa-FIA are associated with negative influences on the health of females. The sGnRHa-FIA injections proved to exhibit the same efficacy as the DI protocol with sGnRHa-PS. Although no statistical differences were found in ovulation dynamics, we do not recommend the use of SI with sGnRHa-PS in brook char.

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Keywords Brook char · Freund′s incomplete adjuvant · GnRHa · Synchronization of ovulation

Abbreviations

BW	Body weight
FIA	Freund′s incomplete adjuvant
FCR	Food conversion ratio
sGnRHa-FIA	Salmon gonadotropin-releasing hormone analog diluted in physiological saline and homogenized in FIA
sGnRHa-PS	Salmon gonadotropin-releasing hormone analog diluted in physiological saline
LH	Luteinizing hormone
MIS	Maturation-inducing steroid
MPF	Maturation-promoting factor
pGSI	Pseudo-gonadosomatic index
PS	Physiological saline
SGR	Specific growth rate

Introduction

Synthetic analogs of gonadotropin-releasing hormone neuropeptide (GnRHa) are effective in stimulating secretion of luteinizing hormone (LH) from the pituitary (Zohar et al. 2010). Luteinizing hormone stimulates ovarian production of maturation-inducing steroids (MIS), for example 17α,20β-DP and maturation-promoting factors (MPF), followed by ovulation (Nagahama and Yamashita 2008). The potential for using GnRHa to control reproduction has been well known for more than 3 decades. Its successful experimental use in salmonids has been widely reported for purposes such as limiting handling, stress reduction, shortening the spawning period, and better control of production at hatcheries (Breton et al. 1990; Goren et al. 1995; Gillet et al. 1996, Park et al. 2007). The simplest method of GnRHa delivery is with injection of GnRHa peptide dissolved in physiological saline. Since the GnRHa is rapidly degraded by endo-enzymatic processes (Goren et al. 1990; Zohar et al. 1990a), a single injection does not provide the prolonged and controlled LH secretion from the pituitary necessary for gonadotropin control of gametogenesis in salmonid species (Breton et al. 1983). Consequently, a double injection (DI) is needed, increasing the frequency of handling of the broodstock. To avoid this, sustained release preparations have been developed, including cholesterol pellets, ethylene–vinyl acetate implants, and biodegradable polyesters of lactic acid and glycolic acid (Mylonas and Zohar 2001). The high cost of these preparations, however, limits their practicality for brook char fry producers, since the mean size of cultivated broodstock often does not exceed 1.0–1.5 kg, which makes implantation difficult and dramatically raise the cost of treatment.

Adjuvants are used to initiate and augment the inflammatory reaction required for induction of an optimal innate and adaptive immune response to vaccines, as well as to ensure long-lived immunity (Safari et al. 2011). Adjuvants can also allow a lower dose and thereby increase the potency of antivenoms (Pratanaphon et al. 1997) and reduce vaccine costs (Singh and O'Hagan 1999). Freund′s incomplete adjuvant (FIA) mechanism of action is promotion of the formation of depots of antigen at the site of immunization (Guy 2007).

In salmonid species, the evidence for the efficacy of FIA as a carrier for GnRHa is restricted to rainbow trout (*Oncorhynchus mykiss*) (Arabaci et al. 2004; Vazirzadeh et al. 2008) and chum salmon (*Oncorhynchus keta*) (Park et al. 2007). There is, however, a wide range of sources describing the use of FIA in salmonid aquaculture for vaccination (Poppe and Breck 1997). Although the use of water-in-oil emulsion with GnRHa in FIA (GnRHa-FIA) appears promising, post-spawning broodstock mortality has been found in rainbow trout (Arabaci et al. 2004).

Egg quality from hormone-treated broodstock is still questionable. Salmonids are, in general, a low fecundity species. Reduction in egg quality through hormonal manipulation would dramatically increase the costs of treatment. A number of studies describe a negative effect of hormone intervention on egg quality in salmonid fishes (Fitzpatrick et al. 1984; Mylonas et al. 1992; Taranger et al. 1992; Olito et al. 2001; Mikolajczyk et al. 2005; Bonnet et al. 2007; Noori et al. 2010). Others have reported that egg quality following hormonal treatment remained unaffected (Hunter et al. 1978, 1981; Donaldson et al. 1981a, 1981b; Sower et al. 1984; Zohar et al. 1990a, b; Slater et al. 1995; Arabaci et al. 2004; Park et al. 2007; Vazirzadeh et al. 2008). Egg quality is influenced by many common exogenous factors, for example stress, water temperature, age of broodstock, so may not be the result of hormone treatment alone. Several markers can be used to determine the quality of egg batches (Craik and Harvey 1984; Lahnsteiner et al. 1999a, b; Lahnsteiner 2007). Ovarian fluid pH and egg weight and size are parameters easily detectable in the field (Kallert 2009) and potentially useable for determination of reproductive quality (Lahnsteiner et al. 1999b; Aegerter and Jalabert 2004; Mansour et al. 2008). We used these parameters as the main indicators of egg quality.

The brook char (*Salvelinus fontinalis*, Mitchill 1814) was introduced into the Czech Republic in the late nineteenth century, and its importance in Czech salmonid aquaculture is currently increasing. It has proven to be a suitable species for recirculation aquaculture systems because of its aggressive food intake, satisfactory growth rates, relative tolerance to a changing environment, and popularity among consumers. Demand for its interspecific hybrid with arctic char (*Salvelinus alpinus*), the so-called Alsatian char, is rising in Czech neighborhoods, especially in Germany. Increase in its production requires greater amounts of high-quality eggs. As in other salmonids, the spawning period of brook char can extend over more than 4 weeks. It is sensitive to fungal diseases such as saprolegniosis throughout the spawning period, which results in extensive loss of broodstock. Hormone treatments in artificial reproduction of brook char could be an effective tool to minimize these problems and to better control egg production.

The goal of this study was to investigate the efficacy of sGnRHa-FIA treatment on induction and synchronization of ovulation. Since this is the first investigation of GnRHa treatment in artificial reproduction of brook char, the efficacy of single and double injection (DI) of sGnRHa-PS was also evaluated. Additionally, the influence of all treatments on egg quality was assessed. Survival rate to the eyed stage was monitored in egg samples. Broodstock mortality and health was observed during a 6-month post-spawning period.

Materials and methods

Experimental design

At the beginning of October 2010, sexually maturing 2-year-old first spawning brook char females (350 ± 24 g) were transported from a commercial farm to the experimental

facility of the Faculty of Fisheries and Protection of Waters in Vodnany (South Bohemia, Loc: 49°N, 14°E) approximately 5 weeks prior to the peak of the natural spawning period. Females were randomly selected and divided into 5 groups ($n = 10$) each placed into a 0.8-m^3 raceway supplied with running water from the Blanice River for a 7-day acclimatization period. The oxygen level was 11.8 ± 0.5 mg l^{-1}. Mean water temperature decreased during the experiment from 9.6 °C initially to 5.5 °C and subsequently rose to 9 °C at the end of October. Fish were kept under a natural photoperiod regime for our geographic location. The experimental groups were intraperitoneally injected as follows:

Group A: sGnRHa-FIA at 50 μg kg^{-1} BW
Group B: sGnRHa-FIA at 25 μg kg^{-1} BW
Group C: DI of sGnRHa at 25 μg kg^{-1} BW with 3-day interval
Group D: SI of sGnRHa at 25 μg kg^{-1} BW
Group E: injected with physiological saline only

GnRHa preparation

Salmon D-Arg^{6}Pro^{9}NEt-GnRHa (Bachem AG, Germany) was used. To prepare GnRHa-FIA treatments, the GnRHa was dissolved in 0.9 % NaCl physiological saline (PS) and mixed with Freund′s incomplete adjuvant (FIA, Sigma Aldrich) 1:1v/v using an Ika T-10 homogenizer. Each female received a total volume of 0.5 ml GnRHa-FIA preparation. GnRHa-PS treatments were prepared by dilution of sGnRHa in 0.9 % NaCl to the required concentration. Concentration of sGnRHa in solution was 25 μg ml^{-1}. Females treated with GnRHa-PS injections (Group C and D) received 1 ml of solution per 1 kg BW.

Egg quality

Immediately after stripping, ovarian fluid was collected and pH was measured using a multifunctional inoLAB 720 pH meter (WTW, 823 62 Weilheim, Germany). Pseudogonadosomatic index (pGSI) was calculated as: pGSI = weight of stripped eggs × 100 × fish weight^{-1}. The diameter ($n = 35$) and absolute weight ($n = 35$) to the nearest 0.01 g (KERN 572-33) of non-hardened eggs randomly selected from each female were determined. A small sieve and fine filter paper was used to remove remaining ovarian fluid to ensure accurate measurements. Egg diameter was measured using Quick PHOTO CAMERA 2.2 software (Olympus, Hamburg, Germany) from photographs taken with a binocular microscope Olympus BX51 fitted with an Olympus E-510 digital camera.

Egg sampling, fertilization and incubation

A sample of 200 ± 10 eggs was taken from each female and fertilized with an equal volume of a sperm mixture from 4 to 5 males. Milt was collected at the time of fertilization using a 1-ml syringe for each male. Sperm was mixed in a small dish during the process of fertilization and examined for motility before use. Eggs were incubated in separate small incubators (see Kallert 2009, p. 22) equipped with independently controllable inflow. Incubators were placed in a recirculation system with a self-cooling system maintaining the water temperature at 6.1 ± 0.4 °C throughout the incubation period. Oxygen content during incubation was maintained at 11.4 mg l^{-1}, and pH level was in the range of 7.80 ± 0.02. Other water parameters such as Cl^-, Fe, NH_3, NH_4, N–NO_2, and N–NO_3 were well below limits that could negatively impact egg development. Dead white eggs

were removed and counted from the incubators using glass tube when eyed stage of living eggs was reached. Survival to the eyed stage was calculated in all samples as the percent of eyed eggs in the total number of eggs (survival to the eyed stage = number of dead eggs × 100 × total number of eggs^{-1}).

Post-spawning mortality observation

When the second injection was administered to group C, fish in all groups were monitored every 3 days for ovulation by manual stripping. Each stripped female was identified by a fin cut and removed from the group into a 54-m^3 concrete pond to observe post-spawning mortality for a 6-month period. The concrete pond was supplied with running water from the Blanice River. Mean water temperature was initially 8.9 ± 1.1 °C, decreasing during the winter to 0.5 ± 0.8 °C, and subsequently rising gradually to 12 ± 1.5 °C in April 2011 when observation was terminated. The mean oxygen content throughout this period was 12 ± 1.3 mg l^{-1}. Approximately 3–4 days after stripping, females began to accept food and were fed with a commercial diet for trout (Biomar, EFICO Enviro 920, 3 mm) (1.0–1.5 % of total biomass). Food conversion ratio (FCR) was calculated as: FCR = Food consumed × $(W_T - W_t)^{-1}$. Specific growth rate (SGR, % day^{-1}) was: SGR = [(ln W_T − ln W_t) × T^{-1}] × 100, where W_T and W_t represent fish weight at the start and the end of the observation period (T = 160–180 days). At the end of the observation period (April 14, 2011), fish were counted and killed and dissected to evaluate health status.

Statistical analysis

All data were analyzed by Statistica 9 Cz (StatSoft, Tulsa, USA). Ovulation dynamics and percent of ovulated females were analyzed using survival analysis (Z test). Differences in mean time to ovulation were analyzed using nonparametric multicomparison Kruskal–Wallis test. One-way ANOVA was used to characterize differences in pH levels of ovarian fluid and egg weight. Differences in egg diameter were assessed by hierarchical (nested) ANOVA with individual females nested in treatment. Linear regression analysis was applied to correlate egg weight and survival to the eyed stage. Nonlinear and linear regression model was used to correlate ovarian fluid pH and survival to the eyed stage. Differences in percent of eyed eggs were assessed using one-way ANOVA or Student′s t test after arcsin transformation. If significant differences were found by ANOVA, Tukey′s HSD test was applied for detailed multicomparison assay. The Kolmogorov–Smirnov test was used to prove normal distribution of the data following homoscedasticity verification by Chochran–Bartlett′s test. Broodstock mortality was compared by χ^2 test. A significance level (α) of 0.05 was applied to all tests except where indicated. Data are presented as mean ± SEM.

Results

Synchronization and advancement of the ovulation

First ovulations occurred on day 9 post first injection in all sGnRHa-treated groups. Ovulation rate in groups A, B, C, and D was 30, 60, 40, and 30 %, respectively (Fig. 1). On day 12, the percent of ovulated females increased to 70, 80, 100, and 50 % in groups A, B,

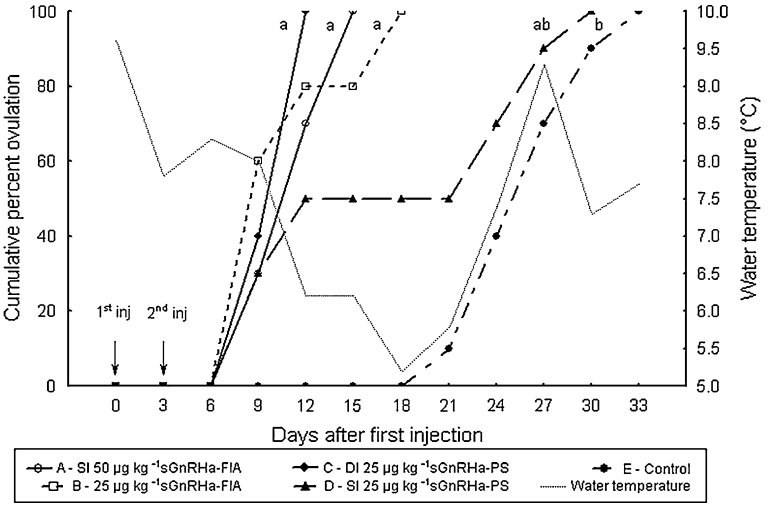

Fig. 1 Ovulation dynamic in brook char after four hormone treatments (**a** sGnRHa-FIA 50 µg kg^{-1}; **b** sGnRHa-FIA 25 µg kg^{-1}; **c** sGnRHa-PS DI 25 µg kg^{-1}; **d** sGnRHa-PS SI 25 µg kg^{-1}; **e** Control). Arrows indicate first and second hormone injection on day 0 and 3, respectively. *Curves* with the different *letters* are significantly different (survival analysis, $P < 0.05$)

C, and D, respectively, with 100 % of the fish in group C stripped within 3 days (Fig. 1). On day 15, 100 % of fish in group B were ovulated, and on day 18 100 % of fish in group A were ovulated. Ovulation was arrested from day 12 to day 21 in group D. Fish in this group were observed to be once again ovulating on day 24, once more reaching 100 % ovulation on day 30. In the control group E, first ovulation was recorded on day 21. The ovulation rate in this group increased gradually, and 100 % of females were stripped by day 33 of the experiment. The advancement of ovulation in hormone-treated groups compared to the control was shown to be highly significant with survival analysis ($\chi^2 = 23.5$; $df = 4$; $P = 0.0001$). Mean time to ovulation in groups A, B, and C was 12 ± 2.4; 11.4 ± 3.7, and 10.8 ± 1.5 days, respectively, and differed significantly from the control group E (26.7 ± 3.7 days) using Kruskal–Wallis multicomparison test ($n = 50$; $df = 4$; $P < 0.01$) (Fig. 2). Mean time to ovulation in group D was an intermediate value, 18.3 ± 8.8 days, and no statistical differences were found between group D and groups A, B, C, or E (Fig. 2).

Egg quality

The highest ovarian fluid pH levels were found in groups with the most advanced ovulation, A, B, and C (8.46 ± 0.07, 8.38 ± 0.09, and 8.38 ± 0.17, respectively). Significant differences were found only between group A and groups D and control group E (8.29 ± 0.09 and 8.25 ± 0.13, respectively) using Tukey′s HSD test ($P = 0.025$ and $P = 0.003$ for group D and E, respectively) (Table 1).

Generally, egg weight tended to be lower in the hormone-treated groups. However, significantly lighter eggs than in group E (25.2 ± 1.8 mg) were detected in group B (20.3 ± 2.9 mg) ($P < 0.01$). In addition, egg weight in group A (21.7 ± 1.7 mg) compared to group E appeared to be close to the level of significance ($P = 0.054$), suggesting

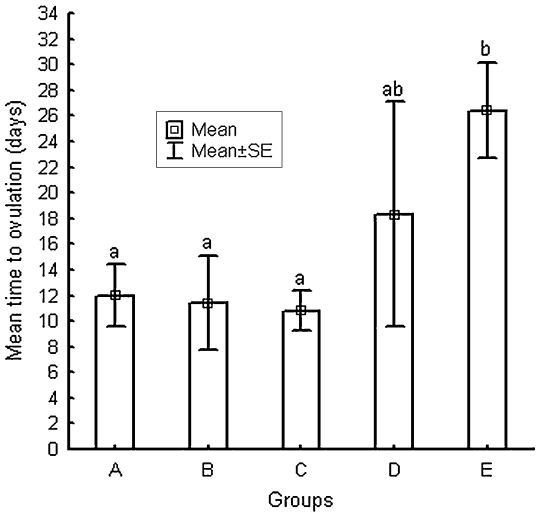

Fig. 2 Mean time to ovulation dependent on hormone treatment (**A** sGnRHa-FIA 50 μg kg^{-1}; **B** sGnRHa-FIA 25 μg kg^{-1}; **C** sGnRHa-PS DI 25 μg kg^{-1}; **D** sGnRHa-PS SI 25 μg kg^{-1}; **E** Control). Means (± SE, $n = 10$) with the different *letters* are significantly different (Kruskal–Wallis test)

Table 1 pGSI, ovarian fluid pH, egg weight, egg diameter, and eyed egg values of brook char females treated with GnRHa treatments

Treatment	pGSI (%) Mean ± SE $n = 10$	Ovarian fluid pH Mean ± SE $n = 10$	Egg weight (mg) Mean ± SE $n = 10$	Egg diameter (mm) Mean ± SE $n = 350$	Eyed eggs (%) Mean ± SE $n = 10$
A	8.8 ± 2.3^{a}	8.46 ± 0.07^{c}	21.7 ± 1.7^{ab}	3.65 ± 0.24^{b}	27 ± 22^{b}
B	7.3 ± 2.9^{a}	8.38 ± 0.09^{bc}	20.3 ± 2.9^{a}	3.61 ± 0.18^{a}	39 ± 24^{b}
C	6.5 ± 3.4^{a}	8.38 ± 0.17^{bc}	22.5 ± 3.0^{ab}	3.72 ± 0.25^{c}	30 ± 18^{b}
D	8.6 ± 2.2^{a}	8.29 ± 0.09^{ab}	22.7 ± 2.9^{ab}	3.73 ± 0.26^{c}	22 ± 24^{ab}
E	9.1 ± 2.8^{a}	8.25 ± 0.13^{ab}	25.2 ± 1.8^{bc}	3.81 ± 0.17^{d}	5 ± 5^{a}

Values with the different letters are significantly different (one-way ANOVA followed by Tukey′s HSD multiple comparison, $P < 0.05$)

that if more cases were obtained, statistical differences may be demonstrated also in this group. There were, however, no egg weight differences found between groups C and D (22.4 ± 2.9 mg and 22.7 ± 2.9 mg, respectively) in comparison with any of the other groups. No significant differences were found in pGSI level among the treatments (Table 1).

Egg diameter was found to correspond to the trends in egg weight (Table 1). Comparison of the data by hierarchical (nested) ANOVA detected significant differences among both individual females and treatment groups ($P < 0.01$ for both). The analysis of egg diameter with Tukey′s HSD test showed significant differences among all treatments (MSE = 0.0165; $df = 1632$; $P < 0.01$), except between groups C and D (Table 1), with the smallest eggs being found in groups A and B (3.65 ± 0.24 mm and 3.61 ± 0.18 mm, respectively) and largest eggs in the control group E (3.81 ± 0.17 mm).

Significantly higher survival to the eyed stage was seen in groups A, B, and C (27 ± 22 %, 39 ± 24 %, and 30 ± 18 %, respectively) compared to 5 ± 5 % in the control group, E ($P < 0.05$, 0.01, and 0.01 for groups A, B, and C, respectively, with

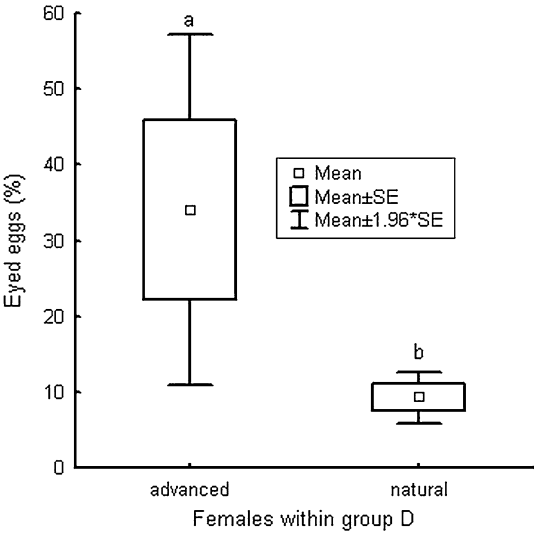

Fig. 3 Differences in survival to the eyed stage between eggs obtained from hormonally advanced females (advanced) and naturally ovulating females (natural) in group D sGnRHa-PS SI 25 μg kg^{-1}. Means (± SE, $n = 5$) with the different *letters* are significantly different (Student's t test)

Tukey's HSD test). Percent of eyed eggs in group D was 22 ± 24 % and did not differ from either control group E or other treatment groups (Table 1). Analysis with Student's *t* test showed that the 50 % of fish in group D that ovulated in advance (days 9 and 12) (Fig. 1) exhibited a significantly higher percent of eyed eggs (41 ± 24 %) than did those that ovulated beginning on day 24 (9 ± 4 %) ($P = 0.035$) (Fig. 3). This discrepancy was not observed in the other groups. In addition, the females in the advanced ovulation groups tended to produce lighter and smaller eggs, had higher pH levels of ovarian fluid (Table 1), and higher survival rates to the eyed stage. However, after plotting the data from all females into a single linear regression model (Fig. 4), we found a low negative relationship between egg weight and survival to the eyed stage ($R^2 = 0.26$). Those eggs that weighed less than 23 mg exhibited slightly higher survival to the eyed stage than eggs weighing more (Fig. 4.). The relationship between ovarian fluid pH and the percent of eyed eggs was low ($R^2 = 0.12$), as analyzed using a nonlinear regression model (Fig. 5). Detailed regression analysis within individual groups revealed that some females producing eggs at the extreme small end of the range exhibited higher ovarian fluid pH with decreased survival to the eyed stage, for example group A (Tables 2, 3). In addition, in some groups, neither the non-hardened egg weight nor the ovarian fluid pH showed an association with the percent of eyed eggs (Tables 2, 3).

Post-spawning mortality of broodstock

No mortality was recorded throughout the 6-month period in any of the groups. In counting the fish in April 2011, however, we found fewer than expected. The percent of remaining fish in groups A, B, C, D, and E was 90, 100, 90, 90, and 80 %, respectively. All surviving females were in excellent condition, and mean final weight (720 ± 47 g) did not differ among the groups. No differences were detected in either FCR or SGR levels at the end of the monitoring period. Specific growth rate was approximately 0.47 % day^{-1}, and FCR levels were 1.1–1.2 in all groups. Fish willingly accepted food even when water temperature decreased to below 1 °C and the holding pond was fully covered in ice. Normally developing ovaries were found in each dissected fish, and no visible abnormalities

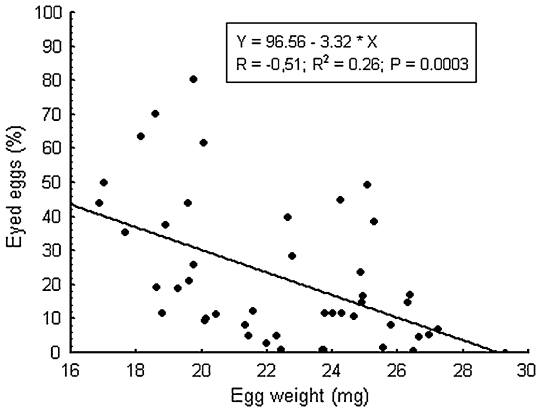

Fig. 4 Relationship between egg weight and survival to the eyed stage from all experimental females (linear regression model)

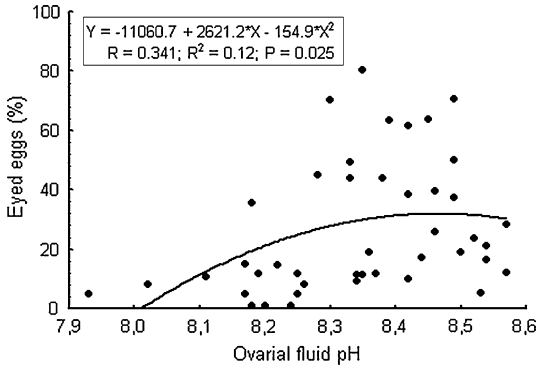

Fig. 5 Relationship between ovarian fluid pH and survival to the eyed stage from all experimental females (nonlinear regression model)

Table 2 Simple regression models describing the relationship between eyed egg percent and ovarian fluid pH

Treatment	R	P	R^2	Equation
A	0.22	0.55	0.05	$y = -799.6 + 97.96x$
B	−0.03	0.94	0.001	$y = 103.14 - 7.08x$
C	−0.61	0.08	0.37	$y = 976.1 - 111.9x$
D	0.32	0.4	0.1	$y = -537 + 67.21x$
E	−0.42	0.25	0.18	$y = 147.4 + 17.31x$

(intra-abdominal lesions, bruises, hematomas, or granulomas) were observed in either body wall or organ epithelium.

Discussion

Synchronization and advancement of the ovulation

In our study, all sGnRHa (D-Arg^6,Pro^9,NEt-GnRHa) treatments significantly advanced and synchronized ovulation of brook char compared to the control. These results are consistent with results of other authors using different or the same synthetic GnRHas in salmonids

Table 3 Simple regression models describing the relationship between eyed egg percent and weight of non-hardened eggs

Treatment	R	P	R^2	Equation
A	0.90	0.0008	0.82	$y = -162.73 + 9.1x$
B	0.53	0.22	0.28	$y = 117.43 + 3.7x$
C	−0.21	0.58	0.05	$y = 59.18 + 1.15x$
D	−0.61	0.08	0.37	$y = 126.51 + 4.65x$
E	−0.007	0.99	0.0	

such as (D-Ala^6,Pro^9,NEt-GnRHa) (Erdahl and McClain 1987; Taranger et al. 1992; Mylonas et al. 1992; Haraldsson et al. 1993), (D-Arg^6,Pro^9,NEt-GnRHa) (Jansen 1993) and ([D-Nal(2)^{6}aza-Gly^{10}]-GnRHa) (Mikolajczyk et al. 2008). Both sGnRHa-FIA (25 or 50 μg) and sGnRHa-PS double injection of 25 μg were highly effective in accelerating and synchronizing ovulation in brook char. An ovulation rate of 100 % was recorded within 3 days in those females treated with DI of 25 μg. Fish in both 25 μg and 50 μg sGnRHa-FIA treated groups showed nearly the same ovulation pattern as those treated with the DI protocol. These results are similar to previous experiments conducted in rainbow trout (*Oncorhynchus mykiss*) using FIA as a carrier of ([D-Ser(tBu)^6,Pro^9-NEt]-GnRHa) and (D-Arg^6,Pro^9,NEt-GnRHa) (Arabaci et al. 2004; Vazirzadeh et al. 2008).

A SI of sGnRHa-PS is economically the most advantageous. However, a SI of sGnRHa-PS at 25 μg in our study evoked synchronization of ovulation in only 50 % of the females. Subsequently, ovulation in this group was arrested for 12 days and recurred concomitant with the first natural ovulations in the control group beginning day 24. This corresponds with other experiments in salmonids and/or coregonids, in which a single GnRHa injection of ([D-Ser(tBu)^6,Pro^9-NEt]-GnRHa) (Arabaci et al. 2004), ([D-Nal(2)^{6}aza-Gly^{10}]-GnRHa) (Mikolajczyk et al. 2005), (D-Tle^6,Pro^9,NEt-GnRHa) (Svinger et al. 2010) and (D-Ala^6,Pro^9,NEt-GnRHa) (Noori et al. 2010) was only partially successful in inducing ovulation. This failure of SI in some individuals may be due to rapid clearance of effective GnRHa from the circulation profile (Zohar et al. 1990b), leading to insufficient LH plasma levels (Breton et al. 1990). Ovulation in such individuals is interrupted and continues some time later in response to natural secretion of native GnRH peptide from the hypothalamus.

Our results suggest that the SI of 25 μg is insufficient to induce a high ovulation rate compared to other assessed treatments in brook char. However, Taranger et al. (1992) report that even 1 μg kg^{-1} BW of GnRHa was effective in Atlantic salmon (*Salmo salar*) if given close to natural ovulation time. Similar results were obtained by other authors (Crim and Glebe 1984; Crim et al. 1986; Fitzpatrick et al. 1984). In our experiment, hormone treatments were administered approximately 4 weeks prior to natural ovulation time at 9–9.5 °C water temperature, which is approximately 3.5–4.0 °C above normal spawning temperature of brook char in our region. There is strong evidence that elevated temperatures impede peri-ovulatory physiological readiness of females via an inhibitory effect of dopamine on LH release from pituitary in Arctic char (*Salvelinus alpinus*) (Gillet et al. 1996; Gillet and Breton 2009). Impairment of ovarian steroidogenic activity at elevated temperatures has been reported in Atlantic salmon (King and Pankhurst 2004a, b; King et al. 2003; Watts et al. 2004; Vikingstad et al. 2008; Pankhurst and King 2010) and rainbow trout (Pankhurst et al. 1996; Pankhurst and Thomas 1998). Whether these mechanisms were, at least partially, in effect during the first and second injection periods, at 9 °C, remains unclear. If so, they seemed to have been fully counteracted by GnRHa-FIA treatment and the double injection, while the single injection of 25 μg did not have such a strong counteraction. It has to be noted that higher effectiveness of DI applied in

group C could be caused by the higher total dose. Nevertheless, the theory of low counteraction ability of single injection seems to be more probable since double injection of 12.5 μg sGnRHa-PS and 12.5 μg of sGnRHa-FIA was much more effective than single injection of 50 μg sGnRHa-PS at 8.5 °C in an additional experiment carried out 1 year later (in 2011) in the same strain of brook char (Svinger, unpublished results). Similar phenomenon was observed in Northern whitefish (*Coregonus peled*), where double injection of 5 μg mGnRHa was far more effective compared to single injection of 25 μg (Svinger et al. 2010), and Breton et al. (1990) reported 100 % ovulation in rainbow trout treated with GnRHa-sustained release form, despite dosages of 12.5–50.0 μg, whereas 100 % ovulation rate was never achieved in those females treated with acute injections at dose of 20 μg. These suggest that hormone delivery method (sustained release or multiple injections versus single injection) is more important than the dose of hormone used.

Exposure of salmonids to colder water appears to accelerate steroidogenic activity (Vikingstad et al. 2008) and suppress dopamine inhibitory tone on LH secretion (Gillet et al. 1996; Gillet and Breton 2009) allowing onset of natural ovulation time. This is supported by the fact that females in the control group began to ovulate some days after a sharp drop in water temperature to 5.5–6.0 °C (Fig. 1). Thus, we cannot exclude the possibility that if SI is given later at lower water temperatures, the physiological system of the fish will respond more favorably. Thorough research to determine temperature levels that affect or modulate final stages of the reproductive cycle in brook char is necessary.

Egg quality

The percent of eyed eggs was low in all groups. This was probably due to utilization of first spawners (Kallert 2009) and, in addition, extremely high late summer temperatures. This heat resulted in elevated water temperatures (19–20 °C) at the production farm from which the char was obtained. High temperature during vitellogenesis in salmonids is commonly known to result in reduced egg quality in the ensuing spawning season (Gillet 1991; Taranger and Hansen 1993; Pankhurst et al. 1996).

However, the most GnRHa-accelerated groups showed significantly higher survival to the eyed stage than the control group. The 50 % of the females in the SI group that ovulated in advance produced a significantly higher percent of eyed eggs than did the fish that released eggs simultaneously with the control group. This is in direct contradiction with other reports where the early stripped females of brown trout (*Salmo trutta*) (Mylonas et al. 1992), Atlantic salmon (Crim and Glebe 1984), coho salmon (*Oncorhynchus kisutch*) (Fitzpatrick et al. 1984), and chum salmon (Park et al. 2007) produced lower fertilization and survival rates than did later stripped individuals. On the other hand, a number of studies have reported that hormone treatment did not result in alteration of egg quality (Jalabert et al. 1978; Donaldson et al. 1981a; Hunter et al. 1981; Billard et al. 1984; Arabaci et al. 2004; Vazirzadeh et al. 2008), or that eggs obtained from hormone-treated females had higher survival rates than those from spontaneously ovulating females (Sower et al. 1984). Mylonas et al. (1992) explain these discrepancies as slight asynchrony between the process of meiotic maturation regulated by the maturation-inducing steroids and the process of ovulation regulated by prostaglandins. Gillet et al. (1996) found the percent of eyed eggs in Arctic char to be negatively correlated with the plasma LH level, which could be the major factor in the asynchrony causing reduction in egg quality. This was also suggested by Billard et al. (1984). In addition, Gillet and colleagues report that plasma LH level is not the only factor involved in the control of egg quality, citing variable responsiveness of females to GnRH injection and the difference in maturation stage of

females at the time of treatment. Since plasma LH levels were not measured in our study, we are unable to say whether any of the hormone treatments elicited such phenomenon in brook char or whether it influenced egg quality, since eggs of treated females exhibited higher survival rates.

Nevertheless, if each brood fish at the time of hormone treatment is at a slightly different physiological and maturation stage, there should be accompanying measurable parameters by which the sexual product quality is being influenced. Both egg size and ovarian fluid pH levels are usable and easily detectable parameters for egg quality assessment. Decreased pH of ovarian fluid caused by water inflow into the coelomic cavity, ovarian secretion changes, or injection of intracellular content of broken eggs into ovarian fluid (Lahnsteiner et al. 1999b; Dietrich et al. 2007; Wojtczak et al. 2007) reduces sperm motility and promotes premature egg hydration resulting in lower egg fertilization (Aegerter and Jalabert 2004).

In our study, females in the most GnRHa accelerated groups (A, B, and C) produced smaller and lighter eggs and had higher ovarian fluid pH levels. The significance was most noted in egg diameters, where the highest number of observations was made ($n = 350$ for each group). These groups exhibited also higher percent of eyed embryos compared to the control group. The most comparable results to these were reported by Mansour et al. (2008), who found that, in Arctic char held at 7 °C, good-quality eggs exhibited lower absolute non-hardened egg weight and egg diameter accompanied by higher ovarian fluid pH in comparison with poor-quality eggs. Similar results were found by Aegerter and Jalabert (2004) in rainbow trout. Mansour et al. (2008) considered poor-quality eggs as over-ripe. We found, in contrast, that there was a strong positive relationship between egg weight and eyed egg percent ($R^2 = 0.81$) in group A. Further, some females of this group exhibited the highest levels of ovarian fluid pH followed by low survival to the eyed stage, suggesting that some non-ripe females in this group may have been stripped. Elevated spawning water temperatures, which occurred at the time of the first ovulation of females in the control group, might have also accelerated the over-ripening process in some individuals in group D and controls, which resulted in low survival rates at the eyed stage.

When data from all females were plotted into simple regression models, only low relationships between non-hardened egg weight, ovarian fluid pH, and survival to the eyed stage ($R^2 = 0.26$ and $R^2 = 0.12$ for egg weight and ovarian fluid pH, respectively) were found. In contrast, Lahnsteiner et al. (1999b) found a much higher regression coefficient ($R^2 = 0.46$) in a simple regression model describing the relationship between ovarian fluid pH and eyed egg percent in lake trout (*Salmo trutta lacustris*). When detailed regression analysis was applied in our study, the highest correlations were found in groups C and E, $R^2 = 0.37$ and $R^2 = 0.18$, respectively. This may be due to more accurate pH measuring or to other factors involved in survival to the eyed stage (Lahnsteiner et al. 1999b; Lahnsteiner 2007). The limited number of observations could also have played a role. Further, most ovarian fluid pH levels were in the range of 8.2–8.4, which could diminish the possibility of detection of distinct relationships, since this is considered to be the most suitable level in salmonid species (Wilcox et al. 1984; Lahnsteiner et al. 1999b; Mansour et al. 2008).

The hormone-treated females were stripped in a short window of time, which markedly reduced the frequency of anesthetizing and handling necessary when monitoring for ovulation, in comparison with group D and control group E. Enhanced stress at the final stage of reproductive development can not only result in disruption of reproductive endocrinology, but can also affect egg size and survival of the progeny in rainbow trout and brown trout (Campbell et al. 1992, 1994). Stress response differences are found among

salmonids and even within different strains within a salmonid species (Fevolden et al. 1991; Pottinger and Pickering 1992; Heath et al. 1993). How, and to what extent, the increased handling influenced measured parameters and survival rates in brook char in this study remains unclear.

Mortality of broodstock

Since we did not find dead fish throughout the 6-month post-treatment period, 100 % survival was expected in all groups. However, after counting the broodstock in April 2011, we observed that numbers were reduced in all groups with the exception of group B (25 μg sGnRHa-FIA), which had 100 % survival rate. These missing fish were most probably eaten by gray herons (*Ardea cinerea*), which often visit our experimental facility before the ponds are covered by ice. Nevertheless, there were no statistical differences found among all groups in terms of post-spawning mortality, which is in agreement with other studies where GnRHa treatments were found to improve survival of the broodstock (Mikolajczyk et al. 2005; Goren et al. 1995). In contrast, GnRHa-FIA treatment has been reported to inflict high post-spawning mortality in rainbow trout (Arabaci et al. 2004). The authors do not present any rationale for the high mortality. In a more recent study, GnRHa-FIA was not associated with post-spawning mortality in rainbow trout 3 months post-injection (Vazirzadeh et al. 2008). These authors suggest that this was due to a lesser amount of FIA used compared to the study by Arabaci et al. (2004). In our current experiment, the ratio of FIA to weight of treated females was even higher than that used by Arabaci et al. (2004); hence we cannot confirm this suggestion.

In salmonid aquaculture, adjuvants are widely used in vaccines against various pathogens, for example *Vibrio salmonicida* and *Aeromonas salmonicida*. Most reported side effects induced by mineral oil adjuvant vaccines are lesions at the injection site, abdominal adhesions, post-vaccination mortality, poor feed uptake and conversion, retarded growth, and downgrading at slaughter (Lillehaug et al. 1992; Press and Lillehaug 1995; Midtlyng et al. 1996; Poppe and Breck 1997). In the current study, no abnormalities were found in any GnRHa-FIA-treated brook char. The fish appeared to be in excellent condition throughout the observation period and possessed active appetite even at low temperatures 0.2–3.5 °C from late November until late February. However, both lower FCR (0.8–0.9) and higher SGR (0.6–0.7 % day^{-1}) levels than in our experiment are usually observed in intensively reared brook char (Reiter 2006). These discrepancies may be unrelated to GnRHa-FIA treatment and may more likely be associated with fish in our study being kept for long periods at very low temperatures and/or at low stocking density (Jørgensen et al. 1993; Jobling et al. 1993), which was only 0.3–0.65 kg m^{-3}. There were no differences found in either FCR or SGR among any of the groups. Based on our results, neither sGnRHa-PS nor sGnRHa-FIA are associated with negative influences on the health of brook char females.

Conclusions

All hormone treatments were effective tools to advance and synchronize ovulation in brook char except the single acute injection, which cannot be recommended for these purposes if administered 3–4 weeks prior to natural spawning period and at water temperature above 8 °C. Both sGnRHa-FIA treatments exhibited strong advancing and synchronizing effects similar to the double acute injection and thereby provided effective single injection

protocols. Handling of broodstock was also minimized by these treatments, which can limit stress. Nevertheless, our results suggest that hormone treatment may have a partial capacity to impact characteristics of ovarian fluid and thereby influence survival of eggs in brook char. Further, although survival rates to the eyed stage were higher in advanced ovulation groups, egg size in these groups was significantly decreased with unknown subsequences for hatching and the progeny. Other studies are necessary for more detailed specification of these changes, which could help to develop more efficient hormone therapies for brook char.

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CHAPTER 3

SYNCHRONIZATION OF OVULATION IN CULTURED NORTHERN WHITEFISH (*COREGONUS PELED*, GMELIN 1788) USING [D-Arg^{6}Pro^{9}Net]-sGnRH ANALOGUE AND ITS EFFECT ON EGG QUALITY

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Synchronization of ovulation in cultured northern whitefish (*Coregonus peled*, Gmelin 1788) using [D-Arg^{6}Pro^{9}Net]-sGnRH analogue and its effect on egg quality

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Abstract

Female northern whitefish were treated with salmon [D-Arg^{6}Pro^{9}Net]-sGnRHa emulsified in Freund's incomplete adjuvant as a sustained release treatment (sGnRHa-FIA) or with [D-Arg^{6}Pro^{9}Net]-sGnRHa dissolved in physiological saline as acute release treatment (sGnRHa-PS). Females in four experimental groups (A, B, C, D) and one control group (E) were injected intraperitoneally as follows: A and B – sGnRHa-FIA at doses of 50 and 25 μg kg^{-1}, respectively; C – double injection (DI) of sGnRHa-PS at 25 μg kg^{-1} administered 3 days apart; D – single injection (SI) of sGnRHa-PS at 25 μg kg^{-1}; and E – control group receiving a 1:1 mixture of FIA and physiological saline. All treatments induced and synchronized ovulation. Mean time to ovulation was significantly reduced ($P < 0.05$) in GnRHa-treated groups compared with control. Hormone treatments did not affect the relative fecundity. Slight differences were found in ovarian fluid pH between group A and D ($P < 0.05$). Except for group C, egg size was significantly reduced ($P < 0.01$) in hormonally synchronized groups compared with the control. Survival to the eyed stage of eggs from females in groups A, B and C was significantly lower ($P < 0.01$) than in groups D and E. Per cent hatched alevins was lower ($P < 0.01$) in groups A, B and C than in groups D and E. We conclude that synchronization of ovulation in northern whitefish can be induced by a single acute injection of [D-Arg^{6}Pro^{9}NEt]-sGnRHa at 25 μg kg^{-1} BW if given near the natural spawning time determined by water temperature below 2°C.

Keywords: induced ovulation, *Coregonus peled*, [D-Arg^{6}Pro^{9}Net]-sGnRHa, Freund's incomplete adjuvant, egg quality

Introduction

Synthetic analogues of gonadotropin-releasing hormone (GnRHa) have proven effective in inducing and synchronizing ovulation in salmonids (Crim & Glebe 1984; Erdahl & McClain 1987; Kouřil, Barth, Štěpán, Fila, Příhoda & Flegel 1987; Breton, Weil, Sambroni & Zohar 1990; Mylonas, Hinshaw & Sullivan 1992; Gillet, Breton & Mikolajczyk 1996; Olito, Loopstra & Hansen 2001; Haffray, Enright, Driancourt, Mikolajczyk, Rault & Breton 2005; Mikolajczyk, Skolowska-Mikolajczyk, Szczerbik, Duc, Goryczko, Dobosz, Glogowski, Epler & Enright 2008; Noori, Amiri, Mirvaghefi & Baker 2010). In contrast to cyprinids, hormone therapy in salmonids mostly does not involve the use of dopamine inhibitors to suppress the inhibitory tone of dopamine on luteinizing hormone (LH) secretion from the pituitary (Podhorec & Kouřil 2009). Salmonids require a more prolonged LH surge, which can be achieved through sustained-release preparations (Breton *et al.* 1990; Goren, Gustafson & Doering 1995; Zohar & Mylonas 2001) or by multiple acute injections of the peptide (Mylonas *et al.* 1992). These methods of GnRHa delivery are not always compatible with fish farming practice due

to high cost, limited availability and broodstock properties such as small size or fragility.

In immunology, adjuvants are used to initiate and augment the inflammatory reaction necessary for induction of optimal innate and adaptive immune responses to vaccines, as well as to ensure long lasting immunity (Safari, Dekker, Rijkers & Snippe 2011). Adjuvants can also increase the potency of antivenoms, thereby allowing a lower dose (Pratanaphon, Akesowan, Khow, Sriprapat & Ratanabanangkoon 1997) and reducing costs (Singh & O'Hagan 1999). In salmonid artificial reproduction, Freund's incomplete adjuvant (FIA) has been used as a GnRHa carrier. Such GnRHa-FIA mixtures have shown an effect similar to that of commercial sustained release formulas when administered to rainbow trout (*Oncorhynchus mykiss*) (Arabaci, Diler & Sari 2004; Vazirzadeh, Hajimoradloo, Esmaeili & Akhlaghi 2008) and chum salmon (*Oncorhynchus keta*) (Park, Lee, Lee, Kim, Kim, Tamaru & Sohn 2007). Most recently GnRHa-FIA was successfully used for advancement and synchronization of ovulation in small-size and sensitive broodstock of brook char (*Salvelinus fontinalis*) (Svinger, Policar, Polakova, Steinbach, Jankovych & Kouril 2012).

Coregonid farming has a long history in the Czech Republic. In 1890, European whitefish (*Coregonus lavaretus* L.) were introduced to be extensively reared in polyculture with common carp (*Cyprinus carpio*) in deep ponds with cool water conditions. Northern whitefish (*Coregonus peled*), able to tolerate warmer water than the European whitefish, were introduced from Siberia 80 years later (Hochman 1987). In 1997, market-size whitefish production ranged approximately 140 tons (Annual report 2002, Czech Ministry of Agriculture). In the past decade, however, coregonid farmers have been challenged by expansion in the population of cormorants (*Phalacrocorax carbo sinensis*), and annual production decreased dramatically to 24 tons (Annual report 2011, Czech Ministry of Agriculture). The harvested survived fish are often severely wounded and exhibit signs of stress (Adamek, Kortan & Flajshans 2007). This is especially seen in the fragile northern whitefish.

Northern whitefish mature at 2 years. Similar to some salmonid species, spontaneous ovulation is extended over several weeks, and broodstock must be frequently checked for ovulatory status, due to early occurring arthretic processes occurring in eggs as early as 2–3 days after ovulation (Hochman 1987). In addition, unpredicted mass ovulation may occur in this species especially following a sharp drop in winter water temperatures to below 2°C (Hochman 1987). The combination of wounded broodstock and frequent handling results in high pre-spawning mortality, making artificial reproduction of this species difficult and unprofitable.

The first use of GnRHa to optimize coregonid broodstock management was reported by Mikolajczyk, Kuźmiński, Dobosz, Goryczko and Enright (2005) using [D-Nal(2)^{6}aza-Gly^{10}]-GnRHa azagly-nafarelin (Gonazon®) in European whitefish. Svinger, Kouril and Pavlista (2010) attempted to synchronize ovulation in northern whitefish using [D-Tle^{6}Pro^{9}NEt]-GnRHa Lecirelin (Supergestran®). While single acute injections of 16 and 32 μg kg^{-1} body weight (BW) of the Gonazon induced and synchronized ovulation in European whitefish (Mikolajczyk *et al.* 2005), a single acute injection of 25 μg kg^{-1} BW was not effective in northern whitefish, and a double injection protocol had to be employed to achieve the desired effect. This suggests that the northern whitefish requires the more prolonged stimulation of continuously elevated LH levels, which are ensured by GnRHa emulsified in FIA.

Good quality eggs are usually defined as those yielding low levels of mortality at fertilization, eying, hatch and first-feeding and those that produce the fastest-growing and healthiest fry and older fish (Bromage, Jones, Randall, Thrush, Davies, Springate, Duston & Barker 1992). Although the effectiveness of GnRHa on synchronizing ovulation in salmonids has been widely reported, thorough investigations of the impact of hormone treatments on egg quality and progeny are lacking, not only in northern whitefish, but in the subfamily Coregoninae in general.

The objectives of the present study were to investigate efficacy of GnRHa treatments on induction and synchronization of ovulation and its effect on egg quality in northern whitefish.

Material and methods

The experiment was carried out at the experimental facility of Faculty of Fisheries and Protection of Waters (FFPW). On November 24, 2010, 160 sexually mature 3-year-old northern whitefish of the same strain were transported to FFPW from Kinsky JSC Fish Farm, Czech Republic, Zdar nad

Sazavou (49°35'1"N, 15°56'13"E). After transport, fish were sorted by sex, and 60 females (599 ± 73 g) were randomly divided into five flow-through raceways (0.8 m^3) supplied with water from the Blanice River and held 10 days for acclimatization under the natural photoperiod. Sixty spermiating males (550 ± 126 g) were kept in a single raceway (4 m^3) under similar water conditions. Only healthy and non-injured size-balanced fish were chosen for the experiment. Water temperature during acclimatization was gradually decreased from 3°C to 0.5°C at hormone administration. Oxygen content was 12.1 ± 0.8 mg L^{-1} throughout the experiment. Fish were not fed during the experiment.

On December 5 (approximately 14–21 days before natural ovulation occurs in northern whitefish at this latitude), females were anaesthetized (clove oil, 0.03 mL L^{-1}) and intraperitoneally treated with synthetic salmon gonadotropin-releasing hormone analogue [D-Arg^{6}Pro^{9}NEt]-sGnRHa (Bachem AG, Germany) as follows:

Group A: sGnRHa-FIA at 50 μg kg^{-1} BW
Group B: sGnRHa-FIA at 25 μg kg^{-1} BW
Group C: acute double injection (DI) of sGnRHa at 25 μg kg^{-1} BW given 3 days apart
Group D: acute single injection (SI) of sGnRHa at 25 μg kg^{-1} BW
Group E: control, injected with 0.9% NaCl mixed with FIA 1:1v/v (0.5 mL per individual)

To prepare GnRHa-FIA, GnRHa was dissolved in 0.9% NaCl and mixed with FIA (Sigma Aldrich, Czech Republic) 1:1 v/v using an Ika T-10 homogenizer. Each female received a total volume of 0.5 mL GnRHa-FIA preparation. For acute treatments, sGnRHa was diluted in 0.9% NaCl to the required concentration (25 μg mL^{-1}). Females treated with acute injections (Group C and D) received 1 mL of solution per 1 kg BW. A quantity of 1 mL insulin in syringes fitted with a 0.7 × 30 mm needle was used for the administration of sGnRHa-FIA treatments. Acute treatments were made using a 1 mL insulin syringe fitted with a 0.33×12 mm needle.

After the second injection in group C, the ovulation status of each female was checked every 3 days by manual stripping. Females were considered to have ovulated if eggs were released with gentle manual pressure on the abdomen. Ovulating females were weighed and eggs were stripped. The total amount of eggs obtained from each female was weighed to calculate relative fecundity (RF = weight of stripped eggs × 100 × g^{-1} fish weight). Stripped females were tagged by fin cut for group identification and held for a short time in a weak potassium permanganate ($KMnO_4$) bath, after which they were placed into 54 m^3 concrete pond to observe post-spawning mortality for a 3 month period. At the end of the observation period (31 March 2011) fish were counted, killed and dissected to evaluate health status.

Immediately after stripping, ovarian fluid was collected and pH was measured using a multifunctional inoLAB 720 pH meter (WTW, 823 62 Weilheim, Germany). Egg diameter was measured to the nearest 0.001 mm using Quick PHOTO CAMERA 2.2 software (Olympus, Hamburg, Germany) from photographs taken with a binocular microscope Olympus BX51 fitted with an Olympus E-510 digital camera. Three samples of 150 ± 10 eggs were taken from each female and fertilized with an equal volume of pooled sperm from three males. Milt was collected at the time of fertilization using a 1 mL syringe for each male. Sperm was mixed in a small dish during the process of fertilization and examined for motility before use. Eggs were incubated in separate small incubators (see Kallert 2009, p. 22) with independently controllable inflow. Incubators were placed in a recirculation system equipped with a thermostatic cooler maintaining the water temperature at 2.5 ± 0.3°C throughout the incubation period. Shortly before hatching, the temperature was raised to 5°C based on information gained from hatchery practices (Hochman 1987). Oxygen content during incubation was maintained at 10.1 mg L^{-1}, and pH level was 7.80 ± 0.02. Other water parameters such as Cl^-, Fe, NH3, NH4, N-NO2 and N-NO3 were well below limits that could negatively impact egg development. Survival to the eyed stage and hatching rate was calculated in all samples as the per cent of eyed eggs and hatched alevins in the total number of eggs in the incubator.

All data were analysed by Statistica 9 Cz (StatSoft, Tulsa, USA). Ovulation course and per cent ovulated females were analysed using survival analysis (Z test). Differences in mean time to ovulation were analysed using non-parametric multicomparison Kruskal–Wallis test. One-way ANOVA was used to characterize differences in ovarian fluid pH. Differences in egg diameter, per cent of eyed eggs and hatched alevins were assessed by

hierarchical (nested) ANOVA with individual females nested in treatment. Arcsin transformation was applied for all per cent values. If significant differences were found by ANOVA, Tukey's HSD test was applied for detailed multicomparison assay. The Kolmogorov–Smirnov test was used to confirm normal distribution of the data following homoscedasticity verification by Chochran–Bartlett's test. Linear regression analysis was applied to correlate per cent of eyed eggs and hatched alevins and latency time. A significance level (α) of 0.05 was applied to all tests except where indicated. Data are presented as mean ± SEM.

Results

Induction and synchronization of ovulation

Compared with control group, all treatments significantly induced synchronized ovulation, whereas no significant differences were found among the treated groups (Fig. 1). First ovulation occurred on day 9 after the first injection in groups C and D, when 8.3% (1 of 12) of females ovulated in both groups. On day 12 after first injection 25% of females ovulated in groups A and B and 58.3%, 16.7%, 8.3% ovulated in groups C, D and E respectively (Fig. 1). On day 15, 66.7% of females had ovulated in groups A and B, and 100%, 75% and 25% in groups C, D and E respectively. On day 18, 91.7%, 100%, 91.7% and 66.7% of females were ovulated in group A, B, D and E respectively (Fig. 1). On day 21, all females in treated groups were ovulated, whereas 25% of females in group E remained unovulated. A 100% ovulation was reached on day 30 in group E. The DI of 25 μg kg^{-1} was the most effective treatment, reducing the mean time to ovulation to 13±1.9 days. Other treatments significantly reduced the mean time to ovulation to 15.5±2.7, 15.3±2.3 and 15.3±2.9 days in groups A, B, C and D, respectively, compared with 19.5±5 days in the control group, E ($P < 0.05$).

Effect of the treatments on egg quality

Relative fecundity was not influenced by the hormone treatments and no significant differences were found among groups. The relative fecundity reached 13.8 ± 2.9, 15.2 ± 1.6, 14.8 ± 2.6, 13.7 ± 3.2 and 14.8 ± 2.4% in groups A, B, C, D and E respectively (data not shown).

Statistical differences were found in ovarian fluid pH levels between groups A (8.08 ± 0.07) and D (8.17 ± 0.06) ($P < 0.05$) (Fig. 2). Among other groups, ovarian fluid pH levels were not significantly different. The measured levels were 8.13 ± 0.06 in groups B and C, and 8.11 ± 0.05 in group E. These values were also not different from those in groups A and D (Fig 2). No relationship between ovarian fluid pH level and survival to the eyed stage and hatching rate was found. Ovarian fluid pH remained constant throughout the experiment, and no relation between ovarian fluid pH and latency time was recorded in any of the groups.

Egg size was significantly reduced in all GnRHa-treated groups compared with the control except

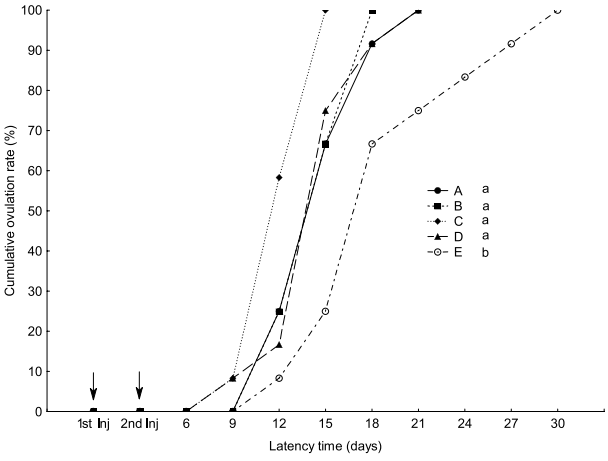

Figure 1 Ovulation rate in respective groups of northern whitefish. The same superscript letters denote groups that were not significantly different (P < 0.05). Arrows indicate days of GnRHa administration.

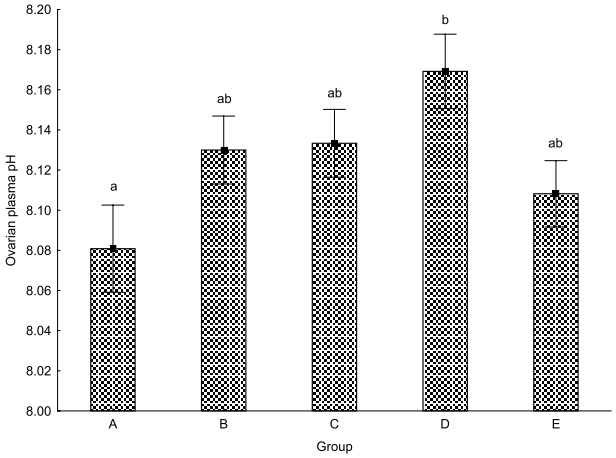

Figure 2 Ovarian plasma pH in northern whitefish. The same superscript letters denote groups that were not significantly different ($P < 0.05$).

group C ($P < 0.01$). Egg diameter was smaller in groups A, B and D (2.031 ± 0.005, 2.026 ± 0.005 and 2.016 ± 0.005 mm, respectively), compared to 2.087 ± 0.005 and 2.084 ± 0.005 mm in groups C and E respectively (Fig. 3). No relationship between egg size and survival to the eyed stage, hatching rate or latency time was found.

Survival to the eyed stage of eggs from females in groups A, B and C (43.5 ± 21.3, 44.0 ± 27.0 and 38.1 ± 31.1% respectively) was significantly lower ($P < 0.01$) than in groups D and E (63.2 ± 15.6 and 62.0 ± 25.5% respectively) (Fig. 4). A significant relationship was found if survival to the eyed stage was plotted against the latency time ($P < 0.01$, $R^2 = 0.18$) (Fig. 5). Females with longer latency time appeared to produce slightly higher numbers of eggs at the eyed stage. The relationship was not strong, thus detailed regression analysis was applied within individual groups. This analysis revealed a significant relationship between eggs at the eyed stage and latency time in groups C and E ($P < 0.01$, $R^2 = 0.34$ and $P < 0.01$, $R^2 = 0.31$, respectively), with no relationship found in groups A, B and D ($R^2 = 0.05$, $R^2 = 0.05$ and $R^2 = 0.01$) (Fig. 6).

Per cent of hatched alevins was lower ($P < 0.01$) in groups A, B and C (20.7 ± 13.1, 20.2 ± 13.5 and 17.8 ± 16.2% respectively) than in groups D and E (30.4 ± 14.4 and 35.3 ± 24.7%, respectively), whereas the per cent of

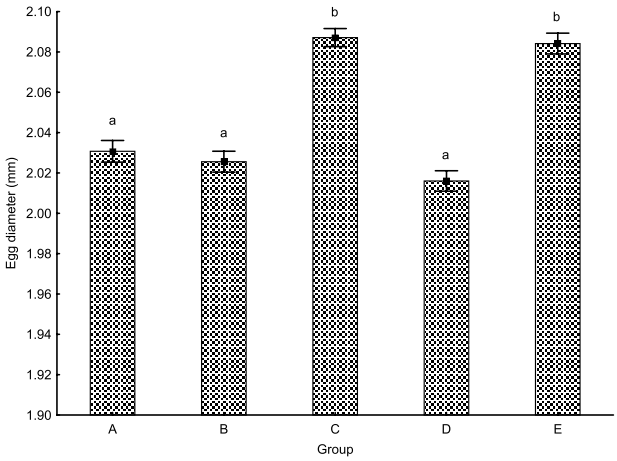

Figure 3 Egg size (mm) of northern whitefish treated with various hormone treatments. The same superscript denotes groups that were not significantly different ($P < 0.01$).

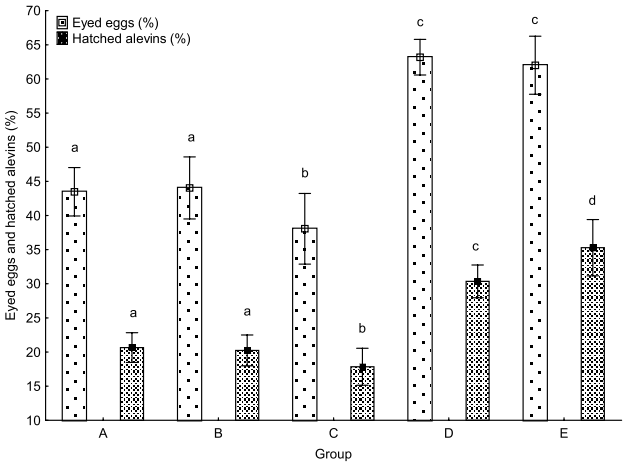

Figure 4 Survival to the eyed stage (light bars) and hatching rate (dark bars) in northern whitefish. The same superscripts denote groups that were not significantly different ($P < 0.01$).

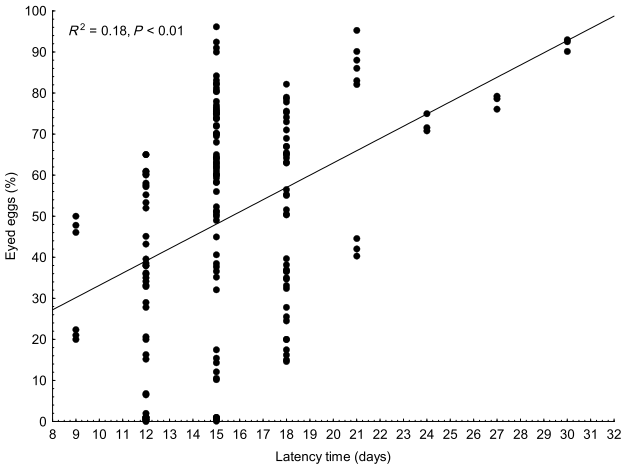

Figure 5 Correlation plot of per cent eyed eggs versus latency time in northern whitefish. Data from all groups are included (n = 180).

hatched alevins was significantly lower in group D compared with group E ($P < 0.05$) (Fig. 4). A significant relationship was found when per cent of hatched alevins was plotted against the latency time ($P < 0.01$, $R^2 = 0.33$) (Fig. 7). Those females with longer latency time seemed to have a slightly higher per cent of hatched alevins. As in data on survival to the eyed stage, detailed regression analysis was applied within individual groups. The analysis revealed a significant relationship between per cent of hatched alevins and latency time in groups C and E ($P < 0.01$, $R^2 = 0.25$ and $P < 0.01$, $R^2 = 0.71$), with no relationship found in groups A, B and D ($R^2 = 0.03$, $R^2 = 0.05$ and $R^2 = 0.02$) (Fig. 8). In all groups, survival to the eyed stage was highly predictive of the per cent of hatched alevins ($R^2 = 0.68$, $R^2 = 0.81$ $R^2 = 0.84$, $R^2 = 0.42$ and $R^2 = 0.55$ for groups A, B, C, D and E, respectively, $P < 0.001$ for all) (Fig. 9).

No significant differences in post-spawning broodstock mortality were found in any group. In the 3 month post-spawning period 2, 1, 3, 1 and 2 females died in groups A, B, C, D and E respectively. Normally developing ovaries were found in the dissected fish, and no visible abnormalities (intra-abdominal lesions, bruises, haematomas or

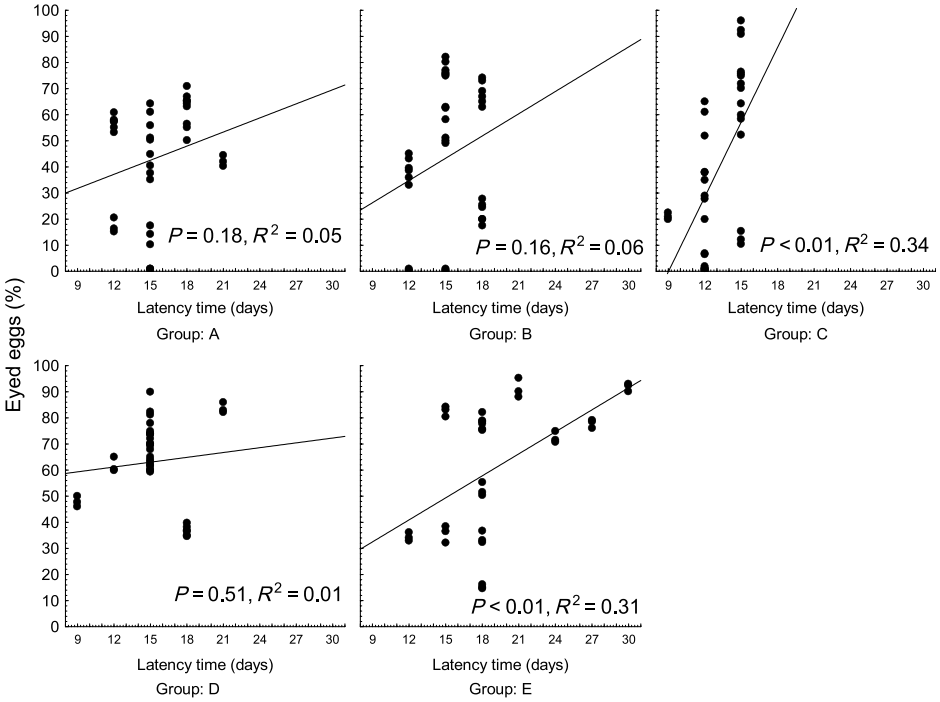

Figure 6 Correlation plots of per cent eyed eggs versus latency time in each group of northern whitefish.

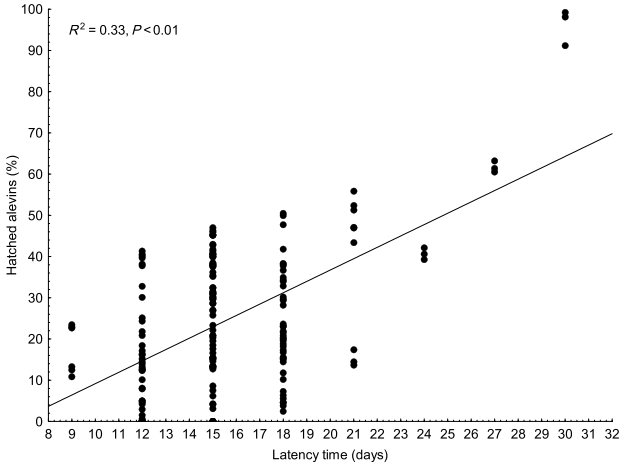

Figure 7 Correlation plot of hatching rate versus latency time in northern whitefish. Data from all groups are included (n = 180).

granulomas) were observed in either body wall or organ epithelium. Small residues (white flakes 1–1.5 mm diameter) of GnRHa-FIA emulsion were found in body cavity of all females treated with GnRHa-FIA.

Discussion

Synthetic analogues of gonadotropin releasing hormone neuropeptide (GnRHa) are effective in stimulating secretion of luteinizing hormone (LH) from

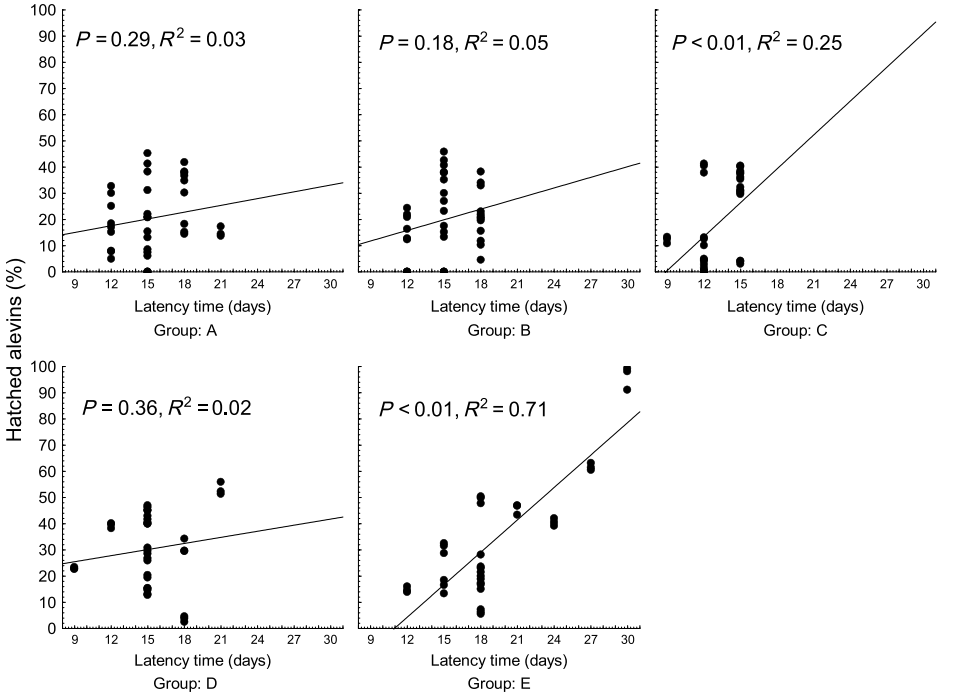

Figure 8 Correlation plots of hatching rate versus latency time in each group of northern whitefish.

the pituitary (Zohar, Muñoz-Cueto, Elizur & Kah 2010). Luteinizing hormone stimulates ovarian production of maturation-inducing steroids (MIS), e.g. 17α,20β-DP, and maturation-promoting factors, followed by ovulation (Nagahama & Yamashita 2008). The results from this study demonstrate that sGnRHa is effective for induction and synchronization of ovulation in northern whitefish. All utilized hormone treatments in this study significantly induced and synchronized ovulation compared with the control. These results are comparable with those obtained in other salmonid species using sustained GnRHa treatments (Breton *et al.* 1990; Arabaci *et al.* 2004; Park *et al.* 2007; Vazirzadeh *et al.* 2008; Svinger *et al.* 2012) or acute GnRHa treatments (Mylonas *et al.* 1992; Taranger, Stefansson & Hansen 1992; Mikolajczyk *et al.* 2008; Noori *et al.* 2010). On the other hand, our results indicate no differences between sustained release and acute treatment in northern whitefish. This is contrary to results of other authors reporting the efficacy of sustained release preparations in salmonids as much higher than that of acute release preparations. Breton *et al.* (1990) reported 100% ovulation in rainbow trout females treated with GnRHa sustained release form (using commercial microencapsulated form, in a polygylcolic polylactic biodegradable matrix), despite dosages of 12.5-50.0 μg kg^{-1}, whereas 100% ovulation rate was never achieved in those females treated with acute injections at dose of 20 μg kg^{-1}. Arabaci *et al.* (2004) and Vazirzadeh *et al.* (2008) used sustained GnRHa-FIA treatment at dosages of 25–50 μg kg^{-1} in rainbow trout, and its efficacy was again far above that of acute hormone injections. Furthermore, no significant differences were found between single or double acute injection in northern whitefish in the present study, although the double injection seemed to be slightly more effective. This is in contrast to previous results by Svinger *et al.* (2010) in northern whitefish using acute injections of mammalian [D-Tle^{6}Pro^{9}NEt]-GnRHa at the same dosages as those of the salmon [D-Arg^{6}Pro^{9}NEt]-GnRHa used in the present study. A single injection of 25 μg kg^{-1} of [D-Tle^{6}Pro^{9}NEt]-mGnRHa (Lecirelin,

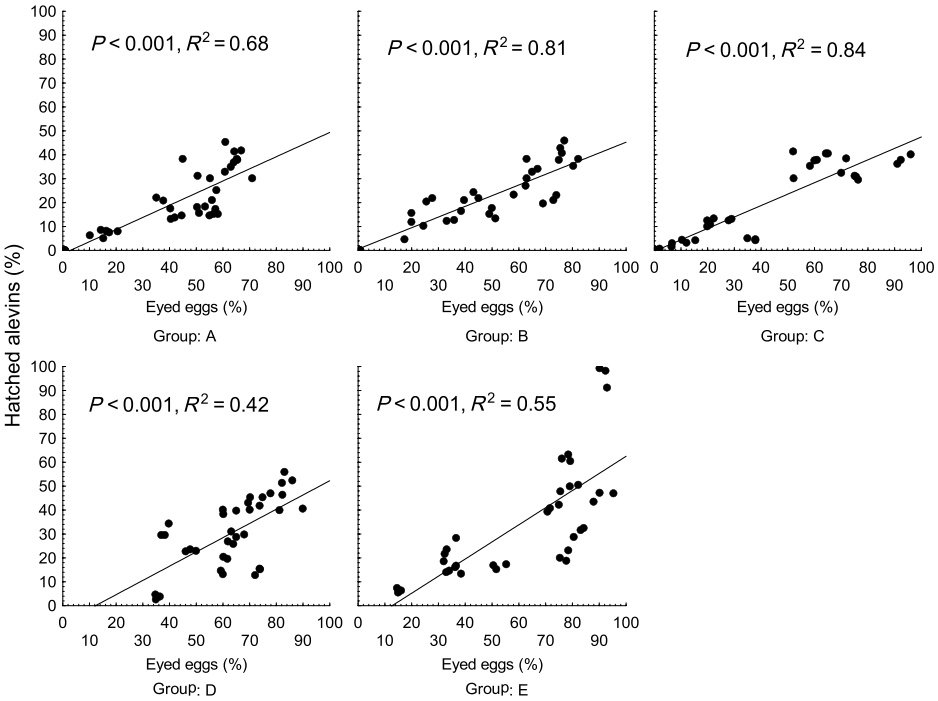

Figure 9 Correlation plots of per cent eyed eggs versus hatching rate in each group of northern whitefish.

Supergestran®) induced ovulation only in a small number of the treated females, and the double injection at 25 μg kg^{-1} induced ovulation in only 75% of females. Similar to the results of the present study, single acute injections of [D-Nal(2)^{6}Pro^{9}Aza-Gly]-GnRHa at 16 μg kg^{-1} and 32 μg kg^{-1} have been reported to effectively induce and synchronize ovulation in European whitefish (Mikolajczyk *et al.* 2005) and European grayling (*Thymallus thymallus*) (Mikolajczyk *et al.* 2008). Distinct GnRHa molecules are known to have different LH release potency. [D-Arg^{6}Pro^{9}NEt]-GnRHa has been shown to be the most effective of GnRHa formulations tested in rainbow trout (Breton *et al.* 1990) and coho salmon (*Oncorhynchus kisutch*) (Kraak, Donaldson, Dye, Hunter, Rivier & Vale 1987). In the case of [D-Nal(2)^{6}Pro^{9}Aza-Gly]-GnRHa (Azaglynafarelin, Gonazon™), Haffray *et al.* (2005) reported a much longer half-life in rainbow trout than other GnRHa molecules. Therefore, potential differences between [D-Tle^{6}Pro^{9}NEt]-mGnRHa and [D-Arg^{6}Pro^{9}NEt]-sGnRHa in LH release potency may have led to the discrepancies in these two experiments in northern whitefish.

Exposure to reduced water temperatures during the weeks prior to ovulation appears to enhance steroidogenic activity (Vikingstad, Andersson, Norberg, Mayer, Stefansson & Taranger 2005) in salmonids and induce or stimulate ovulation, perhaps acting as a late-stage environmental cue (Taranger & Hansen 1993; Taranger, Stefansson, Oppedal, Andersson, Hansen & Norberg 2000; Vikingstad, Andersson, Norberg, Mayer, Klenke, Zohar, Stefansson & Taranger 2008). Results of Pankhurst and Thomas (1998) in rainbow trout and King and Pankhurst (2004) in Atlantic salmon (*Salmo salar*) suggest that maintenance of salmonids at elevated temperatures may impair pituitary responsiveness to the hormone treatment. The northern whitefish in a trial of Svinger *et al.* (2010) were injected at temperatures of nearly 5°C prior to the sharp drop in winter water temperature, while the fish in the present study were injected at 0.5°C, 1 week after the temperature drop. Whether this played a role in the high effectiveness of the single

acute injection [D-Arg^{6}Pro^{9}NEt]-sGnRHa remains unclear, as we did not measure either the LH or steroid levels.

Relative fecundity is a useful working index for the farmer, as it allows egg production to be directly related to stocking levels, feeding rates, water supply, effluent constraints and the age and number of broodfish (Bromage *et al.* 1992). In our study, relative fecundity in hormone-treated females remained unchanged compared with control, and its values fell within the normal range for northern whitefish fecundity in the Czech Republic (Hochman 1987, Fish Farm Kinsky JSC).

Ovarian fluid pH level is considered one of the main parameters of ovarian fluid quality. Poor quality ovarian fluid is characterized by pH less than 7.4 in rainbow trout (Wojtczak, Dietrich, Slowinska, Dobosz, Kuzminski & Ciereszko 2007), whereas high quality ovarian fluid pH is 8.44–8.57 in lake trout (*Salmo trutta* m. *lacustris*) (Lahnsteiner, Weismann & Patzner 1999). Decreased ovarian fluid pH (to 7.29–7.67) negatively influenced spermatozoa motility, velocity and duration of movement in rainbow trout (Wojtczak *et al.* 2007). A decline in ovarian fluid pH (pH 6.5–6.55, Krishna 1953; pH 6.47, Dietrich, Wojtczak, Slowinska, Dobosz, Kuzminski & Ciereszko 2007) may occur if egg content escapes into the ovarian fluid, which may be due to over-ripening (Lahnsteiner 2000) or broken eggs (Dietrich *et al.* 2007). As no measurements of ovarian fluid in northern whitefish have been made before, we can only compare our values to those observed in other salmonid species and state that they are most similar to those observed in rainbow trout (Aegeter & Jalabert 2004, Wojtczak *et al.* 2007). The pH levels measured in northern whitefish in the present study were 8.00–8.23, and significant differences were found only between groups A and D, with no differences found between hormone-treated groups and control. Only one female of the 60 had ovarian fluid pH less than 8.0 at 7.93. In contrast to results of Lahnsteiner *et al.* (1999), no relationship between ovarian fluid pH and survival to the eyed stage was found, as narrow distribution of ovarian fluid pH values and high variability of survival to the eyed stage made the detection of any relation impossible. This suggests that optimum ovarian fluid pH in northern whitefish may lie within the range 8.00–8.23.

Egg size is implicated as a likely indicator of egg quality in a number of studies dealing with hormone-induced ovulation in salmonids, regardless of controversy over other egg quality determinants. Some researchers report that hormone treatment did not affect egg size (Hunter, Donaldson, Stone & Dye 1978; Donaldson, Hunter & Dye 1981a; Hunter, Donaldson & Dye 1981; Haraldsson, Sveinsson & Skúlason 1993; Bonnet, Fostier & Bobe 2007), whereas Donaldson, Hunter and Dye (1981b) reported reduced egg size with hormone treatment. In the present study, females subjected to three of four hormone treatments exhibited reduced egg size compared with the control group. Egg size in rainbow trout was reported to be reduced from 3.9 ± 0.1 to 3.7 ± 0.2 mm after ovulation was synchronized using similar GnRHa and GnRHa-FIA treatments (Arabaci *et al.* 2004), although the authors considered the differences non-significant. However, the measurement method for the large rainbow trout egg diameter might be questionable, as significant differences in our study could be observed on the order of hundredths of millimetre in small northern whitefish eggs. No relationship of egg size to survival at the eyed stage, hatching and latency period was found, suggesting that size does not affect on egg quality in northern whitefish. This is in accordance with other studies in salmonids (Glebe, Appy, T.D. & Saunders 1979; Kato & Kramler 1983; Thorpe, Miles & Keay 1984; Bromage & Cumaranatunga 1988). It has been demonstrated, however, that larger eggs produce larger first feeding fry (Pitman 1979; Springate & Bromage 1985; Springate, Bromage & Cumaranatunga1985), so it is possible that smaller fry might suffer higher mortality due to a smaller mouth gape if they do not receive enough food or are fed on large pellets or particles (Bromage *et al.* 1992). This might be crucial for northern whitefish fry needing minuscule food particles for first feeding. Thus, further experiments regarding the effect of hormone synchronization of ovulation on egg size are needed, especially as egg size was not reduced in the most synchronized and advanced acute double injection group.

Survival to the eyed stage was reduced in GnRHa-treated groups except for group D, treated with single acute injection of 25 μg kg^{-1}, while hatching rate was reduced in all GnRHa-treated groups compared with controls. Many authors relate decrease in egg quality with the amount of GnRHa administered. Mylonas *et al.* (1992) recorded lowered eying when a double injection of 10 μg kg^{-1} was applied. Crim and Glebe (1984),

Haraldsson *et al.* (1993), Taranger *et al.* (1992) and Olito *et al.* (2001) report that egg quality was reduced with a dose of 100–150 μg kg^{-1}, and some authors have reported that egg quality following hormone treatment remained unaffected (Billard, Reinaud, Hollebecq & Breton 1984) or was better than in untreated fish (Sower, Iwamoto, Dickhoff & Gorbman 1984; Jansen 1993; Svinger *et al.* 2012). Results of the present study suggest that factors other than GnRHa dose affected egg quality, as eying and hatching was also reduced in GnRHa-FIA treatment at the 25 μg kg^{-1} GnRHa dose.

Survival to the eyed stage and hatching rate were positively correlated with latency time. Fish that ovulated later tended to have higher survival to the eyed stage as well as higher hatching rates. This is consistent with results of Mylonas *et al.* (1992), who obtained similar results in brown trout (*Salmo trutta*), suggesting time to ovulation as the major factor determining fertility regardless of GnRHa treatment. Crim and Glebe (1984) also recorded lowered viability of Atlantic salmon eggs stripped early in the course of their experiment. Closer analysis of our data in separate groups revealed that latency time influenced eying and hatching only in treated group C and control E, while no relationship was found among the other groups. This compares favourably with results obtained in coho salmon by Fitzpatrick, Suzumoto, Schreck and Oberbillig (1984), where low fertility was higher in GnRHa-treated fish, and egg fertility was lowest in females that ovulated early in the season in both GnRHa-treated and control groups. Mylonas *et al.* (1992) suggest that hormone treatment may increase natural asynchrony of the two processes, mediated by different reproductive hormones that take place at the completion of oocyte development: final oocyte maturation controlled by MIS and ovulation regulated by prostaglandins (Bradley & Goetz 1994; Goetz & Garczynski 1997), which may lead to ovulation of under-ripe and unfertilizable oocytes. This phenomenon was, however, also observed in rainbow trout stripped without hormone treatment, (Springate, Bromage, Elliot & Hudson 1984) and eggs stripped between 4 and 10 days following ovulation at 10°C consistently achieved high rates of fertilization. Under culture conditions, the eggs of rainbow trout are ovulated but not oviposited; they remain in the body cavity until they are artificially stripped from the fish. During the period of retention in the body cavity, the eggs undergo a ripening process (Bromage *et al.* 1992), including meiotic maturation, which may not be completed until some time after ovulation (Nagahama 1983). Thus, the reduction in survival to the eyed stage and hatching in other treated groups (A, B, C) and some individuals in group C and E might have resulted from stripping eggs immediately after ovulation and some of slightly immature or under-ripe eggs might have been forced out by the manual stripping (Bromage *et al.* 1992). In fact, one might ask what the results might be if the individuals with lower eying and hatching would have been stripped a day later, since the physiological and egg developmental stage of individual females may differ at the time of artificial stripping as well as at the time of hormone administration (Billard *et al.* 1984; Gillet *et al.* 1996). This may explain high and consistent survival to the eyed stage in group D, suggesting that these females were stripped at the appropriate time. Further experiments are needed to confirm this, since data on fertilization rates are lacking in our study and we are unable to state if egg size played a role in fertilizability of the eggs. On the other hand, Springate *et al.* (1984) and Bromage *et al.* (1992) reported that eggs with moderate or low fertilization rates invariably experience similar low rates of survival to eying and hatching. Thus, we assume that fertilization rate in our study may also have been reduced in those groups with low eying and hatching rate. This is supported by a strong relationship between survival to the eyed stage and hatching rate found in all groups in the present study. Increased mortality between the eyed stage and hatching was observed in group D, which resulted in significantly reduced hatching compared with the control group regardless of comparable survival to the eyed stage. This is in accordance with results of Bonnet *et al.* (2007) who concluded that spawning induction treatment in rainbow trout can produce limited but significant egg quality defects, which may be characterized by embryonic mortalities occurring after eying and possibly after hatching.

GnRHa-FIA treatment has been reported to be associated with high post-spawning mortality in rainbow trout broodstock (Arabaci *et al.* 2004). Although some females died in the post-spawning period in our experiment, no differences were found among the groups, and loss of fish was related to the intensive handling. This is in accordance with more recent studies where no

post-spawning mortality was associated with GnRHa-FIA in rainbow trout and brook char (Vazirzadeh *et al.* 2008; Svinger *et al.* 2012). However, experiments focused specially on endocrine and immunological action of GnRHa-FIA treatments should be carried out in the future to exclude the possibility that GnRHa-FIA pose a risk for the broodstock. After 3 months, small residues of white GnRHa-FIA emulsion were still visible in the body cavity, which might call into question the suitability of the GnRHa-FIA-treated northern whitefish for human consumption. No such residue was detected in brook char 6 months post spawning (Svinger *et al.* 2012).

Conclusion

This study demonstrates that synchronization of ovulation in northern whitefish can be induced by a single acute injection of [D-Arg^{6}Pro^{9}NEt]-sGnRHa at 25 μg kg^{-1} BW if given near the natural spawning time determined by water temperature below 2°C. Induction and synchronization of ovulation by sGnRHa was associated with reduced egg size, survival to the eyed stage and hatching rate. Thus, further experiments are needed to clarify the cause of these changes and to determine whether reduction in egg size might influence egg fertilizability and survival of the fry.

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CHAPTER 4

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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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

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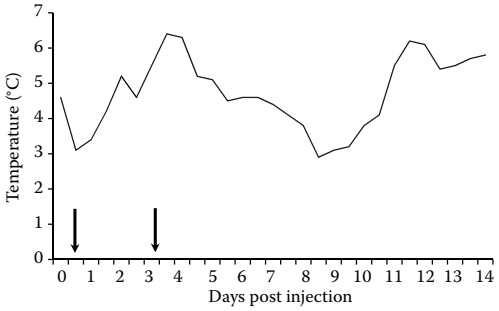

Figure 1. Temperature fluctuation during the experiment. Arrows indicate days of hormone administration

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

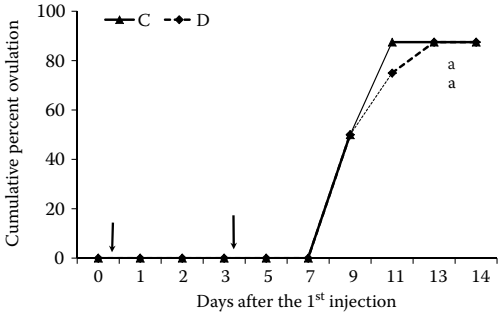

Figure 2. Ovulation progress in groups treated with double injection (DI) (group C – 2 × 25 µg/kg, group D – 2 × 10 µg/kg). Arrows indicate days of hormone administration. Because no ovulation was recorded either in group A or B, these are not included in the figure

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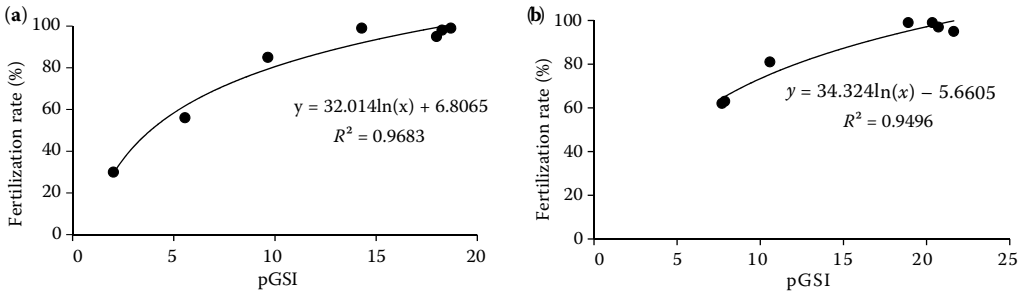

Figure 3. Relationship between pGSI level and fertilization rate in group C (**a**) and group D (**b**) ($P < 0.01$)

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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CHAPTER 5

EFFECTS OF SALMON GONADOTROPIN-RELEASING HORMONE ANALOG (GnRHa) ON REPRODUCTIVE SUCCESS AND EGG SIZE IN RAINBOW TROUT AND BROOK TROUT

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ARTICLE

Effects of Salmon Gonadotropin-Releasing Hormone Analog (GnRHa) on Reproductive Success and Egg Size in Rainbow Trout and Brook Trout

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Abstract

Rainbow Trout *Oncorhynchus mykiss* were treated with a 100- or 25-μg/kg dose of salmon gonadotropin-releasing hormone analog (sGnRHa) that was emulsified in Freund's incomplete adjuvant (FIA). Controls were treated with a mixture of FIA and physiological saline. Latency (number of days from the first injection to the date of detected ovulation) was significantly shortened in treated groups compared with the control group. Egg weight, eyeing rate, and hatching rate were significantly reduced in treated groups; mortality after the eyed stage was significantly higher in treated groups. The relationship between latency and the eyeing rate or hatching rate was positive, but the relationship between latency and mortality after the eyed stage was negative. Brook Trout *Salvelinus fontinalis* were treated with a single injection of sGnRHa–FIA at 12.5 μg/kg, double acute injections of sGnRHa at 12.5 μg/kg administered 3 d apart, or a single acute injection of sGnRHa at 50 μg/kg. Controls were injected with FIA and physiological saline. Latency was significantly shortened in treated groups except the group that received the single acute injection of 50 μg/kg. Egg weight and alevin weight were significantly lower in treated groups. Egg mortality after the eyed stage was significantly higher in the double-injected group than in the 12.5-μg/kg sGnRHa–FIA group and the control group. Latency had a negative relationship with the eyeing rate and hatching rate. This result for Brook Trout is opposite that observed for Rainbow Trout. Latency and mortality after the eyed stage were not correlated. It is evident that the experimental designs currently used in salmonid research should be updated to determine reliable means of avoiding egg mortality, particularly in Rainbow Trout, after GnRHa treatment.

Gonadotropin-releasing hormone analogs (GnRHa) are used in salmonid reproduction to precisely control the timing of the final stages of the reproductive cycle. Application of GnRHa allows for accurate prediction of ovulation dates in many species. Such treatments reduce the handling of broodstock, make broodstock handling more flexible over the season, may advance

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ovulation by more than 5 weeks compared with the natural spawning time, and markedly shorten the spawning season (which often extends over a period of 3 months in salmonids).

Gonadotropin-releasing hormone analogs have been successfully applied in a wide range of salmonids, such as Rainbow Trout *Oncorhynchus mykiss* (Breton et al. 1990; Arabacı et al. 2004; Vazirzadeh et al. 2008), Arctic Char *Salvelinus alpinus* (Haraldsson et al. 1993; Jansen 1993; Gillet et al. 1996), Atlantic Salmon *Salmo salar* (Crim et al. 1983a; Taranger et al. 1992; Vikingstad et al. 2008), Brown Trout *Salmo trutta* (Mylonas et al. 1992; Noori et al. 2010), European Grayling *Thymallus thymallus* (Kouřil et al. 1987; Mikolajczyk et al. 2008), Chinook Salmon *O. tshawytscha* (Olito et al. 2001), Sockeye Salmon *O. nerka* (Slater et al. 1995), Chum Salmon *O. keta* (Park et al. 2007), Coho Salmon *O. kisutch* (Fitzpatrick et al. 1987), Brook Trout *Salvelinus fontinalis* (Svinger et al. 2013), Lake Trout *Salvelinus namaycush* (Erdahl and McClain 1987), Powan (European Whitefish) *Coregonus lavaretus* (Mikolajczyk et al. 2005), and Northern Whitefish *Coregonus peled* (Svinger and Kouřil, in press).

Most of these studies have focused on optimization of the hormone administration method (acute versus sustained release of GnRHa) or refinement of effective GnRHa doses. Other studies have assessed the endocrine response of salmonids to GnRHa injection outside the thermal optimum, especially at elevated temperatures (Gillet et al. 1996; Pankhurst and Thomas 1998; Taranger et al. 2003; King and Pankhurst 2004, 2007; Vikingstad et al. 2008; Gillet and Breton 2009). There is limited information regarding the effect of hormone treatment on egg quality. Studies that have examined egg quality after hormone treatment have produced inconsistent and controversial results. Crim et al. (1983b, 1986), Crim and Glebe (1984), Taranger et al. (1992), Haraldsson et al. (1993), Olito et al. (2001), and Noori et al. (2010) reported reduced fertility or a reduced eyeing rate in salmonids that were treated with GnRHa doses of 100 μg/kg of body weight (BW) or higher. Mylonas et al. (1992) reported that fertility and eyeing were reduced when a double injection of only 10 μg/kg BW was used, whereas other authors have found no differences in eggs obtained from treated and untreated females or from females treated with differing doses of GnRHa (Jalabert et al. 1978; Donaldson et al. 1981; Billard et al. 1984; Erdahl and McClain 1987; Slater et al. 1995; Arabacı et al. 2004; Vazirzadeh et al. 2008; Vikingstad et al. 2008). Regardless of GnRHa dose, egg viability improved in GnRHa-treated females compared with naturally ovulating female Brook Trout (Svinger et al. 2013) and steelhead (anadromous Rainbow Trout; Sower et al. 1984).

In salmonids, the induction of ovulation several weeks prior to their natural spawning time shortens the final stages of vitellogenesis, which may have negative consequences for eggs and fry. Reduced egg size after GnRHa treatment has been incidentally recorded in Coho Salmon (Donaldson 1981), Chinook Salmon (Olito et al. 2001), and recently in Northern Whitefish (Svinger and Kouřil, in press). No further detailed reports describing GnRHa treatment effects on egg size are available.

We conducted on-farm experiments with two of the most farmed salmonid species in central Europe—Rainbow Trout and Brook Trout—by using differing doses and administration methods of D-Arg^{6}Pro^{9}NEt-sGnRHa, the superactive analog of native salmon GnRH (sGnRH). To identify potential side effects of using GnRHa in a hatchery, we determined ovarian fluid pH, egg weight, eyeing rate, rate of mortality between eyeing and hatch, hatching rate, weight of yolk sac fry (measured for Brook Trout only), and rate of deformities in yolk sac fry. We chose D-Arg^{6}Pro^{9}NEt-sGnRHa for use in this study because it is a standard active substance in many commercial hormone preparations used for induction of ovulation in fish (e.g., Ovaplant, OvaRH, Dagin, and Ovaprim).

METHODS

Experimental Design

Rainbow Trout.—For the Rainbow Trout experiment, 3-year-old females of a spring-spawning, eastern Bohemian strain were used. One week prior to hormone injection, the Rainbow Trout broodstock were randomly divided into two experimental groups (A_{RT} and B_{RT}) and a control group (C_{RT}) of similar mean weight. The weight was 1,806 ± 57 g (mean ± SD) for group A_{RT}, 1,924 ± 35 g for group B_{RT}, and 1,861 ± 56 g for group C_{RT}. Each group contained 20 individuals, which were placed into three 4-m^3 raceways supplied with water from the Loučná River (part of the Elbe River system) in the Czech Republic. Water temperature was 9.5°C at the beginning of the trial and rose continuously, reaching 13.4°C on the final day of the experiment. Temperature and oxygen were measured daily using a Handy Polaris oxygen meter (OxyGuard International A/S, Denmark). Oxygen content in the outlet was maintained at 9.5 mg/L throughout the experiment by using an aerator (Linn Gerätebau GmbH, Germany). The flow-through rate was maintained at 65 L/min. All fish were kept under the natural photoperiod, and feeding was ceased 4 d before the expected date of stripping. Fish were not fed during the experimental period until they had been stripped. The experiment began with hormone injections on 24 February 2011 and continued until 17 May 2011, when all females in the control group had been stripped. Fish were intraperitoneally injected with sGnRHa emulsified in Freund's incomplete adjuvant (FIA) as follows: group A_{RT} received 100 μg of sGnRHa–FIA/kg of BW; group B_{RT} received 25 μg of sGnRHa–FIA/kg of BW; and group C_{RT} (the control) received 1:1 FIA and 0.9% physiological saline.

Brook Trout.—For the Brook Trout experiment, 3-year-old females of the Czech *Klatovy* strain were used. One week before hormone injection, broodstock were randomly divided into three experimental groups (A_{BT}, B_{BT}, and C_{BT}) and a control group (D_{BT}). The weight of fish was 587 ± 25 g (mean ± SD) in group A_{BT}, 635 ± 28 g in group B_{BT}, 587 ± 16 g in group C_{BT}, and 568 ± 16 g in group D_{BT}. Each group ($n = 25$) was placed in a separate 4-m^3 raceway supplied with water from the Loučná River. Water temperature was 11°C at the beginning of the trial and continually decreased to 4.9°C on the last day of the

experiment. Oxygen content and flow-through were maintained at levels similar to those in the Rainbow Trout experiment and by using the same equipment. Fish were kept under the natural photoperiod during the entire experimental period. The experiment was carried out from 4 October 2011 (when hormones were injected) to 25 November 2011 (when all females in the control group had been stripped). Feeding was ceased 4 d before the expected date of stripping, and fish were not fed until they had been stripped. Females were intraperitoneally injected with sGnRHa–FIA or received an acute injection of sGnRHa as follows: group A_{BT} received a single injection of 12.5 μg of sGnRHa–FIA/kg of BW; group B_{BT} received double acute injections of 12.5 μg of sGnRHa/kg of BW, with injections administered 3 d apart; group C_{BT} received a single acute injection of 50 μg of sGnRHa/kg of BW; and group D_{BT} (control) received 1:1 FIA and 0.9% physiological saline.

Hormone doses in Brook Trout were chosen based on preliminary findings. An sGnRHa–FIA dose of 25 μg/kg BW and an acute double injection of 25 μg/kg BW were previously found to be highly effective in synchronizing ovulation in Brook Trout (Svinger et al. 2013). These doses were selected to lower the costs of treatment. Experiments were carried out under Czech Republic law number 246/1992 and under the supervision of a person licensed for performing experiments with animals (Ministry of Agriculture license code CZ 00786).

Hormone Preparation

Superactive analog D-Arg^{6}Pro^{9}NEt-sGnRHa was purchased from Bachem AG (Weil am Rhein, Germany). To prepare sustained-release sGnRHa–FIA treatments for both trout species, sGnRHa was dissolved in 0.9% NaCl and mixed with FIA (Sigma) at 1:1 (volume/volume) using an Ultraturrax Ika T-10 homogenizer. Each female received a total volume of 0.5 mL of sGnRHa–FIA preparation, which was administered using a 1-mL insulin syringe fitted with a 0.7- × 30-mm needle. For acute treatments in Brook Trout, sGnRHa was diluted with 0.9% NaCl to achieve the required concentration. Females that were given acute injections received 1 mL of this solution per kilogram of BW. Acute treatments were administered by using a 1-mL insulin syringe fitted with a 0.33- × 12-mm needle.

Detection of Ovulation

Females in both experiments were evaluated for ovulation twice weekly by following the common procedures used in this hatchery. Females were considered to have ovulated if eggs were released upon gentle pressure on the abdomen. Latency was counted as the mean number of days from the first sGnRHa injection until detection of ovulation.

Egg Quality Assessment

Eggs from females that had ovulated were stripped into separate dishes. Three egg samples were taken from each female, and each sample was placed in a glass beaker. Ovarian fluid pH was measured using a WTW 3310 pH meter fitted with a Sentix MIC-D microelectrode (WTW, Weilheim, Germany). From each female, 15 randomly chosen eggs were placed into a small sieve, and the remaining ovarian fluid was carefully removed by using paper towels. Egg weight was recorded to the nearest 1 mg with a precision balance (KERN 572-31). A sample of 300 ± 10 eggs from each female was used for fertilization. Egg samples were fertilized with an excess of sperm from three to four males that had previously been assessed for sperm motility by a drop test (Linhart et al. 2011). A separate syringe was used for sperm collection from each male, and sperm was not pooled until addition of the activation medium (6 g of NaCl, 0.075 g of KCl, 0.15 g of $CaCl_2.2H_2O$, and 0.1 g of $NaHCO_3$ per liter) during egg fertilization. After fertilization, eggs were left 1 h for water hardening and then were placed into miniature salmonid egg incubators with separate and controllable water flow-through as described by Kallert (2009).

The final ovulating female Rainbow Trout in the 100-μg/kg sGnRHa (A_{RT}) group was detected at 18 d postinjection, when the cumulative ovulation rate had reached 65%. The remaining seven females in group A_{RT} were sacrificed on day 49, when no ovulation could be detected. A sample of 30 eggs was taken from each of the seven females and was cleared in a mixture of 6:3:1 ethanol : formaldehyde : glacial acetic acid to identify the germinal vesicle position.

Average water temperature during incubation was 9.4 ± 0.5°C for the Rainbow Trout trial and 6.0 ± 0.5°C for the Brook Trout trial. For both species, the oxygen level in incubators was maintained at 11 ± 1 mg/L and flow-through was maintained at approximately 0.25 L/min. Other water characteristics (e.g., pH, Cl^-, Fe, NH_3, NH_4, nitrite [NO_2], and nitrate [NO_3]) were well below limits that have been shown to negatively impact egg development. During incubation, dead (white) eggs were collected and counted. At the eyed stage, eggs were shocked by shaking to sort out and count the unfertilized or unprecipitated dead eggs. Remaining eyed eggs were incubated until hatch, and eggs with dead embryos were removed and counted. Embryo eyeing rate, hatching rate, mortalities between the eyed stage and hatch, and rate of deformities were calculated as a percentage of the number of eggs that were placed into the incubator at the start of incubation. Weight of freshly hatched yolk sac fry in relation to hormone treatment was determined only for Brook Trout. Ten individual hatchlings were randomly taken from the clutch representing each female and were weighed to the nearest 1 mg.

Statistical Analysis

All data were analyzed by using Statistica 9 Cz (StatSoft, Tulsa, Oklahoma). Data describing the course of ovulation and the percentage of females that ovulated were explored by a survival analysis (*Z*-test). Differences in latency interval were analyzed using the nonparametric Kruskal–Wallis multiple comparison test. Two-way ANOVA was used to characterize differences in ovarian fluid pH, with treatment as a main factor.

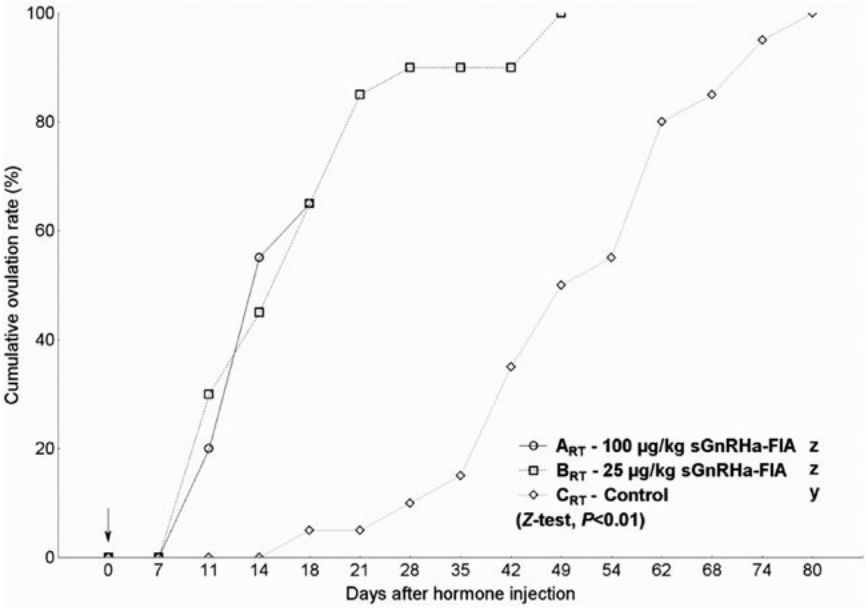

FIGURE 1. Course of ovulation (cumulative ovulation rate, %) in the respective groups of Rainbow Trout that were intraperitoneally injected with salmon gonadotropin-releasing hormone analog (sGnRHa) emulsified in Freund's incomplete adjuvant (FIA; A_{RT} = 100 µg of sGnRHa–FIA/kg of body weight [BW]; B_{RT} = 25 µg sGnRHa–FIA/kg BW; C_{RT} = control, 1:1 FIA and 0.9% physiological saline). Groups without a letter in common are significantly different ($P < 0.05$). The arrow indicates the date of sGnRHa administration.

Differences in egg weight, fry weight, and measures of embryo survival were assessed by one-way ANOVA. Arcsine transformation was applied for all percentage values. If significant differences were found by ANOVA, Tukey's honestly significant difference test for unequal sample sizes was applied for detailed multiple comparison testing. The Kolmogorov–Smirnov test was used to confirm the normal distribution of data after homoscedasticity verification by the Cochran–Bartlett test. Linear and nonlinear regression analyses were applied to correlate latency and embryonic survival variables. A significance level α of 0.05 was applied to all tests except where indicated. Data are presented as mean $\pm$ SE.

RESULTS

Rainbow Trout

In Rainbow Trout, both treatments significantly synchronized ovulation and shortened the spawning period in comparison with the control ($P < 0.01$). The cumulative percentage of females that ovulated during the study period is displayed in Figure 1. For all seven of the nonovulating females in group A_{RT}, the germinal vesicle remained in the center of the oocyte, with no sign of the onset of peripheral migration to the animal pole. Latency was 13.7 $\pm$ 0.7 d in group A_{RT} and 19.5 $\pm$ 2.5 d in group B_{RT}; these latency periods were significantly shorter than the latency of 52.8 $\pm$ 3.6 d for group C_{RT} ($P < 0.01$). Latency in group A_{RT} was calculated only based on the 13 females that ovulated; the sacrificed females were not included in the calculation.

Differences among treatments in ovarian fluid pH, egg weight, survival to the eyed stage, mortality between the eyed stage and hatch, hatching rate, and percentage of deformities in yolk sac fry are shown in Table 1. Regression analysis did not reveal a correlation between ovarian fluid pH and egg viability characteristics. Egg weight was not predictive of egg viability variables or latency. Survival to the eyed stage plotted against latency showed a low but significantly positive correlation in group B_{RT} ($R^2 = 0.22$, $P < 0.05$; Figure 2). The lack of a correlation between survival to the eyed stage and latency for group A_{RT} is likely due to the lower number of females included in the regression model, as the structure of the relationship was identical to that seen in group B_{RT} (Figure 2). A significant correlation between mortality after the eyed stage and latency was found only for group A_{RT} ($R^2 = 0.50$, $P < 0.05$) and was related to the lower number of fish in that group (Figure 3). Generally,

TABLE 1. Mean (±SE) ovarian fluid pH, egg weight, eyeing rate, mortality between the eyed stage (ES) and hatch, hatching rate, and yolk sac fry deformity rate for Rainbow Trout (n = number of females in each group) after injection with salmon gonadotropin-releasing hormone analog (sGnRHa) that was emulsified in Freund's incomplete adjuvant (FIA; A_{RT} = 100 μg of sGnRHa–FIA/kg of body weight [BW]; B_{RT} = 25 μg sGnRHa–FIA/kg BW; C_{RT} = control, 1:1 FIA and 0.9% physiological saline). Within a column, means with different lowercase letters are significantly different (one-way ANOVA followed by Tukey's multiple comparison test for unequal sample sizes, $P < 0.05$).

Group	n	Ovarian fluid pH	Egg weight (mg)	Eyed eggs (%)	Mortality, ES to hatch (%)	Hatching rate (%)	Deformities (%)
A_{RT}	13	8.18 ± 0.06 z	55.26 ± 2.55 z	67.85 ± 6.12 z	11.28 ± 3.94 z	61.76 ± 6.42 z	0.12 ± 0.12 z
B_{RT}	20	8.02 ± 0.05 y	53.12 ± 2.06 z	71.34 ± 4.93 z	11.50 ± 3.18 z	64.58 ± 5.17 z	0.15 ± 0.09 z
C_{RT}	19	8.21 ± 0.05 z	62.22 ± 2.11 y	87.80 ± 5.06 y	1.60 ± 3.26 z	86.61 ± 5.31 y	0.18 ± 0.10 z

only eggs from treated specimens with the shortest latencies showed high mortality after the eyed stage (Figure 3). Hatching rate showed a low but significant positive correlation with latency for group B_{RT} ($R^2 = 0.33$, $P < 0.05$; Figure 4). A low hatching rate was found, especially in treated groups with the shortest latency (Figure 4). Regression analysis did not reveal a correlation between the percentage of yolk sac fry deformities and egg weight or latency.

Brook Trout

With the exception of the single acute injection of 50 μg/kg (group C_{BT}), all treatments significantly induced and advanced ovulation in Brook Trout and shortened the spawning period compared with the control ($P < 0.05$; Figure 5). All treatments showed low synchronization effects. The cumulative percentage of females that ovulated over the study period is presented in Figure 5. On November 25, 2011, when the experiment was terminated, the percentage of females that had been stripped was 88% in group A_{BT}, 68% in group B_{BT}, 72% in group C_{BT}, and 76% in group D_{BT} (Figure 5). All remaining Brook Trout females were lost due to saprolegniasis, a common phenomenon in Brook Trout during the spawning season. Mean latency reached 29.0 ± 2.8 d in group A_{BT} and 24.1 ± 3.3 d in group B_{BT}; these latencies were significantly shorter than the latency of

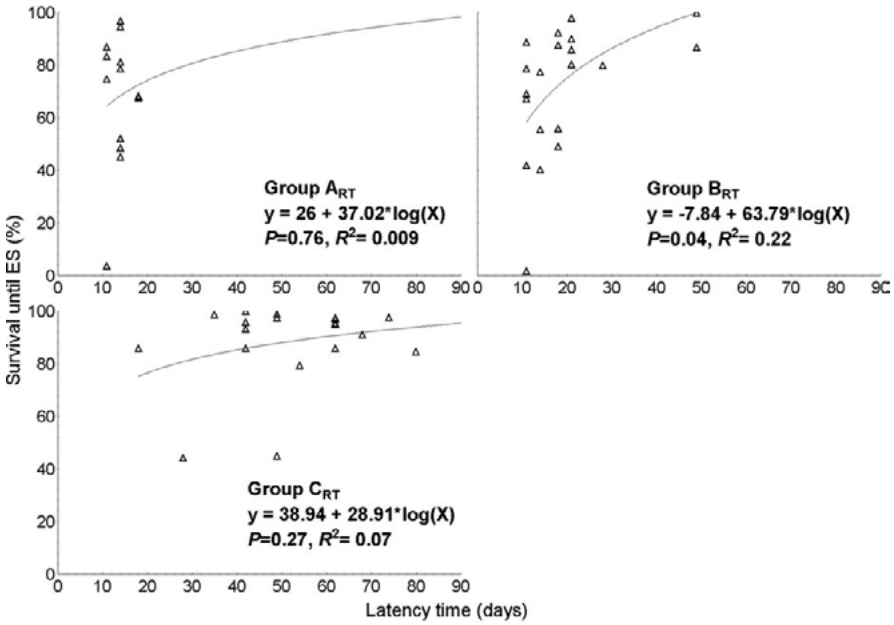

FIGURE 2. Correlation between the percentage of eyed eggs (i.e., survival to the eyed stage [ES]) and latency (number of days from the first injection to the date of detected ovulation) for each group of Rainbow Trout (groups are defined in Figure 1).

TABLE 2. Mean (± SE) ovarian fluid pH, egg weight, yolk sac fry weight, eyeing rate, mortality between the eyed stage (ES) and hatch, and hatching rate for Brook Trout (n = number of females in each group) after intraperitoneal injection with salmon gonadotropin-releasing hormone analog (sGnRHa) emulsified in Freund's incomplete adjuvant (FIA) or with sGnRHa only (A_{BT} = single injection of 12.5 µg of sGnRHa–FIA/kg of body weight [BW]; B_{BT} = double acute injection of 12.5 µg sGnRHa/kg BW, with injections administered 3 d apart; C_{BT} = single acute injection of 50 µg sGnRHa/kg BW; D_{BT} = control, 1:1 FIA and 0.9% physiological saline). Within a column, means with different lowercase letters are significantly different (one-way ANOVA followed by Tukey's multiple comparison test for unequal sample sizes, $P < 0.05$).

Group	n	Ovarian fluid pH	Egg weight (mg)	Fry weight (mg)	Eyed eggs (%)	Mortality, ES to hatch (%)	Hatching rate (%)
A_{BT}	22	8.02 ± 0.02 z	39.21 ± 0.88 z	43.98 ± 0.99 z	82.05 ± 3.26 z	4.34 ± 0.88 z	77.71 ± 3.43 z
B_{BT}	17	8.07 ± 0.03 z	39.12 ± 1.00 z	43.93 ± 1.12 z	82.29 ± 3.71 z	7.12 ± 1.01 y	75.17 ± 3.90 z
C_{BT}	18	8.07 ± 0.03 z	38.46 ± 0.97 z	42.34 ± 1.09 z	74.53 ± 3.60 z	6.35 ± 0.98 zy	68.18 ± 3.79 z
D_{BT}	19	8.07 ± 0.02 z	44.74 ± 0.94 y	48.24 ± 1.06 y	79.44 ± 3.51 z	3.94 ± 0.95 z	75.50 ± 3.69 z

42.6 ± 2.2 d for group D_{BT} (the control, $P < 0.01$). Latency for group C_{BT} was 36.3 ± 3.6 d and did not significantly differ from that of any other group.

Among-group comparisons of data on ovarian fluid pH, egg weight, yolk sac fry weight, survival to the eyed stage, mortality after the eyed stage, and hatching rate are shown in Table 2. Regression analysis did not reveal a correlation between ovarian fluid pH and egg viability variables. No correlation was found between egg weight, yolk sac fry weight, and egg viability measures. Egg weight was significantly predictive of yolk sac fry weight ($r^2 = 0.66$ for group A_{BT}, 0.82 for group B_{BT}, 0.77 for group C_{BT}, and 0.69 for group D_{BT}; $P < 0.01$ for all groups). No correlation between egg weight and latency was observed; the exception was the control group, in which a small but significant negative relationship was observed ($r^2 = 0.30$; $P < 0.05$). Survival to the eyed stage was plotted against latency; although groups A_{BT} and D_{BT} showed constant survival levels over the monitored period (A_{BT}: $r^2 = 0.13$, $P = 0.10$; D_{BT}: $r^2 = 0.15$, $P = 0.10$), a significant negative relationship was found for groups B_{BT} and C_{BT} (B_{BT}: $r^2 = 0.42$, $P < 0.01$; C_{BT}: $r^2 = 0.55$,

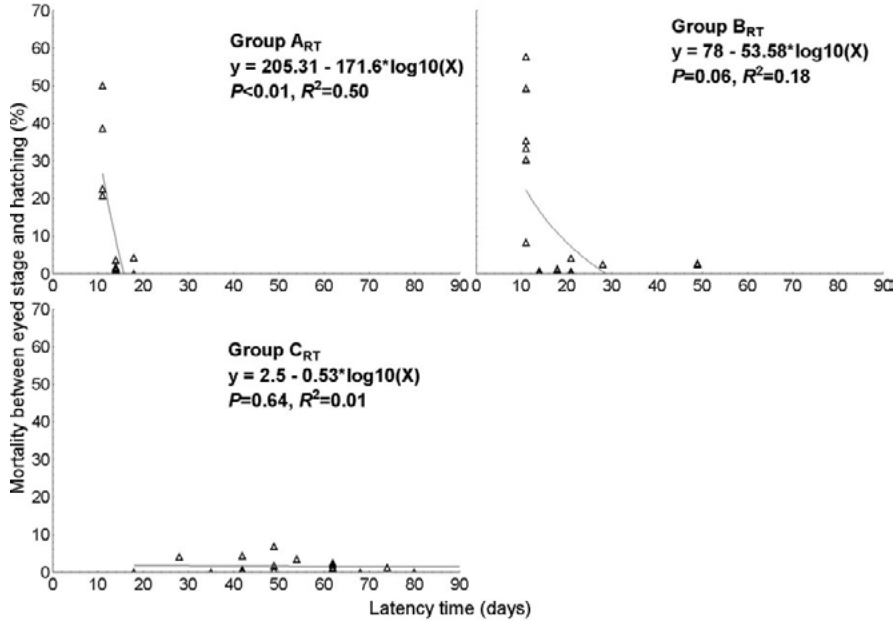

FIGURE 3. Correlation between mortality (eyed stage to hatch) and latency for each group of Rainbow Trout (groups are defined in Figure 1).

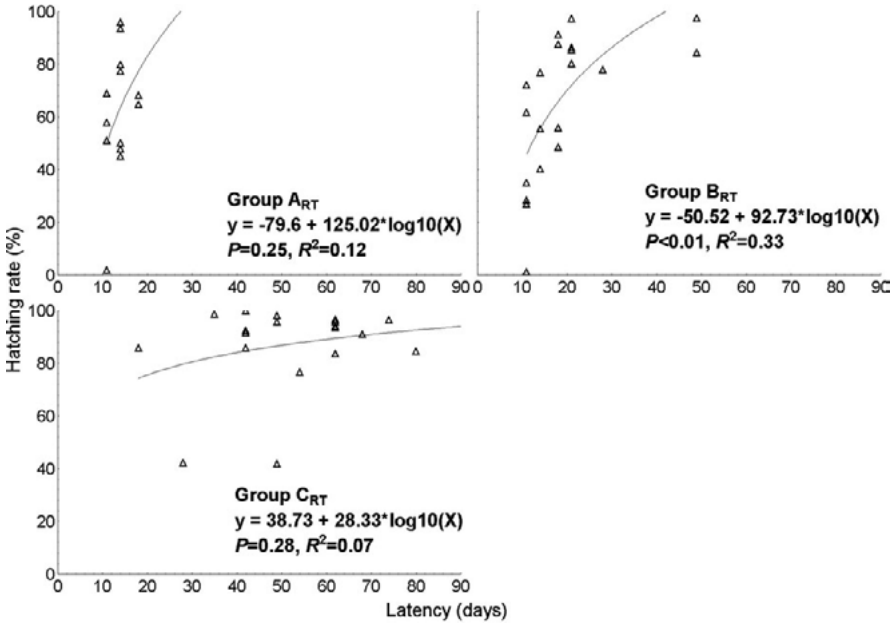

FIGURE 4. Correlation between hatching rate and latency for each group of Rainbow Trout (groups are defined in Figure 1).

$P < 0.01$; Figure 6). Mortality between the eyed stage and hatch showed no correlation with latency for groups A_{BT} and C_{BT} ($P > 0.05$). For group B_{BT}, the mortality value decreased with increasing latency, whereas in control group D_{BT} the opposite trend was observed. However, the correlation was weak in both groups (B_{BT}: $r^2 = 0.36$, $P < 0.05$; D_{BT}: $r^2 = 0.24$, $P < 0.05$; Figure 7). A significant negative correlation between hatching rate and latency was found for group C_{BT} ($r^2 = 0.56$, $P < 0.01$; Figure 8). No relationship between hatching rate and latency was observed in other groups, for which hatching rate remained almost constant regardless of latency (Figure 8). No deformities of hatched Brook Trout yolk sac fry were observed.

DISCUSSION

All of the sGnRHa treatments in our study induced and/or advanced ovulation to a greater or lesser extent in both species; this has been previously demonstrated for other salmonid species. However, in Rainbow Trout, a high dose of sGnRHa during sustained-release FIA treatment (i.e., group A_{RT}) was unexpectedly less effective than a dose that was equivalent to 25% of that level (group B_{RT}). Autopsy and subsequent examination of ova from the nonovulating individuals revealed that the high-dose sGnRHa–FIA treatment did not result in induction of final oocyte maturation. Treatments with GnRHa–FIA at doses ranging from 25 to 50 μg/kg have reportedly been highly effective inducers and synchronizers of ovulation in Rainbow Trout if administered some weeks prior to the spawning season (Arabacı et al. 2004; Vazirzadeh et al. 2008). This range of sGnRHa–FIA doses has also been shown to be effective in Brook Trout, European Grayling, and Northern Whitefish (Svinger and Kouřil 2012, in press). In Brown Trout *Salmo trutta* m. *fario*, doses from 15 to 60 μg/kg were effective when D-Arg^{6}Pro^{9}NEt-sGnRHa was used (V. W. Svinger and D. M. Kallert, unpublished data). Use of the high dose of FIA-emulsified sGnRHa (i.e., as administered to group A_{RT}) in salmonids has not been previously reported, although a number of studies have applied GnRH analogs at doses exceeding 100 μg/kg to Arctic Char (Haraldsson et al. 1993), Atlantic Salmon (Weil and Crim 1983; Crim et al. 1986; Taranger et al. 1992), Caspian Brown Trout *Salmo trutta caspius* (Noori et al. 2010), and Coho Salmon (Donaldson et al. 1981). Since these studies differed in terms of study species, GnRH analogs, GnRHa delivery systems, and experimental conditions, it is difficult to identify common denominators. It can be stated that in these studies, high rates of ovulation and spermiation were obtained when a high dose of GnRHa was applied, which is in contrast to our present results for Rainbow Trout.

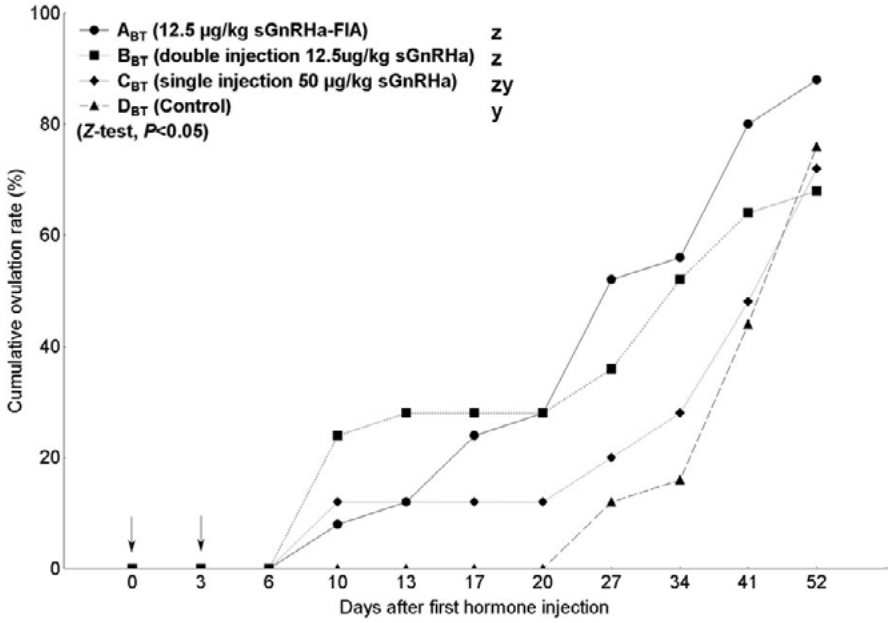

FIGURE 5. Course of ovulation (cumulative ovulation rate, %) in the respective groups of Brook Trout that were intraperitoneally injected with salmon gonadotropin-releasing hormone analog (sGnRHa) emulsified in Freund's incomplete adjuvant (FIA) or with sGnRHa only (A_{BT} = single injection of 12.5 µg of sGnRHa–FIA/kg of body weight [BW]; B_{BT} = double acute injection of 12.5 µg sGnRHa/kg BW, with injections administered 3 d apart; C_{BT} = single acute injection of 50 µg sGnRHa/kg BW; D_{BT} = control, 1:1 FIA and 0.9% physiological saline). Groups without a letter in common are significantly different ($P < 0.05$). Arrows indicate the date(s) of GnRHa administration.

Billard et al. (1984) reported that sustained-release treatment with (des-Gly^{10}, D-Ala^{6}-NEt)-luteinizing hormone (LH)–releasing hormone analog in silicone rubber implants containing a dose of 10–20 µg/kg delayed ovulation in Brown Trout if administered close to the natural spawning time—that is, when simultaneous natural stimulation of LH pituitary secretion was likely to be taking place. In teleosts, Habibi (1991) demonstrated that *in vitro* exposure of Goldfish *Carassius auratus* pituitary to a continuous dose of native sGnRH or chicken GnRH-II resulted in desensitization and refractoriness of the pituitary to stimulation by these same hormones. The pituitary desensitization phenomenon was later observed in Black Porgy *Acanthopagrus schlegeli* (Yen et al. 2002). Whether treatment with continuous-release GnRHa at an unknown or inappropriate physiological stage could impair LH secretion via pituitary desensitization *in vivo* is unclear, especially since neither the Billard et al. (1984) study nor our study addressed this question. It remains speculative whether the effects of high GnRHa doses in sustained-delivery systems are influenced by specific physiological events during the period of hormone release. Nevertheless, it is likely that such cases may occur, and this should be studied in the future.

The present results for Brook Trout expand on the findings of our previous study (Svinger et al. 2013) and clearly demonstrate that a single acute injection (i.e., group C_{BT}) does not evoke high ovulation rates in this species if injected approximately 1 month prior to the natural spawning time. Generally, a synchronization effect was not achieved with any of the treatments, since the doses used in the sGnRHa–FIA treatment and the acute double-injection protocol were too low with respect to injection time. Taranger et al. (1992) demonstrated that even a low acute GnRHa dose of 1 µg/kg BW can be effective in Atlantic Salmon if administered close to the natural spawning time. Thus, the treatments applied to Brook Trout might have been more effective if administered some weeks later. A single acute injection of 25 µg/kg that was given approximately 3 weeks prior to the natural spawning period was effective in up to 50% of Brook Trout females (Svinger et al. 2013). However, higher efficacy of the low-dose (12.5-µg/kg) sGnRHa–FIA treatment compared with the single, acute high-dose (50-µg/kg)

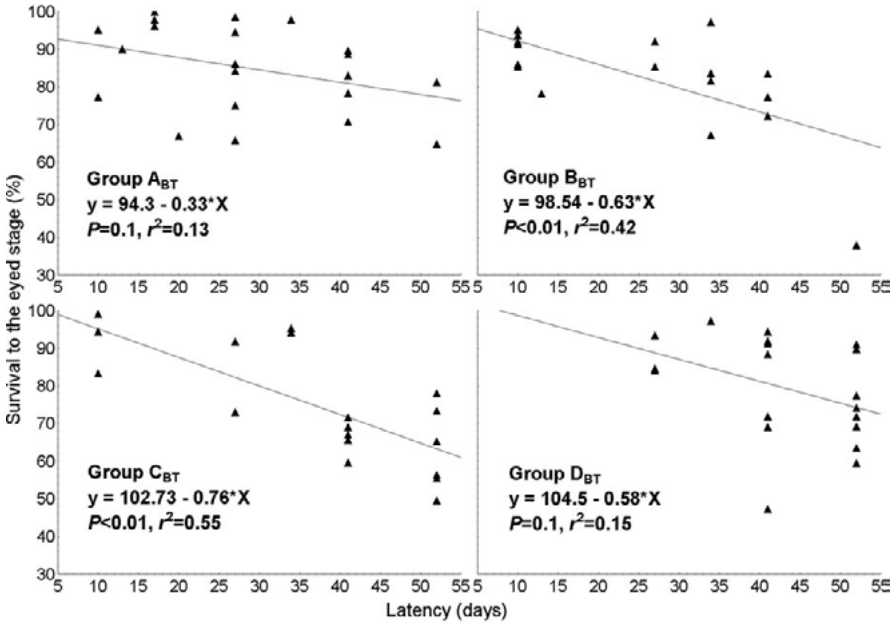

FIGURE 6. Correlation between the percentage of eyed eggs (i.e., survival to the eyed stage) and latency (number of days from the first injection to the date of detected ovulation) for each group of Brook Trout (groups are defined in Figure 5).

sGnRHa treatment underscores the greater importance of sustained GnRHa delivery in salmonids relative to the dose of GnRHa per kilogram of BW per se.

The pH of ovarian fluid is considered a potential marker for egg quality. Poor egg survival was seen when ovarian fluid pH was lower than 7.4 in Rainbow Trout (Wojtczak et al. 2007) or when ovarian fluid pH was less than 8.1 in Lake Trout (Lahnsteiner et al. 1999). Alteration of ovarian fluid pH due to the leaking of proteins into the fluid can indicate the first stages of egg degeneration (overripening), which may also be simultaneously indicated by the presence of certain hydrolytic enzymes in the ovarian fluid (Fauvel et al. 1993; Lahnsteiner et al. 1999; Lahnsteiner 2000). Ovarian fluid pH in our experiments was slightly reduced in Rainbow Trout group B_{RT}, whereas in Brook Trout the ovarian fluid pH was nearly equal among individuals and groups, regardless of sGnRHa treatment. No relationship between ovarian fluid pH and embryo survival measures was found for either species, suggesting that overripening was not the cause of the decreased survival of embryos from treated Rainbow Trout females. This is supported by the fact that females were checked for ovulation twice weekly and that egg wet weight was lower in treated females than in control females. In other species (nonsalmonids as well as salmonids), egg wet weight tends to increase with postovulatory retention in the body cavity, together with ongoing overripening, due to increased water content (Hirose et al. 1977; Lahnsteiner 2000; Aegerter and Jalabert 2004; Mansour et al. 2008; D.M. Kallert, unpublished data). In our study, less-extreme levels of ovarian fluid pH were detected. If we accept the quadratic relationship between the percentage of eyed embryos and the ovarian fluid pH as suggested by Lahnsteiner et al. (1999), it is difficult to find a correlation when ovarian fluid pH lies within the optimum, because survival to the eyed stage does not show a correlation with ovarian fluid pH in the optimum range.

In this study, egg weight upon ovulation was significantly lower with hormone injection in both species, regardless of treatment or dosage. Our results represent the first record of egg diminution in Rainbow Trout after GnRHa treatment. Olito et al. (2001) suggested that GnRHa treatment may stimulate the pituitary to produce LH (gonadotropin hormone [GTH] II), which stimulates final sexual maturation, but not follicle-stimulating hormone (GTH-I), which is responsible for the uptake of egg yolk precursors. This, along with deficiencies in maternal messenger RNA deposition into the ova, could pose a significant problem in later development. Other possible reasons for lower egg weight may be the early stripping of females, thereby

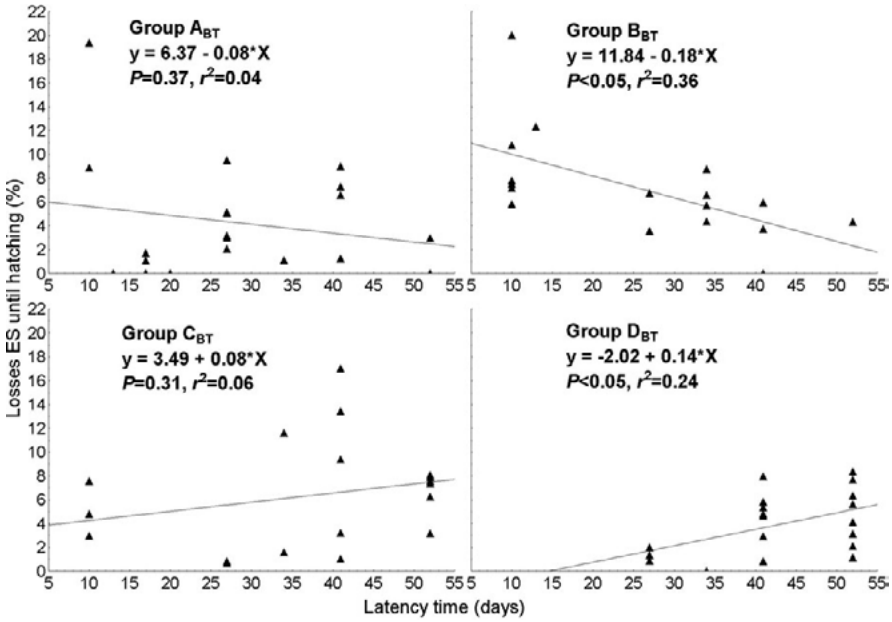

FIGURE 7. Correlation between mortality (eyed stage [ES] to hatch) and latency for each group of Brook Trout (groups are defined in Figure 5).

truncating the final phase of vitellogenesis or causing premature interruption of vitellogenesis via reduction of sex steroid production. Treated fish of both species, particularly groups B_{BT} and C_{BT}, showed reduced egg weight compared with untreated fish, regardless of the similarity in latency.

The reduction in egg weight was not the basis for lower embryo survival in Rainbow Trout, as suggested by Olito et al. (2001), since no correlation between egg size and survival or percentage of deformities was detected in either species. Hence, we can also assume that the reduction in egg weight was probably not accompanied by the absence of specific proteins that Lahnsteiner (2007) strongly correlated with the percentage of eyed embryos in Brown Trout. On the other hand, it is not clear whether the possible absence of these proteins is correlated with reduced egg weight. The ability of GnRHa treatment to alter egg protein composition independent of egg size remains to be investigated. In addition, elevated mortality shortly before hatch was observed in Rainbow Trout groups A_{RT} and B_{RT} and in Brook Trout group B_{BT}. This is in accordance with the report by Bonnet et al. (2007) that the use of D-Arg^{6}Pro^{9}NEt-sGnRHa to induce spawning in Rainbow Trout may result in egg defects that are characterized by embryo mortalities occurring after eyeing. However, the source of these defects remains unclear.

Reduction in egg weight was followed by a decreased weight of yolk sac fry in sGnRHa-treated Brook Trout. This agrees with the results of other authors (Brown 1946; Fowler 1972; Wallace and Aasjord 1984; Hutchings 1991), who reported that salmonid egg size is highly predictive of the size of hatched alevins. Similar to the results of the present study, neither embryo survival nor the percentage of hatched alevins has been reported to be correlated with egg size (Hutchings 1991; Jónsson and Svavarsson 2000). Nonetheless, in Brook Trout, larger eggs have been shown to be positively related to survival throughout the first 50 d of exogenous feeding (Hutchings 1991); furthermore, in Arctic Char, smaller eggs have been found to produce smaller fry with lower initial feeding success and growth relative to larger fry (Wallace and Aasjord 1984). The size advantage may soon be counteracted by other environmental determinants of growth, and under good hatchery conditions, differential egg size is not a primary determinant of reproductive success (see review by Bromage et al. 1992). It is not clear whether a reduction in egg size induced by sGnRHa treatment could result in impaired growth potential of offspring; thus, further examination is needed, especially if fry are intended for use in restocking activities in natural waters.

Our results indicating that egg size is not predictive of embryo survival are in agreement with those of Springate and Bromage

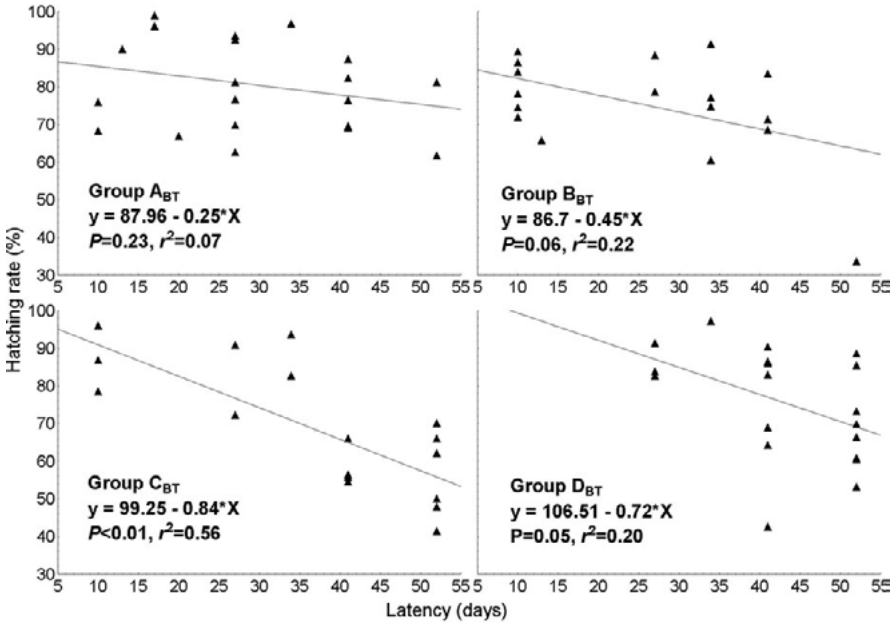

FIGURE 8. Correlation between hatching rate and latency for each group of Brook Trout (groups are defined in Figure 5).

(1985) but are contrary to those of Craik and Harvey (1984), who found a significant positive correlation between egg wet weight and hatching. Inconsistency in results among studies considering egg size as a measure of quality is common (Glebe et al. 1979; Pitman 1979; Kato and Kamler 1983; Thorpe et al. 1984; Wallace and Aasjord 1984). This is mainly due to differences in the age and size of parent fish, variation in culture conditions, and—probably a primary factor—variation in the ripeness stage of the eggs used (Bromage et al. 1992).

When the eyeing rate and hatching rate were plotted against latency in Rainbow Trout, females with the shortest latency had eggs with the lowest embryo survival. Pooling of the data from the Rainbow Trout groups demonstrated a course of embryo survival that was dependent on latency—nearly identical to findings reported by Mylonas et al. (1992) for Brown Trout, Svinger and Kouřil (in press) for Northern Whitefish, and Fitzpatrick et al. (1984) for Coho Salmon. Mylonas et al. (1992) hypothesized that GnRHa treatment caused a reduction in egg quality by disrupting the timing of two distinct processes: (1) final oocyte maturation controlled by maturation-inducing steroid 17α,20β-P and (2) ovulation regulated by prostaglandins. We suggest that this asynchrony is undoubtedly the primary cause of the alteration in egg quality, but there is no conclusive evidence that either GnRHa treatment or dosage is the source. Similar to Fitzpatrick et al. (1984), we suggest that reduced egg survival is chiefly caused by errors in determining the female's oocyte maturation status. There is evidence that Rainbow Trout eggs that are stripped immediately after ovulation have lower survival rates than eggs that are stripped 4–10 d after ovulation (Sakai et al. 1975; Bry 1981; Springate et al. 1984). While conducting experiments with GnRHa-synchronized ovulation, we endeavored to accurately detect the time of ovulation in experimental fish but without considering that underripe eggs might be collected. In autumn 2012, we were able to attain synchronized ovulation in Brown Trout *S. trutta lacustris* broodstock by using acute double injections of D-Arg^{6}Pro^{9}NEt-sGnRHa at 25 μg/kg BW (our unpublished data). In some of the females, ovulation was detected at 9 d postinjection, but manual stripping was delayed to 13 d postinjection. Ovulation in other females was detected at 13 d postinjection, and those fish were immediately stripped. Better fertilization rates were observed in females with the shortest latency but with delayed stripping (99% fertilization) than in females with longer latency that were stripped immediately after ovulation was detected (90% fertilization). Detailed follow-up comparison was not possible in this case, and further assessments are underway.

Variation in ripeness of the eggs being fertilized could also explain the inconsistency in results of previous studies that have

investigated the influence of GnRHa treatments on egg quality. There may also be differences among species, since the survival of eggs from sGnRHa-treated Brook Trout is consistently equal to or better than that of eggs from untreated control fish (Svinger et al. 2013), and the dependence of egg survival on latency in Brook Trout is opposite the pattern observed for Rainbow Trout. In the present study, the sGnRHa dose did not show any influence on egg survival in either species.

In summary, it is evident that the experimental designs that are currently in use in salmonid research must be updated to determine reliable means of avoiding egg mortality after GnRHa treatment. The gross egg mortality observed in our study is unacceptable. For commercial breeders in particular, the savings obtained by reduction of handling and better planning of stripping do not compensate for the potential risk of financial loss associated with low egg survival, investment in GnRHa treatment, and reduced production of eyed eggs.

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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

In all salmonid trials presented in this thesis, GnRHa treatment administered as double acute injection or in sustained release form (GnRHa-FIA) proved to be highly reliable in inducing and synchronizing ovulation. This is in agreement with results of other authors studying Atlantic salmon (Taranger et al., 1992), rainbow trout (Arabaci et al., 2004; Vazirzadeh et al., 2008), Caspian brown trout (Noori et al., 2010), Arctic charr (Gillet et al., 1996), brown trout (Mylonas et al., 1992), and chum salmon (Park et al., 2007).

Acute single injection of GnRHa is the most economically advantageous and the simplest method of GnRHa delivery. However, in this study, a single acute injection of D-Arg^6,Pro^9,NEt-sGnRHa at 25 µg or 50 µg evoked only a low synchronization effect in brook trout ovulation. In Arctic grayling and northern whitefish, a single acute injection of D-Tle^6,Pro^9,NEt-mGnRHa (Supergestran) did not induce ovulation (Svinger et al., 2010). This corresponds with other salmonid experiments in which a single GnRHa injection of D-Ser(tBu)^6,Pro^9,NEt-GnRHa in rainbow trout (Arabaci et al., 2004) and D-Ala^6,Pro^9,NEt-GnRHa in brown trout (Mylonas et al., 1992; Noori et al., 2010) was only partially successful in inducing ovulation. The failure of a single acute injection in some individuals may be due to rapid clearance of effective GnRHa from the circulation (Zohar et al., 1990b), leading to insufficient duration of stimulation of LH secretion (Breton et al., 1990). Ovulation in such individuals is interrupted and continues some time later in response to natural secretion of native GnRH peptide from the hypothalamus. In contrast, no significant differences were found between the single and double acute injection of D-Arg^6,Pro^9,NEt-sGnRHa in northern whitefish (Chapter 3), although the double injection was slightly more effective. This is in contrary to our previous results in northern whitefish using an acute injection of mammalian D-Tle^6,Pro^9,NEt-GnRHa at the same dosage as that of the salmon D-Arg^6,Pro^9,NEt-sGnRHa (Švinger et al., 2010). A single injection of 25 µg.kg^{-1} of D-Tle^6,Pro^9,NEt-mGnRHa induced ovulation in only a small number of the treated females, while the double injection at 25 µg.kg^{-1} induced ovulation in 75% of females. Similar to D-Arg^6,Pro^9,NEt-sGnRHa treatment, single acute injections of D-Nal(2)^6,Pro^9,aza-Gly-GnRHa (Azaglynafarelin, Gonazon) at 16 and 32 µg.kg^{-1} have been reported to effectively induce and synchronize ovulation in European whitefish (Mikolajczyk et al., 2005) and European grayling (Mikolajczyk et al., 2008). Therefore, differences in D-Tle^6,Pro^9,NEt-mGnRHa and D-Arg^6,Pro^9,NEt-sGnRHa treatment (Svinger et al., 2010 and Chapter 3) may be related to differing LH induction potency in northern whitefish

It should be noted that the trial using D-Tle^6,Pro^9,NEt-mGnRHa was carried out at 5 °C compared to the trial using D-Arg^6,Pro^9,NEt-sGnRHa at 0.5 °C, and natural ovulation in northern whitefish occurs one to two weeks after water temperature falls below 2 °C. There is strong evidence that elevated water temperature can modulate the endocrine pathways 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 have the capacity to influence the efficacy of hormone treatment (Pankhurst and Thomas, 1998; King and Pankhurst, 2004b). Further, exposure to reduced water temperatures during the weeks prior to ovulation appears to enhance steroidogenic activity in salmonids and induce or stimulate ovulation, perhaps acting as a late-stage environmental cue (Taranger

and Hansen, 1993; Taranger et al., 2000; Vikingstad et al., 2005; Vikingstad et al., 2008). This may have shifted physiological status of the northern whitefish females in the D-Arg^6,Pro^9,NEt-sGnRHa trial closer to the natural spawning time, making the single acute injection highly effective as reported by Taranger et al. (1992) in Atlantic salmon.

Administration of emulsified D-Arg^6,Pro^9,NEt-sGnRHa in Freund´s incomplete adjuvant proved to be promising technique, ensuring a reliable single injection protocol with high induction and synchronization effect. The results presented in this thesis represent the first reports of utilization of emulsified D-Arg^6,Pro^9,NEt-sGnRHa in northern whitefish and brook trout (Chapter 3 and 4) and correspond with results obtained in rainbow trout (Arabaci et al., 2004; Vazirzadeh et al., 2008), chum salmon (Park et al., 2007), and recently in native Central European stream-resident brown trout (*Salmo t. fario*) and European grayling (Švinger and Kouřil, 2012). GnRHa-FIA treatment has been reported to be associated with high post-spawning mortality of rainbow trout broodstock (Arabaci et al., 2004). Vazirzadeh et al. (2008), in a similar trial, observed no mortality and suggested that the increased mortality found by Arabaci et al. (2004) was due to the higher amount of FIA used in their study. In our current experiments (Chapters 3 and 4, Švinger and Kouřil, 2012), the ratio of FIA to weight of treated females was greater than that used by Arabaci et al. (2004); hence we cannot confirm this suggestion. Although some females died in the post-spawning period in our experiments, the numbers were not unusually high; no differences were found among the groups, and loss of fish was considered related to the intensive handling and bird predation. In salmonid aquaculture, adjuvants are widely used in vaccines against various pathogens, for example *Vibrio salmonicida* and *Aeromonas salmonicida*. Most frequently reported side effects induced by mineral oil adjuvant vaccines are lesions at the injection site, abdominal adhesions, post-vaccination mortality, poor feed uptake and conversion, retarded growth, and downgrading at slaughter (Lillehaug et al., 1992; Press and Lillehaug, 1995; Midtlyng et al., 1996; Poppe and Breck, 1997). No such abnormalities were observed in our experiments, which lead to the conclusion that GnRHa-FIA treatment does not pose a risk to the broodstock. Three months post-treatment, small amounts of white GnRHa-FIA emulsion residue were still visible in the body cavity in northern whitefish, which might call into question the suitability of the GnRHa-FIA-treated fish for human consumption. No such residues were detected in brook trout 6 months post-spawning (Svinger et al., 2013).

A dose of 100 μg.kg^{-1} sGnRHa-FIA was ineffective in a high proportion of rainbow trout broodstock (Chapter 5). This outcome was surprising, and the only similar result was found in brown trout (Billard et al., 1984). Silicone rubber implants containing a dose of 10–20 μg.kg^{-1} delayed ovulation if administered close to the natural spawning time when simultaneous natural stimulation of LH pituitary secretion was likely to be taking place. In teleosts, Habibi (1991) demonstrated *in vitro* that exposure of goldfish (*Carassius auratus*) pituitary to a continuous dose of native sGnRH or cGnRH-II resulted in desensitization and refractoriness of the pituitary to stimulation by these same hormones. The pituitary desensitization phenomenon was later observed in black porgy (*Acanthopagrus schlegeli*) (Yen et al., 2002). It is not clear whether treatment with continuous release GnRHa at an inappropriate physiological stage could impair LH secretion via pituitary desensitization *in vivo*; neither Billard et al. (1984) nor our study addressed this question. Experiments focused on endocrine and immunological action of GnRHa-FIA treatments should be

carried out. In addition, GnRHa release kinetics must be studied to investigate the possibility of reduction of the portion of FIA in the GnRHa-FIA emulsion, which would reduce the GnRHa-FIA treatment costs to even lower levels.

Side-effects of GnRHa treatments on egg size, ovarian fluid pH, and reproductive success were observed in each experiment presented here with the exception of Arctic grayling. This is also the first report of the association of ovarian fluid pH in northern white fish and brook trout with egg survival following GnRHa treatment. In our experiments, however, ovarian fluid pH was shown to have low informative value, and egg viability was influenced by other factors. Egg survival to the eyed stage was reduced in northern whitefish, rainbow trout, and brown trout (Kallert and Švinger, unpublished results) but not in brook trout. Reduction in egg viability following GnRHa treatment has also been reported by other authors in a wide range of other salmonid species (Crim et al., 1983, 1986; Crim and Glebe, 1984; Taranger et al., 1992; Haraldsson et al., 1993; Olito et al., 2001; Noori et al., 2010). Mylonas et al. (1992) explain this as the result of a slight asynchrony between the process of meiotic maturation regulated by the maturation-inducing steroids and the process of ovulation regulated by prostaglandins. Gillet et al. (1996) found the percent of eyed eggs in Arctic char to be negatively correlated with the plasma LH level (high levels elicited either by high GnRHa dose or dopamine inhibitor), which could be the major factor in the asynchrony causing reduction in egg quality. This was also suggested by Billard et al. (1984). Gillet et. al (1996) agree that plasma LH level is not the only factor involved in the control of egg quality, citing variable responsiveness of females to GnRH injection and differences in the physiologic stage of females at the time of treatment. Since plasma LH levels were not measured in our study, we are unable to say whether any of the hormone treatments elicited this phenomenon or whether it influenced egg quality, since eggs of treated females exhibited equal or higher survival rates compared to controls, e. g. in brook charr. Decreased egg survival rates in northern whitefish and rainbow trout were not correlated with higher GnRHa doses in our experiments. When eying rate and hatching rate were plotted against latency in northern whitefish and rainbow trout, eggs with low embryo survival were stripped from females with the shortest latency. Pooling data for our northern whitefish and rainbow trout groups demonstrates a course of embryo survival dependent on latency nearly identical to findings of Mylonas et al. (1992) in brown trout. Based on our results, we suggest that the above mentioned asynchrony is undoubtedly the primary cause of the egg quality deterioration, but there is no conclusive evidence that either GnRHa treatment or dosage (producing enhanced LH levels) is the source. Similar to Fitzpatrick et al. (1984), we suggest that reduced egg survival is chiefly caused by errors in determination of the female's oocyte maturation status. In rainbow trout, eggs must be stripped between fourth and tenth day post-ovulation to ensure high fertilization rates and survival (Sakai et al., 1975; Bry, 1981; Springate et al., 1984). While conducting experiments with GnRHa-synchronized ovulation, we endeavored to accurately detect the time of ovulation in experimental fish, and, when an ovulated female was detected, immediate stripping followed without consideration that under-ripe eggs might be collected. If the individuals with lower eying and hatching had been stripped several days later, results may have been different. Delaying the stripping of ovulating GnRHa-treated brown trout females (*S. t. lacustris*) by several days resulted in significant improvement of fertilization rates, which supports our suggestion. However, further assessments dealing with this issue are needed and shall be considered as priority for the future. This will require new

experimental designs, since those currently used are insufficient to reliably ensure GnRHa-synchronized ovulation without consequences for egg quality.

In all our experiments, GnRHa treatment resulted in reduced egg size. This was previously described only in coho salmon (Donaldson, 1981) and Chinook salmon (Olito et al., 2001). Our results provide the first unequivocal evidence that egg size reduction is a side-effect of using GnRHa in salmonids. Although egg size was not correlated with any of the observed egg survival parameters, it is clear that GnRHa-induced ovulation several weeks prior to natural spawning shortens final stages of vitellogenensis either by truncating the final phase of vitellogenesis or premature interruption of vitellogenesis via reduction in sex steroid production (E_2). Similar to results by Bonnet et al. (2007) in rainbow trout, we observed increased mortality of eggs in late embryonic development (after eyed stage) in northern whitefish and rainbow trout. Lahnsteiner (2007) strongly correlated specific proteins with percent of eyed embryos in brown trout, but it is not clear whether the possible absence of these proteins might be correlated with reduced egg weight. Shortening of vitellogenesis could also cause deficiencies in maternal mRNA deposition into the ova, which could pose a significant problem in later development (Kallert, 2009). Whether GnRHa treatment can alter egg protein composition independent of egg size remains to be investigated.

Based on our results we can recommend GnRHa treatments in all salmonid species addressed here only if synchronization or acceleration of ovulation is essential. A single acute injection should be avoided except in northern whitefish at water temperatures below 2 °C. Hatchery management should be aware that GnRHa treatment causes egg and fry size reduction with unknown consequences, and may reduce egg survival at eying and later embryonic development, especially in northern whitefish and rainbow trout. GnRHa treatment in brook trout improves egg survival, especially when elevated water temperatures at 9–10 °C occur closely prior to, or during, the ovulatory period.

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ENGLISH SUMMARY

Extensive asynchrony of sexual maturation is characteristic of salmonid females, and, for various reasons, the spawning season can be extended for 4 months, although it generally extends over a 3 week to 2 month period, as in brook trout and northern whitefish. In aquaculture, to ensure high fertilization and egg survival rates, it is recommended that identification of ovulating females be undertaken at least twice weekly. This is time-consuming when keeping large numbers of broodstock with an extended spawning season, and risk of stress damage to fish increases. Ensuring sufficient Arctic grayling broodstock relies on capture of wild animals from natural waters before onset of the spawning season. Capture and long-term holding of wild stocks is associated with high pre-spawning mortality. Due to the high ovulation synchronizing effect, GnRHa is practical for induction of ovulation 1–1.5 months prior to the natural spawning season. This work focused on optimization of methods of hormone-controlled reproduction using GnRHa in brook trout, northern whitefish, and Arctic grayling from Siberia. Because this is the first report of use of GnRHa in these species, emphasis was on optimization of the hormone dose and delivery method along with assessment of effects of GnRHa treatment on post-spawning broodstock mortality, egg size, and egg viability.

As in other salmonids, GnRHa treatment effectively synchronizes the onset of ovulation in brook trout and northern whitefish. This gives accurate information about ovulatory status of the broodstock and enables planning of the stripping schedule. The most convenient methods were the double acute injection at 25 $\mu g.kg^{-1}$ D-Arg^6,Pro^9-NHEt-sGnRHa administered 3 days apart and injection of 25 $\mu g.kg^{-1}$ of emulsified D-Arg^6,Pro^9-NHEt-sGnRHa in Freund´s incomplete adjuvant, which prolongs and more effectively stimulates LH secretion and requires only a single injection. Double acute injection at 10 $\mu g.kg^{-1}$ of D-Tle^6,Pro^9-NHEt-mGnRHa (Supergestran) was sufficient to induce ovulation in Arctic grayling 1–1.5 months prior to natural spawning, and enabled accelerated stripping and return of wild broodstock to their natural habitat. Single acute injection proved to be ineffective or unreliable in most cases and therefore cannot be recommended. No negative effect of GnRHa or GnRHa-FIA treatments on post-spawning mortality of broodstock was observed. It has to be noted that that to the extent of law Nr. 166/1999 statute book (Czech veterinary law) injective procedures can be carried out just by the veterinarian or by a veterinarian-authorized person in veterinarian presence.

GnRHa treatment has side-effects and alters egg quality. Apart from in brook trout, egg survival was significantly lower (5–20%) in treated females. Increased hormone dose did not influence egg survival at the levels tested. Egg and hatchling size was lower in treated females compared to untreated specimens irrespective of dose or hormone delivery method. Reduced egg size is, however, not the likely source of reduced viability. In general, our results corroborate results of studies dealing with assessment of effects of hormone treatment on egg quality. Based on current experimental designs it is impossible to identify the cause of the side-effects with certainty. Solving this problem should be priority for future research.

CZECH SUMMARY

Značná asynchronnost pohlavního dozrávání je pro lososovité ryby charakteristická a z různých důvodů může být výtěrová sezóna rozložená až do čtyřměsíčního období (většinou však 3 týdny až 2 měsíce, jako je tomu u sivena amerického či síha peledě). Pro dosažení vysoké oplozenosti a přežití jiker se důrazně doporučuje selektovat ovulující jikernačky z generačního hejna alespoň dvakrát týdně, což znamená při velkém počtu jikernaček a dlouhé výtěrové sezóně značnou spotřebu lidské práce, stresování a riziko poškození generačních ryb. Zajištění generačních ryb pro produkci dostatečného množství násadového materiálu sibiřského lipana arktického je prováděno odlovem generačních ryb z volných vod před nástupem výtěrové sezóny. Odlov a dlouhodobý odchov divokých ryb je značně problematický, stresující a spojený s vysokou předvýtěrovou mortalitou. Kromě vysokého synchronizačního efektu lze GnRHa rovněž využít k indukci ovulace 1–1,5 měsíce před přirozeným termínem výtěrového období. Cílem této práce bylo optimalizovat metody hormonálně řízené reprodukce pomocí GnRHa u sivena amerického, síha peledě a lipana arktického. Protože se jedná o první záznam o využití GnRHa u těchto druhů, důraz byl kladen na optimalizaci dávky hormonu, metody podání a posouzení vedlejšího účinku hormonálního zásahu na povýtěrovou mortalitu jikernaček, velikost a životaschopnost jiker.

Podobně jako u ostatních salmonidů, aplikace GnRHa velmi účinně synchronizuje nástup ovulace u sivena amerického a síha peledě. To dává managementu líhní přesnou informaci, ve kterém dni jikernačky dosáhly ovulace a podle toho lze přesně naplánovat výtěrové práce. K těmto účelům je u obou druhů nejvhodnější použít dvojitou akutní injekci v dávce 25 $\mu g.kg^{-1}$ syntetického analogu D-Arg^6,Pro^9-NHEt-sGnRHa aplikovanou 2–3 dny po sobě, nebo injikaci 25 $\mu g.kg^{-1}$ emulgovaného D-Arg^6,Pro^9-NHEt-sGnRHa ve Freundově inkompletním adjuvanciu, což zaručuje jeho postupné uvolňování s efektivnější stimulací sekrece LH a injikaci není nutné opakovat. U lipana arktického účinně indukovala dvojitá akutní injekce 10 $\mu g.kg^{-1}$ D-Tle^6,Pro^9-NHEt-mGnRHa (Supergestran®) ovulaci 1–1,5 měsíce před přirozeným výtěrovým obdobím, což umožnilo rychlý výtěr a návrat divokých generačních ryb do volné vody. Použití jednorázové akutní (okamžité) dávky se ukázalo ve všech případech jako neúčinné nebo velmi nespolehlivé, a proto jej nelze doporučit. V žádném případě nebyl zjištěn negativní vliv GnRHa nebo GnRHa-FIA na povýtěrovou mortalitu generačních jikernaček. Nutno připomenout, že dle znění zákona č. 166/1999 Sb. ,o veterinární péči a o změně některých souvisejících zákonů (veterinární zákon), musí injekční procedury vykonávat pouze veterinární lekař, anebo ním pověřená osoba v jeho přítomnosti.

Hormonální zásah s využitím GnRHa má vedlejší účinky a mění kvalitu jiker. Kromě sivena amerického bylo přežití jiker vždy signifikantně nižší (o 5–20 %) od injikovaných jikernaček. Zvyšující se dávka hormonu v námi použitých rozmezích neměla na přežití jiker žádný vliv. Bez ohledu na dávku a metodu podání hormonu byla velikost jiker, a tím i velikost váčkového plůdku od hormonálně ošetřených jikernaček salmonidů vždy redukována oproti těm, které pocházejí od jikernaček neošetřených. Snížená velikost jiker však není důvodem jejich horšího přežívání. Obecně naše výsledky jen zvyšují heterogenitu doposud známých výsledků prací, zabývajících se hodnocením vlivu hormonálních zásahů na kvalitu jiker. Na základě dosavadních experimentálních designů nelze s určitostí identifikovat a eliminovat skutečnou příčinu těchto vedlejších účinků. Vyřešení těchto problémů by mělo být prioritou pro další výzkum.

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Ministry of Agriculture of the Czech Republic

3) **NAZV QH91310** – Optimization of methods of hormonal induction of ovulation in commercially important fish species (project leader prof. Dipl.-Ing. Jan Kouřil, Ph.D.)
4) **CZ.1.25/3.4.00/10.00314** – Examination of technology of hormonal synchronization of ovulation in salmonids (project leader prof. Dipl.-Ing. Jan Kouřil, Ph.D.)

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5) **025/2011/Z** – Freund´s incomplete adjuvant as GnRHa carrier in artificial reproduction of northern whitefish (*Coregonus peled*) (project leader Dipl.-Ing. Viktor Švinger)
6) **047/2010/Z** – Breeding and environmental aspects of aquaculture and Hydrocenoses (project leader Assoc. Prof. Dipl.-Ing. Martin Flajšhans, Dr. rer. agr.)
7) **074/2013/Z** – Optimalization of breeding aspects of the pond and intensive aquaculture (project leader Assoc. Prof. Dipl.-Ing. Tomáš Policar, Ph.D.)

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Pond aquaculture	2009/2010
Biostatistics	2009/2010
Intensive fish culture	2009/2010
English language	2011/2012

Scientific seminars	**Year**
Seminar days of RIFCH and FFPW	2009/2010
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	2012/2013

International conferences	**Year**
Švinger, V.W., Kouřil, J., Pavlišta, R., 2010. Induced and synchronized ovulation in Northern whitefish (*Coregonus peled*) using GnRHa (D-Tle^{6},Pro^{9}-NEt) Lecirelin in different dosages. Aquaculture Europe 2010, 5.–8. October, Porto. (oral presentatnion)	2010/2011
Švinger, V.W., Kouřil, J., 2011. The use of different superactive GnRH analogues in artificial reproduction of northern whitefish (*Coregonus peled*). In: Coregonid Symposium, Austria, Mondsee, 26–30 September 2011, p. 62. (oral presentation)	2010/2011

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Scientific Research Institute of Ecology of Fishery Reservoirs, Krasnoyarsk, Russian Federation (Arctic grayling experiments, 1 month)	2009/2010
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