Deliverable Report

Deliverable No:	D5.2	**Delivery Month:**	30
Deliverable Title	An optimized GnRHa therapy protocol to improve spawning performance of F1/F2 Atlantic halibut, and to increase availability of eggs of stable and predictable quality.		
WP No:	5	**WP Lead beneficiary:**	P7. IMR
WP Title:	Reproduction and Genetics – Atlantic halibut		
Task No:	5.2	**Task Lead beneficiary:**	P1. HCMR
Task Title:	GnRHa implant therapy as a means to improve spawning performance		
Other beneficiaries:	P1. HCMR	P22. SWH	
Status:	Delivered	**Expected month:**	30
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Other Scientists participating: Mylonas, C.C. and Papadaki, M. (HCMR), Erstad, B. (SWH)

Objective

The objective of this Deliverable was to describe a protocol for efficient induction of ovulation in Atlantic halibut, based on the use of long-term release implants for gonadotropin releasing hormone agonist (GnRHa). Experiments employed mature farmed F1/F2 generation female Atlantic halibut, implanted either with GnRHa or a sham implant, and then the spawning performance was compared between the implanted and controls as described in *D5.1. Documentation of reproductive performance in wild-captured vs cultured female Atlantic halibut*. The data reported includes appearance and degree of swelling of the ovipore as an estimation of stage of maturation prior to hormone application and spawning parameters (as described in Task 5.1). These parameters were compared by appropriate statistical methods to determine optimum dose and timing of GnRHa treatment.

Background

Atlantic halibut females are periodic spawners that release their eggs in 5-10 batches during the annual spawning season (Norberg et al., 1991). Wild-captured Atlantic halibut females mature and release eggs of good quality in captivity. However, sometimes females of the F1/F2 generation have been reported to display reproductive dysfunctions, including irregular spawning cycles, low and unstable fertilization, low gamete survival and lower realized fecundity than wild females. One way of mitigating these dysfunctions might be to use GnRHa therapy. This has been effective in other teleosts (Mylonas et al., 2010) including flatfish, such as the coldwater batch spawner yellowtail flounder (*Pleuronectes ferrugineus*; Larsson et al., 1997). Thus, we tested GnRHa implantations on F1/F2 halibut females from two populations, one produced and held at P7. IMR, Austevoll Research Station (Norway), and one population that was part of the commercial broodstock at P22. SWH Reipholmen hatchery (Norway).

Materials and methods

Preparation of GnRHa implants

The GnRHa implants were prepared by loading the agonist desGly^{10}, dAla^{6}, Pro^{9}-GnRH-NEthylamide (Alarelin, Bachem, Switzerland) into a matrix of poly [Ethylene-Vinyl Acetate] (EVAc, Dow Corning) according to the procedure of Mylonas et al. (2007). Briefly, 376 or 564 mg of GnRHa (for the 500 and 750 µg per implant, respectively) and 0.43 or 0.24 g of bovine serum albumin (BSA, Sigma, Germany) were dissolved in 7.5 ml dH_2O, and were mixed with 8 ml dH_2O containing 0.8 g of Inulin (Sigma, Germany). The mixture was frozen at –80°C and lyophilized for 48h (Alpha 1-2, Martin Christ, Germany). The dried powder was ground using a glass tissue-grinding rod connected to an overhead mixer (RZR 2020, Heidolph, Germany). Twenty ml of a 15% EVAc solution in $MeCl_2$ were then added to the GnRHa/BSA/Inulin powder, and the mixture was vortexed for 5 min and sonicated for 30s at 30 watts (UP 200S, dr. Hielscher GmbH, Germany). The produced emulsion was poured into a leveled aluminum cast (50 x 50 x 4 mm) placed on a block of dry ice. The solidified plate was then placed in a –20°C freezer for 3 days in order to evaporate the $MeCl_2$, followed by 48 h in a vacuum desiccator to remove any moisture. The implants were punched from the dried GnRHa/BSA/Inulin/EVAC plate using a 2 mm dermal punch (Keyes Punch 3mm, Miltex GmbH, Germany).

The *in vitro* release kinetics of the GnRHa implants were evaluated using the procedure of Mylonas et al. (2007) and Sarter et al. (2006). Briefly, the implants (n=4) were embedded in a 2% solution of low melting agarose (Sigma, Germany) in vitro buffer (3.36 g KH_2PO_4, 11.25 g $NaHPO_4$, 0.4 g sodium azide, 0.4 ml Tween 80 in 2 l of dd H_2O, adjusted to pH 7.0), at the bottom of 7 ml flat-bottom p[ethylene] vials. Once the agarose solidified, 5 ml of the in vitro buffer was added and the vials were placed on a rocking shaker in an incubator at 6°C (Fig. 5.2.1). At various times afterwards, a 500 µl sample of the in vitro buffer was taken from each replicate and stored at –80°C until analysis for GnRHa, using an ELISA developed earlier (Mylonas et al., 2007). The in vitro buffer was replaced with fresh one after each sampling.

The release from the implants has been evaluated using an *in vitro* release system, incubated at 6°C (**Fig. 5.2.1**). The release kinetics were similar in both GnRHa doses, and as expected the implants begin with a

Figure 5.2.1. Set up for the *in vitro* release assay for the GnRHa implants maintained at 6°C (right).

high release as soon as they come into contact with the *in vitro* assay buffer (and hence the fish body fluids when administered *in vivo* (**Fig. 5.2.1**). Thereafter, the release was reduced significantly and after 3-4 days it stabilized to about 3-4 % of the total loaded amount per day. In the *in vitro* assay, the implants continued to release GnRHa for a period of at least 32 days, which was the duration of the assay. The total amount of GnRHa released from the implants during this period was estimated at between 75 – 118% of the estimated loaded hormone (data not shown). The two implants (*i.e.* 500 and 750 µg GnRHa) were used in combination depending on the weight of the treated fish, in order to give an effective dose of ~50 or 100 µg GnRHa kg^{-1}.

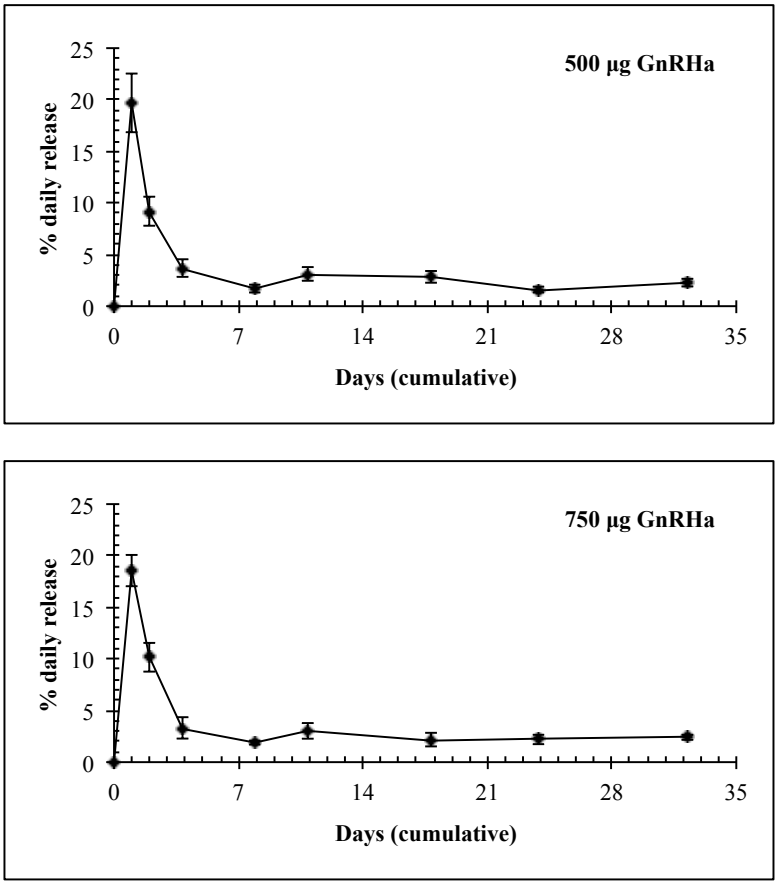

Figure 5.2.2. Mean (±SD) GnRHa released *in* vitro from the GnRHa implants (n = 4) loaded with 500 or 750 µg GnRHa and maintained at 6°C.

Experiment 1 (P7. IMR, Austevoll)

Female Atlantic halibut (n=12) were measured and their weight calculated based on previous data on length/weight correlation in female Atlantic halibut from the same sibling group (**Fig. 5.2.3**). All females had a total length between 100 and 115 cm. Ovarian biopsies were taken in order to estimate ovarian developmental stage. The fish were divided in three groups (n=4), that received either GnRHa implants for an effective dose of 50 µg kg^{-1} or 100 µg kg^{-1}, or were sham injected (Control group). The fish were tagged and held in two 7-m diameter tanks, with a volume of ~50,000 l and were supplied with running seawater from a depth of 155 m. The water temperature was relatively stable, and fluctuated between 7.8 and 8.2°C throughout the year. The GnRHa implanted females were held in one tank and control females in the other. Experimental fish were held together with other untreated Atlantic halibut of both sexes, making a total number of 40 fish in each tank (30 males and 10 females, including those individuals that were used in the experiment). After treatment, fish were left undisturbed for 3 days, and were then checked daily for signs of ovulation.

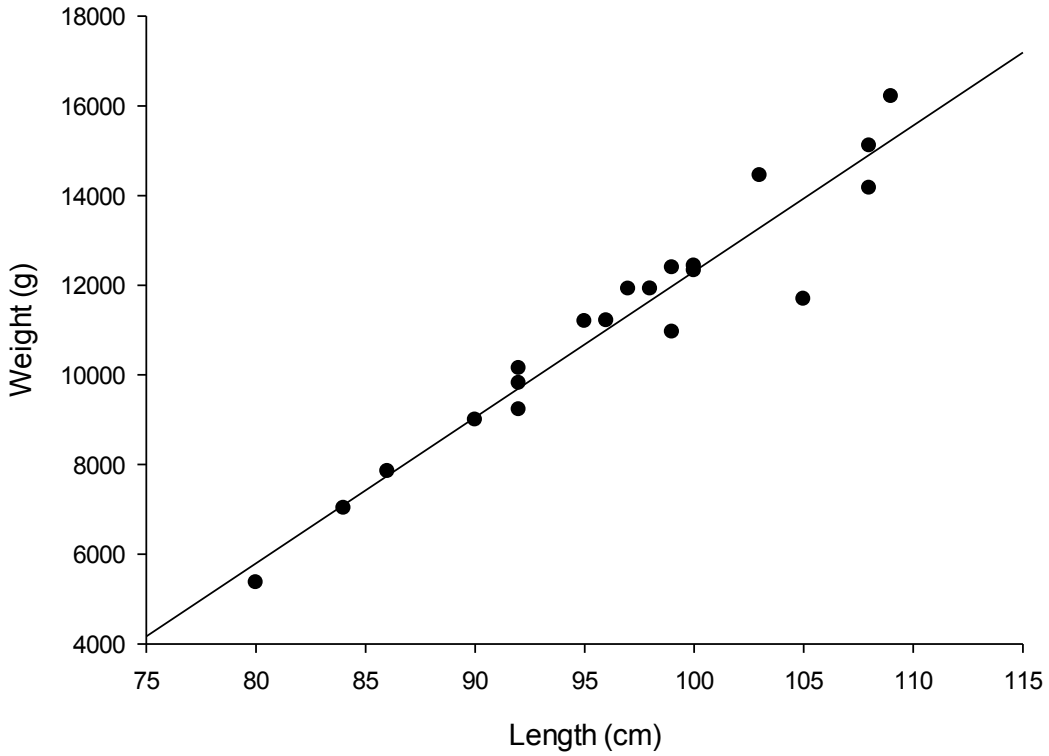

Figure 5.2.3 Weight-length relationship in female Atlantic halibut of the 2007 year-class at IMR, Austevoll Research Station (r^2=0.925; P<0.0001)

Experiment 2, pilot commercial trial (P22. SWH, Reipholmen)

In order to test GnRHa implantation in a commercial system, two trials were run at the Reipholmen hatchery of P22. SWH. Eight females with an average weight of 26 ± 1.3 kg were chosen for implantation based on outer signs of maturation: ovary visible on exterior of fish but not enlarged near the ovipore, degree of swelling and color of ovipore. In the first pilot trial, 4 females were implanted on Jan 16 2015, with 100 µg GnRHa kg^{-1} And 4 females were sham-injected as Controls. All fish were held in the same tank, with a diameter of 11m and a volume of 76m^3. The water supply was from 150 m depth and the temperature throughout the year fluctuated between 7.8 and 8.2°C. One month prior to expected start of spawning, the water temperature was lowered to 5.5-6°C and held constant until all females were spent.

Experiment 3, commercial trial (SWH, Reipholmen)

Ten females were chosen using the same criteria as in the pilot trial, and based on documented spawning performance. All females chosen had given average to low amounts of eggs in previous seasons. On January 14 2016, five females, with an average size of 27 ± 1.4 kg, were implanted with 75 µg GnRHa kg^{-1}. Five females, with an average weight of 32 ± 6.1 kg, were sham-injected as Controls. The fish had not been treated previously, but were held in the same tank and under the same conditions as in the pilot experiment. One Control female died 8 days after treatment and is not included in calculations of fecundity and egg viability (fertilization success).

Results:

Experiment 1 (IMR, Austevoll)

In response to treatment with the GnRHa implants, Atlantic halibut started ovulating after 7 days (**Fig. 5.2.4**). There were no significant differences between fish receiving the two GnRHa doses (50 or 100 µg kg^{-1}) (data not shown), so the data from the two GnRHa doses were pooled. One Control fish ovulated a day later, while the others ovulated 2 to 3 weeks later or not at all. Control fish also ovulated fewer times and over a more prolonged period.

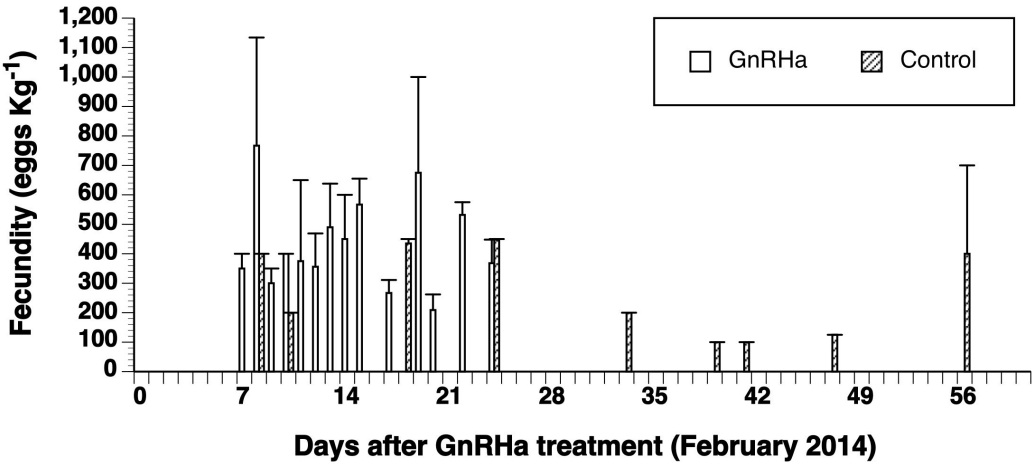

Figure 5.2.4. Mean (±SEM) daily egg production of Atlantic halibut treated with GnRHa implants (50 or 100 µg kg^{-1}) or sham-injected as Controls.

Total egg production was not significantly higher in the GnRHa treated fish (P = 0.069), even though implanted fish spawned more batches than Control fish (**Fig 5.2.5**). Nevertheless, a marked trend towards higher egg production was evident. In all groups, three individuals had only vitellogenic oocytes at the time of implantation, while one individual in each group was close to ovulation, as seen by presence of hyaline oocytes. Implantation did not appear to affect timing of ovulation in females with hyaline oocytes, as the Control fish ovulated for the first time one day after the implanted females. Females implanted while their ovaries were still vitellogenic all ovulated for the first time two to three weeks before Controls at the same stage and had finished spawning by the time the first of these Control fish ovulated.

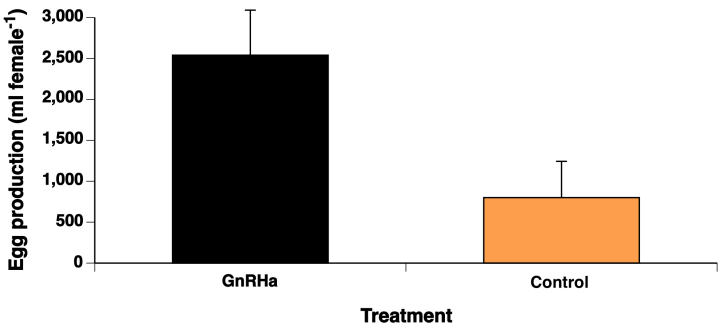

Figure 5.2.5. Total mean (±SEM) egg production of Atlantic halibut treated with GnRHa implants (50 or 100 µg kg^{-1}) or sham-injected as Controls. There was no significant difference between the GnRHa treated and Control fish (ANOVA, P = 0.069).

The GnRHa treatment appeared to synchronize spawning between individuals, as seen when cumulative egg production in the three groups was compared (**Fig 5.2.6**). All GnRHa-implanted fish completed spawning between March 5 and March 22, within a period of 17 days. Control females spawned between March 5 and April 26, a period of 52 days. The spawning periods lasted for 12.8±2.9 days in the group that received 100 µg GnRHa kg^{-1}, for 11±5 days in the group that received 50 µg GnRHa·kg^{-1} and for 14.7±3.2 days in the Control group.

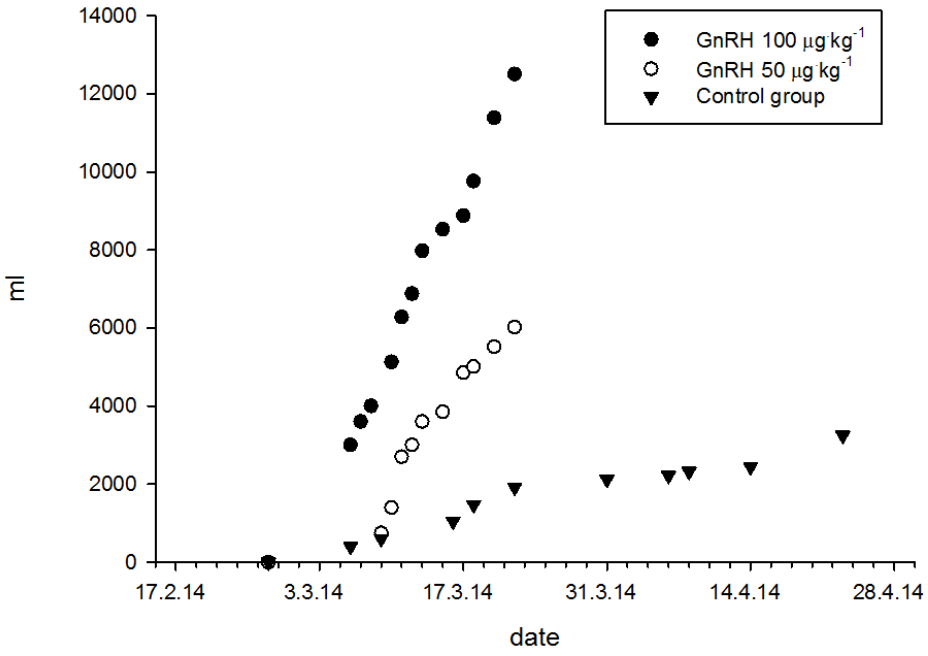

Figure 5.2.6 Cumulative egg production in female Atlantic halibut treated with 100 or 50 µg kg^{-1} GnRHa implants or sham-injected as Controls.

Experiment 2 (SWH Reipholmen)

All implanted females and two Control females ovulated within three weeks of treatment. The remaining Control females did not ovulate during the observation period, which lasted until March 1. There was no difference in start or end date of spawning between implanted females and Control females that spawned. Spawning periods lasted for 17±3.3 days in GnRHa-treated fish and for 17±1.4 days in Control females. Realized fecundity was 294 ± 163 ml·kg^{-1} in implanted fish (n=4) and 403 ± 218 ml·kg^{-1} (n=2) in Control females, respectively. Average fertilization rates were 36.8 ± 18,8% and 24.6 ± 30.8%, respectively. Of the two Control females that spawned, one consistently gave eggs with fertilization rates <4%, while the other had >55% fertilization in 5 out of 7 egg batches. Implanted females spawned between 5 and 8 batches, while the Control female each gave 7 egg batches.

Experiment 3 (SWH Reipholmen)

GnRHa-implanted females ovulated between January 25 and February 17, a period of 23 days and were stripped of eggs for fertilization. The ovulation period in treated females lasted for 16±3.5 days. Measured

fecundity of stripped eggs was 187±72 ml eggs kg^{-1} and average fertilization success was 46.9±0.3%. However, actual realized fecundity may have been higher, as the presence of ovulated eggs in the water indicated at least one of the females released spontaneously several egg batches in the tank. Control females ovulated and were stripped between February 8 and March 16, or during a period of 37 days. The spawning period lasted for 25±9.7 days in Control females. Measured fecundity was 311±176 $ml \cdot kg^{-1}$ and average fertilization success was 53.0±0.3%. Ovulated eggs were also observed in the tank where the control group was held, indicating that at least one female released eggs spontaneously.

Discussion

Although GnRHa implantation did not advance spawning time significantly in Atlantic halibut females, in two of the trials there was an apparent synchronization in spawning time between individuals, as all treated females had completed spawning 1 month before all Control fish were spent. Spawning in Atlantic halibut normally occurs during a period of 2 to 3 months both in captive broodstock and in natural populations (Norberg et al, 1991; Haug1990). This is most likely an adaptation that will ensure production of viable offspring independent of year-to-year fluctuations in temperature and feed availability for larvae. In a commercial production, however, synchronization between individuals can be an advantage as staff efforts can be concentrated to a relatively short period. Atlantic halibut females ovulate and release their eggs (*i.e.* spawn) in captivity, but fertilization of eggs released in the broodstock tank happens only occasionally. Therefore, Atlantic halibut breeders need to be monitored for ovulation and stripped on a regular basis, and eggs are fertilized *in vitro*. Therefore, the use of GnRHa implantation offers a logistic advantage to the commercial broodstock management of the species, by reducing the spawning season.

On the other hand, spawning performance in terms of fecundity per female and fertilization success was not significantly affected by GnRHa treatment in Atlantic halibut females. In the first experiment, which was carried out at P7. IMR, females implanted with GnRHa had a marked trend towards a higher fecundity than Control females. In the 2 commercial trials, however, this trend was not observed. So, at this stage the use of GnRHa therapy to increase fecundity and/or fertilization success is not confirmed. Apparently, spontaneously maturing and ovulating females may produce as many eggs as GnRHa treated individuals. However, GnRHa was demonstrated to be highly effective in ensuring that all females matured and ovulated, as all treated females ovulated at least 3 to 4 egg batches, whereas in all trials some of the Control fish did not ovulate and appeared to resorb their ovaries. So, these results indicate that GnRHa implantation may be a useful tool to ensure that all females in a broodstock group reach maturation and ovulation, increasing parentage contribution to the next generation and increasing overall broodstock fecundity, without having deleterious effects on egg viability.

Deviations: There were no significant deviations in this deliverable.

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