Deliverable Report

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Deliverable Title	Documentation of reproductive performance in wild-captured vs cultured female Atlantic halibut			
WP No:	5		**WP Lead beneficiary:**	P7. IMR
WP Title:	Reproduction and Genetics – Atlantic halibut			
Task No:	5.1		**Task Lead beneficiary:**	P7. IMR
Task Title:	Documentation of reproductive performance in wild-captured vs cultured female Atlantic halibut			
Other beneficiaries:	P17. NIFES	P22. SWH		
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Objective:

The objective of this Deliverable was to provide a systematic documentation of reproductive performance of domesticated (wild-captured) and cultured female Atlantic halibut, and to assess possible differences. Empirical data suggest a significant difference in spawning performance between wild-captured and farmed Atlantic halibut females, but there currently is a lack of systematic documentation. In the present study, individual spawning cycles in cultured and domesticated females, and in reproductive performance were documented for two consecutive years (2015 and 2016). The following parameters were examined: Length of spawning period, number of batches, ovulatory interval, volume and number of eggs per batch, egg diameter, fertilization and hatching rate, cell symmetry and egg steroid concentration (testosterone, cortisol). Moreover, the effect of different photoperiods on the reproductive performance of Atlantic halibut was investigated, and realized and relative fecundity, batch size and average fertilization percentage were assessed.

Introduction

Even though empirical data suggest a significant difference in spawning performance between wild-captured and hatchery-produced farmed Atlantic halibut (*Hippoglossus hippogossus*) females, there currently is a lack of systematic documentation. The Atlantic halibut is a group-synchronous, periodic spawner and in captivity wild-captured females release 6-12 batches of eggs during a period of 2-4 weeks in the spawning season, which lasts from late February to late April in southwestern Norway. In order to obtain eggs with high viability, females have to be stripped according to their individual ovulatory rhythms, to prevent over-ripening and deterioration of the eggs (Norberg et al., 1991). While wild-captured females generally adapt well in captivity, displaying high fecundity with egg batches spawned at regular intervals, hatchery-produced F1/F2 females appear to suffer from a reproductive dysfunction, releasing small batches of eggs at irregular intervals. There is, however, a lack of thoroughly documented evidence to support the hypothesis of such a reproductive dysfunction in farmed females. In the present study, reproductive performance of wild-caught Atlantic halibut and farmed (F1) females was compared. In addition, performance was documented and compared in four groups of F1 broodstock that were photo-manipulated to spawn at four different times of the year. As all wild-caught females that were studied had been held in captivity for >5 years, and were well-adapted to farming conditions, they are referred to as "domesticated" in the present report.

1. Comparison of reproductive performance between wild-captured and cultured Atlantic halibut

1.1 Materials and methods

These experiments were done at the Institute of Marine Research (IMR), Austevoll, Norway and were carried out over the period of two consecutive reproductive seasons.

2015 experiments

Based on the information gathered in the first year of the project (2014), one group of domesticated, wild-captured breeders, held in captivity for at least four years (n=3), and one group of farmed females (F1; n=5) were monitored closely during the spawning season of 2015. All individual females' length was measured in January, before the start of the breeding season. Their weights were estimated based on our previous measurements of length and weight in farmed female Atlantic halibut (**Fig. 5.1.1**) and the weight-length chart provided for Pacific halibut (http://www.iphc.int/publications/bulletins/lenwtmet.pdf) by the International Pacific Halibut Commission.

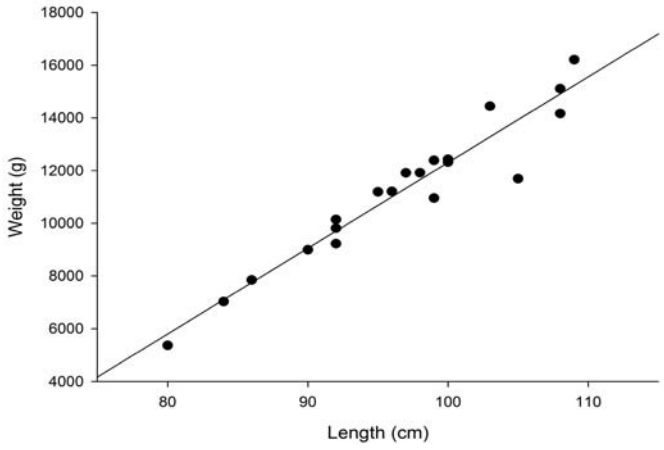

Figure 5.1.1. Weight-length relationship in female Atlantic halibut of the 2007 year-class at IMR, Austevoll Research Station (r^2=0.925; P<0.0001).

Eggs were stripped and fertilized according to previously described procedures (e.g. Norberg et al., 1991). The following parameters were recorded: Egg batch volume, fertilization rate, batch interval (hours between ovulations), number of batches, total and relative fecundity. Fertilization rate was calculated on a subsample of c~200 eggs, using a dissecting microscope (Leica Wild M10).

Egg yolk content of cortisol and testosterone was analyzed by Enzyme-linked immunoassay (ELISA) (Cuisset et al., 1994) in unfertilized eggs from all collected batches. Egg samples were frozen and kept at -80°C until analysis.

2016 experiments

Based on the results obtained in 2015, egg characteristics (fertilization, diameter and cell division symmetry) and hatching success were compared in selected egg batches from the same three 3 domesticated and five 5 farmed females that were used the previous year.

Female Atlantic halibut, either domesticated for at least five years (n=3), or farmed, F1 generation individuals (n=5) were monitored for ovulation, strip-spawned and fertilized during the spawning season of 2016, as described above. As the timing of stripping of the first two batches may not be optimal, the third or fourth egg batch was used in order to ascertain a high fertilization rate. Egg size and dry weight have been documented to decrease over time in batch spawners, so that eggs from the last batches are smaller than eggs from the first (e.g. Kjesbu et al, 1996). This made it necessary to use eggs from similar batch numbers, in order to make a valid size comparison.

Fertilized eggs were photographed at the 8-16 cell stage using a dissecting dark field microscope (Leica MS5; 113.23 pixels/mm), for measurements of egg diameter and blastomere symmetry. Egg diameters were measured automatically in ImageJ (http://rsb.info.nih.gov/ij/) using a custom made plug-in and macros (http://simon.bio.uva.nl/ObjectJ/objectj.html). Eggs were classified according to the following scale:

1. Fertilized, OK
2. Dead
3. Asymmetric cell division
4. From previous batch
5. Unfertilized

Hatching rate was determined in the third or, in three cases, fourth egg batch collected from each female. Eggs were incubated at 6°C under standard hatchery procedures (Mangor-Jensen et al., 1998) for 72 day-degrees (11 days at 6°C). For calculation of hatching percentage, 300 eggs were collected and divided into three beakers containing 500 ml of sterile-filtered seawater (salinity 35‰, temperature 6°C) and incubated in darkness at 6°C for 72 hours. Hatched larvae and dead eggs were counted in a binocular microscope (Leica MS5). Larvae were also photographed in a dissecting microscope (Leica MS5, dark field), in order to document any possible aberrations from normal development.

Validation of ELISA for analysis of egg yolk concentration of cortisol and testosterone

Samples of unfertilized eggs were collected from all batches, and were frozen and stored at -80°C until extraction. For steroid extraction, 500 µg of eggs were homogenized by ultrasonication. The homogenate was centrifuged at 14000 *g* for 2 minutes. Steroids were extracted from 200 µl of the supernatant as described by Pankhurst and Carragher (1992) and Kleppe et al. (2013). Extraction efficiency was determined by addition of a known amount of ^{3}H labelled steroid to the egg homogenate and was 67% for cortisol and 68% for testosterone. Final steroid concentrations were corrected in relation to extraction efficiency. Logit-log binding curves of serial dilutions of steroid standards and plasma samples were parallel

showing that extracted egg samples were suited to the assay conditions. The ED80 and ED20 were 0.004 ng ml^{-1} and 0.08 ng ml^{-1} for testosterone, and 0.07 and 1.2 ng ml^{-1} for cortisol. Detection limits of the assays were 0.008 ng ml^{-1} for testosterone, and 0.07 ng ml^{-1} for cortisol. Intra-assay variation was 6.3% for testosterone (n=6) and 5.4% for cortisol (n=7). Inter-assay variation was 3.4% for testosterone (n=2) and 6.4% for cortisol (n=3). Testosterone and cortisol antisera, acetylcholine esterase-labelled tracers and microplates precoated with monoclonal mouse antirabbit IgG were supplied by Cayman Chemicals (USA). Standard steroids were purchased from Sigma Aldrich (Sigma reference standards). Cross-reactivities for testosterone and cortisol antisera are described by the manufacturer.

1.2 Statistics

Statistica (ver. 12, Statsoft Inc., Tulsa, OK) was used for comparison between groups.

Most of the data had non-homogeneous variances and were subjected to nonparametric analyses. T-tests were used when assumptions were met by the data. Regression analysies was performed in GraphPad Prism (ver. 6.07, GraphPad Software Inc.).

1.3 Results and Discussion

Monitoring of spawning cycles, fecundity and egg viability

The broodstock consisted of 9 individuals, 4 domesticated and 5 farmed females of the F1 generation. The farmed females were second-time spawners, i.e they matured and spawned for the first time in 2014. However, one domesticated female (Frida) developed a large skin lesion and was left undisturbed after the third stripping, because of animal welfare concerns. This individual was not included in the calculations of spawning performance. Biometric data and details on spawning performance are summarized in **Table 5.1.1**. Overall, the domesticated females appeared to spawn fewer, larger egg batches with higher and more stable fertilization success. Relative fecundity, measured as amount of eggs in relation to body weight, did not differ between the two groups. Careful monitoring and timing of stripping, as close to ovulation of the whole batch as possible, was necessary in order to obtain high fertilization of eggs. In cases where the whole egg batch could not be strip-spawned, domesticated females generally released the remaining eggs into the tank. Farmed females, in apparent contrast, tended to leave a small “residue” of eggs, typically 100-250 ml, which were withheld in the ovary. These eggs had to be stripped 6-12 hours after the main batch so that the overripe residue would not have a negative impact on the viability of the next, maturing cohort. Once this was established as an additional routine in strip-spawning eggs from farmed females, the fertilization success stabilized at levels above 75-80% in most individuals, with occasional batches having up to 90-94% fertilization.

Table 5.1.1. Biometric and spawning performance data of domesticated and farmed Atlantic halibut breeders at IMR, Austevoll.

	Domesticated females	Farmed (F1) females
n	3 (4[a])	5
length (cm)	150.7 ± 6.2	113.4 ± 3.9
weight (kg)	48 ± 5.7	19.2 ± 2.3*
number of batches ·female^{-1}	7.3 ± 0.6	9.4 ± 1.7
spawning interval (hours)	82.2 ± 8.4	72.4 ± 22.9
batch volume (ml)	2300 ± 900	700 ± 300*
total fecundity (ml·female^{-1})	16700 ± 420	6800 ± 130*
relative fecundity (ml·kg^{-1})	347 ± 70	349 ± 84
average fertilization (%)	89 ± 7	61 ± 29

[a] One domesticated female was left undisturbed for most of the season, due to a large skin lesion, and was not included in calculations.*Significant difference (P<0.05; Mann-Whitney U-test)

Egg size and viability

Egg diameters were in the range of 2670 to 3700 µm, with average diameters from 3013 to 3104 µm. There were no significant differences in egg diameter between individual fish. However, domesticated females had significantly larger eggs than farmed females and there were significant differences in diameter between viable, dead and unfertilized eggs (p<0.005 and p<0.05, respectively, Kruskal-Wallis test: **Fig. 5.1.2)**.

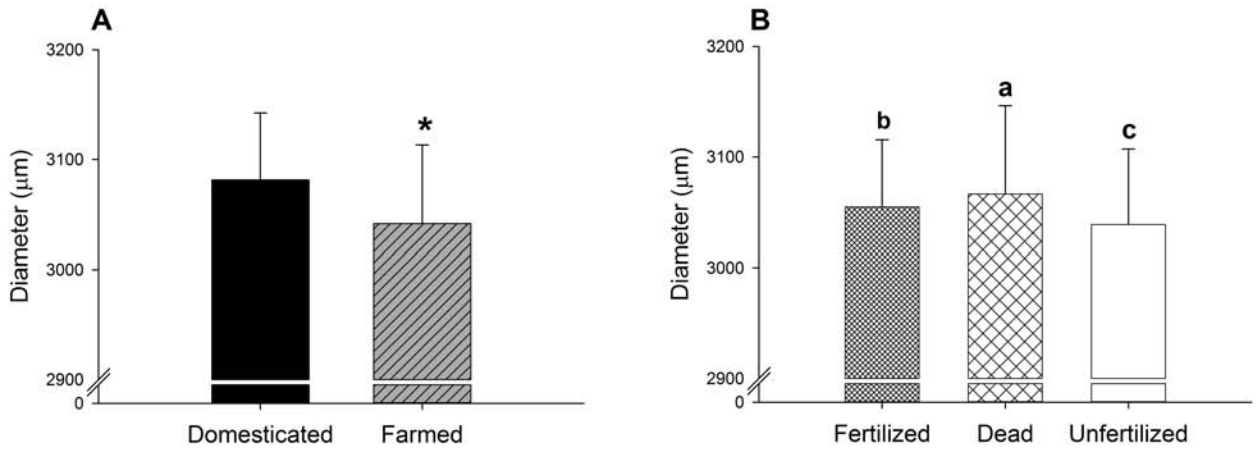

Figure 5.1.2. Diameters of **A)** Eggs from domesticated and farmed Atlantic halibut females (*Indicates significant difference (P<0.05, Kruskal-Wallis test)), and **B)** Fertilized, dead and unfertilized eggs from domesticated and farmed Atlantic halibut females (different letters indicate significant differences (P<0.005, Kruskal-Wallis test)).

When eggs were characterized according to development, most were either in category 1 (fertilized, OK) or 2 (dead). Very few of the fertilized eggs showed signs of asymmetric cell division, and most eggs appeared to be cleared from the ovarian cavity when the batch was spawned (**Fig. 5.1.3**).

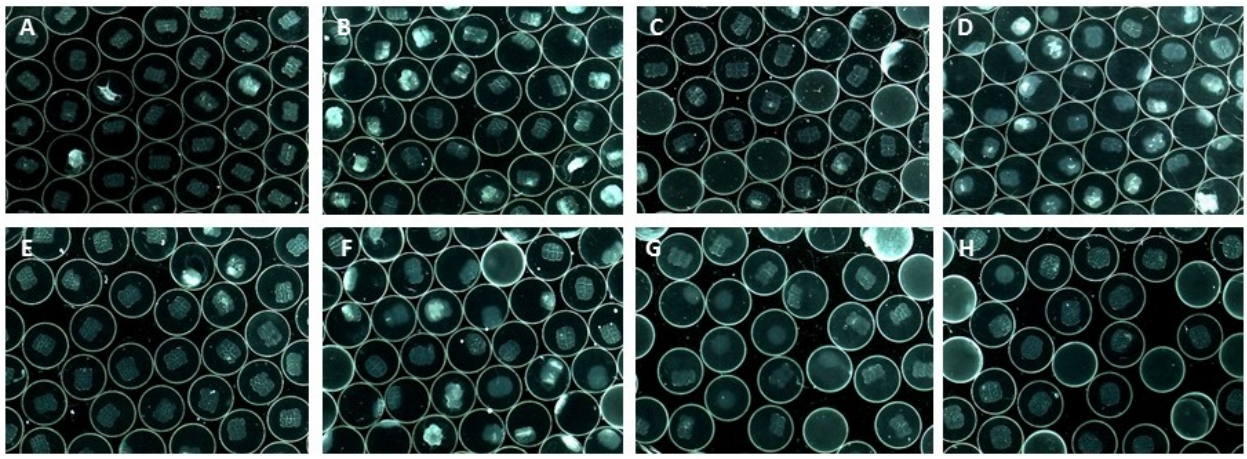

Figure 5.1.3. Fertilized eggs at the 8-16 cell stages from domesticated (A-C) and farmed (D-H) Atlantic halibut.

There was a large variation in the fraction of viable eggs between individuals and between batches from the same individual. Domesticated fish gave eggs that were more viable than those from farmed fish (p=0.04, Mann-Whitney U test). Fertilization and hatching success both showed a tendency to be lower in farmed fish, significant for hatching (p=0.02, Mann-Whitney U test) (**Fig. 5.1.4)**.

Fertilization percentages were 91.3±5.7 and 78.4±13.7 in domesticated and farmed females, respectively, while hatching success was 97.3±0.6% in domesticated females and 76.0±10.9% in farmed fish (**Fig. 5.1.4**A). Overall, eggs from domesticated females tended to have higher fertilization and had significantly higher hatching success, with less individual variation than farmed females.

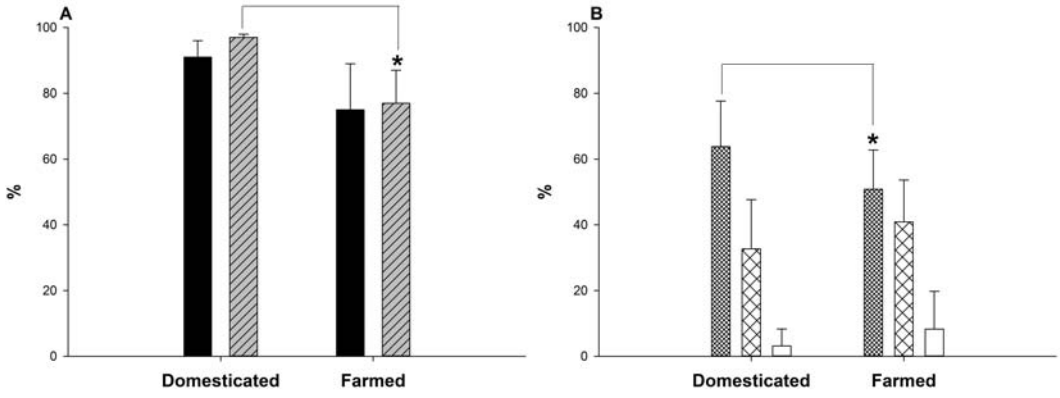

Figure 5.1.4. A) Fertilization (black bars) and hatching (grey bars) success in eggs from domesticated and farmed Atlantic halibut broodstock females. *Indicates significant difference (p=0.02, Mann-Whitney U test). **B)** Percent viable (left), dead (middle) and unfertilized (right) eggs in domesticated and farmed females. *Indicates significant difference (p=0.04, Mann-Whitney U test).

Some morphological differences were observed between eggs and larvae from the different females. Eggs from farmed females generally appeared heavier, and would sink to the bottom of the incubator/beaker, while eggs from domesticated females remained buoyant near the surface. In addition, dead or deformed larvae were observed more frequently when eggs from farmed females hatched (**Fig. 5.1.5)**. It is not clear what caused the deformities, but one possible cause may be mechanical damage of the heavy eggs, that sank and rested at the bottom of the beaker for two days. Further work is needed, however, in order to establish whether this is the cause or if there are genetic/epigenetic factors that may contribute to a higher rate of deformities in larvae from those females.

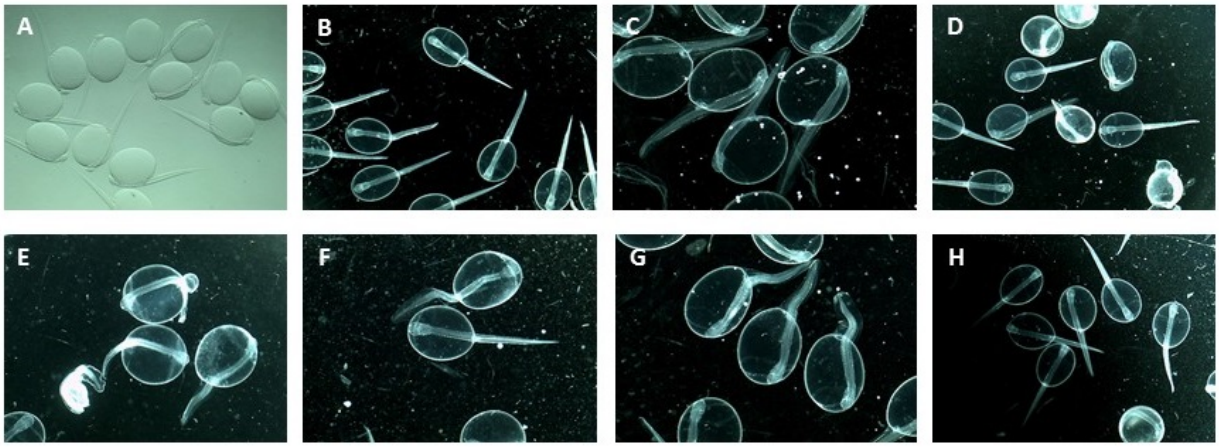

Figure 5.1.5. Newly hatched larvae from domesticated (A-C) and farmed (D-H) Atlantic halibut females. Note spinal deformities in E, F and G.

Egg steroid content

No significant difference was found in average egg steroid content between domesticated and farmed Atlantic halibut (**Fig. 5.1.6**.). There was a general trend towards decreasing egg content of steroids through spawning.

Testosterone levels were low, in the range of 0-11 $pg \cdot egg^{-1}$, especially towards the end of spawning (**Fig. 5.1.7A**). Testosterone levels decreased during the spawning period ($p<0.05$). There was a difference in slopes of testosterone levels between individuals ($p=0.004$) (**Fig. 5.1.7A**) and in average testosterone levels in pooled eggs from wild and farmed fish ($p=0.013$) (**Fig. 5.1.6A**).

The decrease in egg testosterone content observed through the spawning season is in accordance with previously reported changes in plasma concentration (Methven et al. 1992, Björnsson et al., 1998). As spawning progresses, the capacity for sex steroid synthesis becomes lower due to a reduced number of steroid-producing follicle cells. A decrease in egg testosterone content over time would suggest that there is a passive influx of steroids during final maturation, rather than an active sequestration.

Egg cortisol content varied between females, but also between egg batches (**Fig. 5.1.6B**). Overall, egg cortisol content was high, especially in farmed females, ranging from 110 to 450 $pg \cdot egg^{-1}$. Egg cortisol levels in individual fish did not change significantly during the spawning period except in one domesticated female, Rita, where the level decreased ($p=0.007$). However, average levels in individual fish were different ($p<0.0001$) (**Fig. 5.1.7B**). Accordingly, cortisol did not change during the spawning period in pooled eggs of domesticated, wild fish. Data of egg cortisol in pooled farmed fish were best fitted to a second order polynomial ($p=0.0008$), meaning that cortisol levels on average were higher in the middle than in the beginning and end of the spawning period. In a recently published study (Skaalsvik et al., 2015), a similar pattern was found and high cortisol content was correlated with high occurrence of yolk sac edema. Cortisol

implants in female Atlantic cod resulted in increased cortisol concentrations in plasma, oocytes and eggs, but did not affect fertilization, cell division or hatching (Kleppe et al., 2013). However, genes linked to important developmental processes were differentially expressed in oocytes and blastula embryos in response to cortisol. Although no effects were detected in cod egg/embryo viability up until hatching, effects may appear later in development. Our study was limited to egg quality parameters and hatching rate, but future work should address the possible impact of high egg concentrations of cortisol on Atlantic halibut larval development.

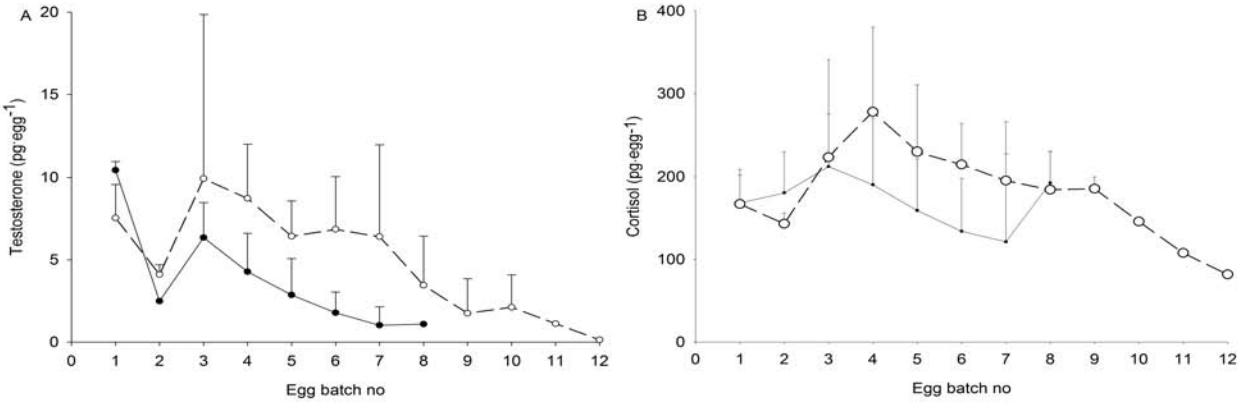

Figure 5.1.6 Average content of testosterone (A) and cortisol (B) in eggs from domesticated and farmed Atlantic halibut broodstock. Domesticated females: Black symbols, solid lines. Farmed females: Open symbols, dashed lines.

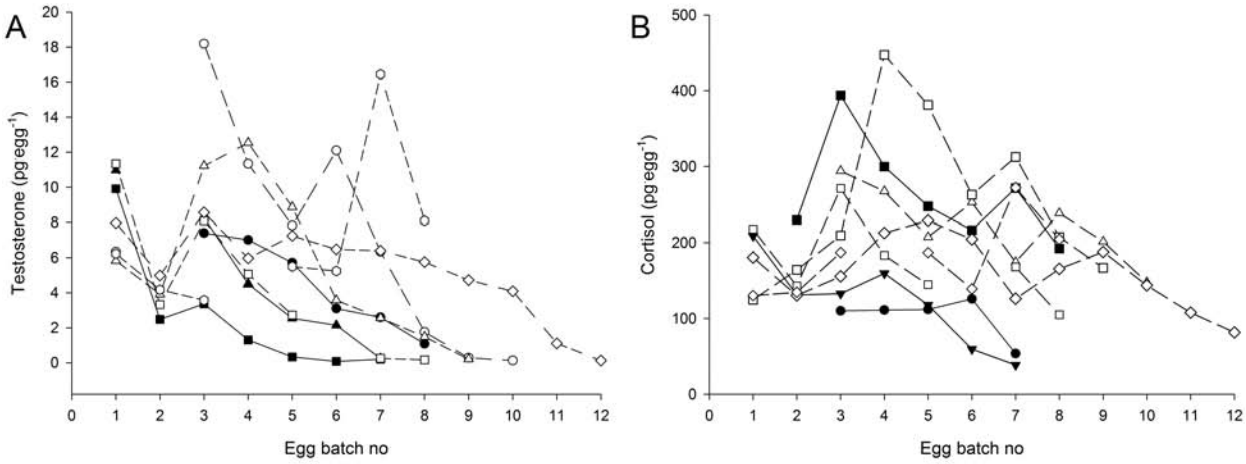

Figure 5.1.7 Individual profiles of testosterone (A) and cortisol (B) content in eggs from domesticated and farmed Atlantic halibut. Domestic females: Black symbols, solid lines. Farmed females: Open symbols, dashed lines.

2. Effect of the application of different photoperiods on Atlantic halibut egg quality

These experiments were held at the commercial hatchery of Sterling White Halibut (SWH), Reipholmen, Norway over two years.

2.1 Materials and methods

Sterling White Halibut no longer uses wild-caught broodstock, but only farmed females. All females used were established spawners of between 12 and 20 years of age. Data was collected from four spawning groups that were held at either normal (C1) or phase-shifted (C2,C3,C4) photoperiod and set to spawn from December 2014 (C4) to August 2015 (C3), respectively (**Fig. 5.1.8**). Individual spawnings were recorded, and females were stripped accordingly, although for practical purposes nighttime stripping was not carried out. Total and relative fecundity, number and size of batches and fertilization rates were individually recorded in all females.

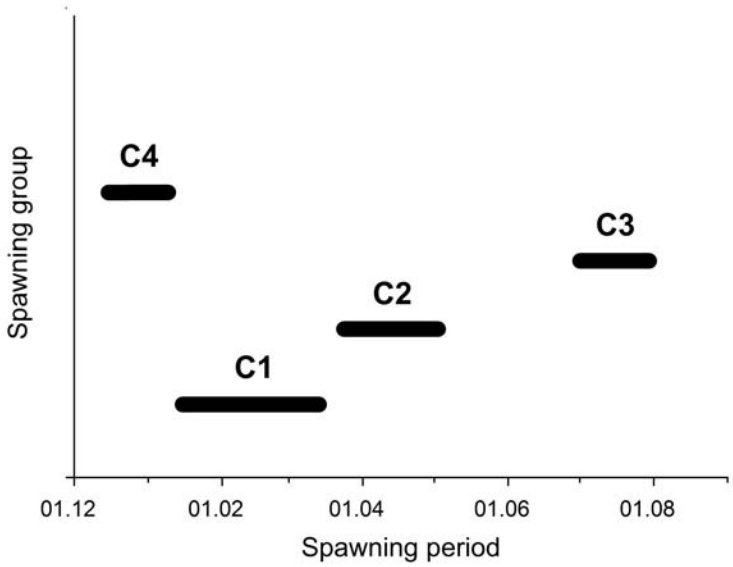

Figure 5.1.8. Spawning periods in the four Atlantic halibut broodstock groups, C1, C2, C3 and C4 that were followed during the study.

2.2 Statistics

Data were tested for homogenous variances using Levenes test and were subjected to one way ANOVA, using spawning time as the grouping variable and Tukey's post hoc test to compare differences between means. Regression analysis was performed to identify an effect of spawning time on fecundity. Statistica (ver. 12, Statsoft Inc., Tulsa, OK) was used for comparison between groups.

2.3 Results and Discussion

Biometric data and data on fertilization success are summarized in **Table 5.1.2**, and fecundity, average and median fertilization are presented in **Fig. 5.1.9**. Fish used in the experiments in task **5.2** were chosen from group C1, based on the information obtained in the present task.

Table 5.1.2. Biometric data and fertilization success for Atlantic halibut broodstock held at SWH and used in Task 5.1. Different letters indicate significant differences (ANOVA and Tukey's post-hoc test; P<0.05).

	C1	C2	C3	C4
n	25	12	21	13
Year class	1995/96 + 2003	1998 + 2002	2002	1995/96 + 2003
Average weight (kg)	27.3 ± 4.9^{ab}	33.3 ± 6.3^{c}	30.6 ± 4.7^{bc}	24.7 ± 4.4^{a}
Number of batches spawned	7 ± 3^{a}	6 ± 2^{a}	6 ± 1^{a}	3 ± 2^{b}
Average fertilization (%)	44.6 ± 28.0	59.5 ± 25.8	57.0 ± 27.0	48.0 ± 30.6
Eggs ≥80% fertilization (%)	13	25	24	18

Overall, total and relative fecundity was similar in all groups except C4, where fewer egg batches were obtained from each female. Individual batch size was, however, similar in all groups. Relative fecundity of the SWH females in groups C1-C3 (355-385 $ml \cdot kg^{-1}$) was similar to that of females held at IMR, which was 347 and 349 $ml \cdot kg^{-1}$ in domesticated and farmed females, respectively. Significant regressions with time of onset of spawning were found for both realized and relative fecundity (p=0.047 and p=0.048, respectively), and in broodstock groups set to spawn late in the year compared to natural spawning, egg yield became lower.

Fertilization success did not differ between the four groups, and was highly variable between batches, as well as between individual females. However, groups C2 and C3 had a larger proportion of high quality eggs, i.e. egg batches with more than 80% fertilization. Overall, the lowest fertilization success, as well as the lowest proportion of high quality eggs were found in group C1, which was the group chosen for treatment with GnRHa in order to induce ovulation (*Deliverable 5.2 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*).

In all, 412 egg batches, with a total volume of 714.6 l were strip-spawned at SWH in 2015. Of those, ~139 l had >80% fertilization and were classified as high quality eggs. A few females in each group had consistently higher fertilization success than the others. The two groups that had the oldest fish also had a lower proportion of eggs <80% fertilization. It was not possible to directly relate high age with low egg quality in the present study. However, concentrating on high-quality breeders may be useful in order to reduce the very considerable effort connected with strip spawning and egg collection of Atlantic halibut.

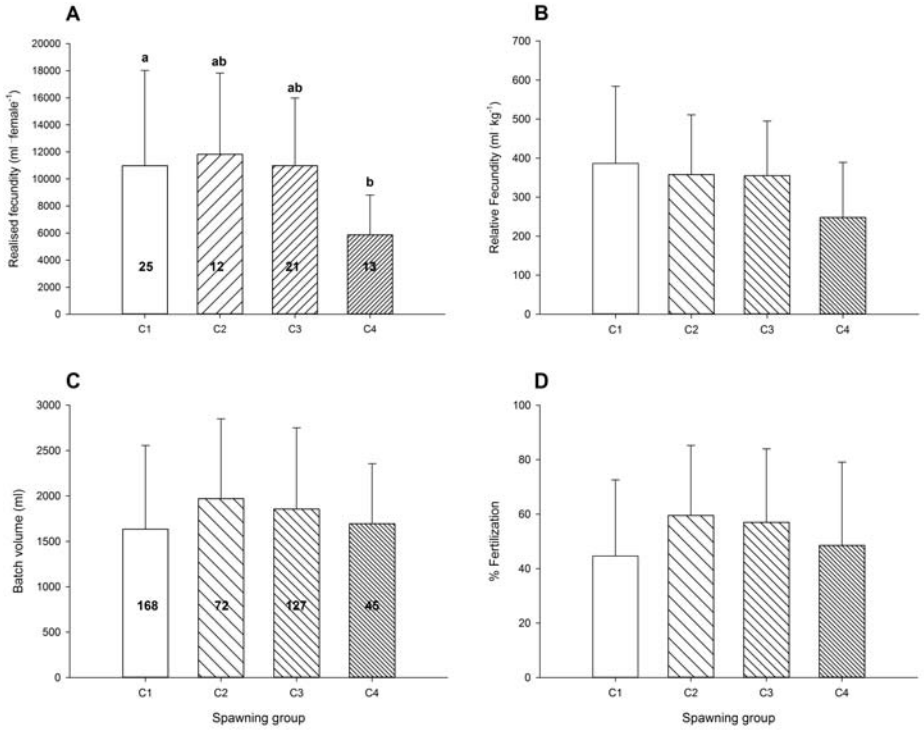

Fig. 5.1.9. Egg quality in four Atlantic halibut broodstock groups with different spawning periods. **A** Realized fecundity (eggs·female^{-1}, number in bars=n (females)), **B** Relative fecundity (eggs·kg^{-1}), **C** Average batch size (ml, number in bars=n (batches)) and **D** Average fertilization. Different letters above bars indicate significant differences (ANOVA and Tukey's post-hoc test, $P<0.05$).

3. Conclusions

Wild-caught, domesticated females were predictable spawners that consistently gave eggs of very high quality (>85% fertilization). Farmed females also produced eggs of high quality when their ovulatory cycles were identified and stripping was carried out close to ovulation. For commercial, as well as breeding purposes, it is not practical to rely on wild-caught females. However, both at IMR and at SWH, relatively few farmed females consistently produced eggs with fertilization rates >80-85%. As a consequence, it may be necessary to include wild-caught broodstock in future breeding groups in order to ensure a broad enough genetic material. Identifying potential high-quality breeders and concentrating the strip-spawning effort on those females may be useful in order to reduce the very considerable workload connected with spawning and egg collection of Atlantic halibut.

References

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Deviations: In the description of Task 5.1, farmed and domesticated (wild-caught) broodstock would be compared at two locations: IMR, Austevoll and SWH, Reipholmen. However, SWH now only holds farmed broodstock females and the following modification was made: Spawning performance was compared between four different broodstock groups held at different photoperiods at SWH, Reipholmen, while the comparison between farmed and wild-caught females was made at IMR, Austevoll. Although this meant that fewer wild-caught individuals were available for our study, we gained useful information on the effects of long-term photoperiod manipulation on spawning output in Atlantic halibut broodstock. All in all, this increased the information gained from Task 5.1, which is presented in this Deliverable.

A minor deviation is that estradiol-17ß (E2) concentration was not measured in the eggs. The extraction efficiency of steroids proved to be low and because of this we had limited amounts of extracted steroids. Preliminary data showed that concentrations of testosterone (T) and cortisol (F) were higher than E2, and for this reason we prioritized measurements of these two steroids.

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