The relationship between sperm density, spermatocrit, sperm motility and fertilization success in Atlantic halibut, *Hippoglossus hippoglossus*

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Received 21 December 1999; received in revised form 15 August 2000; accepted 12 September 2000

**Abstract**

The commercialization of Atlantic halibut, *Hippoglossus hippoglossus*, aquaculture has been hampered by a failure to obtain consistently high fertilization rates. The principal goal of this research was to determine the optimum sperm density for successful fertilization of Atlantic halibut eggs. Sperm densities ranged from $2 \times 10^{11}$ to $6 \times 10^{11}$ spermatozoa/ml at 23% and 99% spermatocrit, respectively, for 36 milt samples collected from 17 males. Regression analysis showed a significant positive linear relationship between sperm density and spermatocrit, supporting the use of spermatocrit as a rapid estimator of sperm density in this species. There was no relationship between sperm density and sperm motility defined as time elapsed from activation until <5% of the spermatozoa maintained forward swimming activity. Implant with a salmon gonadotropin releasing hormone analogue (GnRH(a); Ovaplant™) was followed by a reduction in sperm density, but this affected neither the relationship between sperm density and spermatocrit, nor between sperm density and sperm motility. There was no effect of sperm density on fertilization success within the range of sperm:egg volume ratios of 1:15.6 to 1:8000, equivalent to...
4.6 × 10^8 to 9.4 × 10^5 spermatozoa/egg, respectively. © 2001 Elsevier Science B.V. All rights reserved.

**Keywords:** Hippoglossus hippoglossus; Spermatocrit; Sperm density; Motility; Fertilization success; GnRH(a)

### 1. Introduction

The Atlantic halibut, *Hippoglossus hippoglossus*, is one of the most likely alternatives to Atlantic salmon, *Salmo salar*, for cold-water aquaculture in the North Atlantic (Brown et al., 1995). Successful commercial culture of this flatfish species has, however, been hampered by a variety of problems during early development under culture conditions, prior to weaning and metamorphosis. Among these problems is a failure to obtain consistently high fertilization rates.

The use of high quality gametes is of great importance for ensuring the production of viable larvae, but gamete quality is difficult to assess in a quantitative and meaningful manner (Kjørvik et al., 1990). Techniques used to assess sperm quality in fish include monitoring sperm density and motility, and fertilization success (Billard, 1978; Mounib, 1978; Aas et al., 1991; Methven and Crim, 1991). Egg quality is evaluated by monitoring ovulatory rhythms, blastomere morphology, buoyancy, and fertilization and hatching rates (Norberg et al., 1991; Bromage and Roberts, 1995; Shields et al., 1997). The ratio of spermatozoa to eggs, contact time between gametes, and fertilization method are the other factors that have been evaluated for maximizing fertilization rates (Suquet et al., 1995; Chereguini et al., 1999). Sperm:egg volume ratios used in research with Atlantic halibut have been in the range of 1:100 (Rabben et al., 1986) to 1:1000 (Mangor-Jensen et al., 1998), although recently this ratio was reduced to 1:10,000 without reducing fertilization rate (Vermeirssen et al., 2000b).

The standard method for determining sperm density in fish milt is to count spermatozoa, a time-consuming procedure generally done using a haemocytometer. In the search for faster and more practical ways to estimate sperm density, both centrifugation (to determine spermatocrit) and spectrophotometry (to determine optical density) have been used. A direct relationship between sperm density and spermatocrit has been established for several salmonid species (Bouck and Jacobson, 1976; Baynes and Scott, 1985; Piironen, 1985; Munkittrick and Moccia, 1987; Ciereszko and Dabrowski, 1993; Poole and Dillane, 1998), whitefish, *Coregonus clupeaformis* (Ciereszko and Dabrowski, 1993), yellow perch, *Perca flavescens* (Ciereszko and Dabrowski, 1993) and Atlantic cod, *Gadus morhua* (Rakitin et al., 1999), but not for turbot, *Scophthalmus maximus* (Suquet et al., 1992b), the only flatfish species for which this relationship has been reported. A direct relationship between sperm density and the optical density of fish milt has, on the other hand, been demonstrated for turbot (Suquet et al., 1992a,b), as well as for salmonids, whitefish, yellow perch and seabass, *Dicentrarchus labrax* (Ciereszko and Dabrowski, 1993; Poole and Dillane, 1998; Fauvel et al., 1999). Since both spermatocrit and optical density are easy to measure, the choice of methods has generally been based on access to equipment.

The objectives of this research were (1) to assess the suitability of spermatocrit as a rapid estimator of sperm density in Atlantic halibut, (2) to determine whether sperm
motility is influenced by sperm density in this species, and (3) to determine the optimum sperm density for successful fertilization of Atlantic halibut eggs.

2. Materials and methods

Sperm samples were obtained during the 1998 and 1999 spawning seasons, using wild-caught broodstock held at the St. Andrews Biological Station (Fisheries and Oceans Canada). The mean weight of the fish was $12 \pm 3.3$ kg, and they had been held in captivity for 2 to 7 years. Freshly stripped milt was stored on ice in 150-ml plastic containers until used. Any samples that were obviously contaminated (e.g., with urine) were not kept. A total of 36 usable milt samples were collected from 17 individual males. Seven of these samples were obtained after broodstock had been implanted at the base of the dorsal fin with a gonadotropin releasing hormone analog (GnRH(a); Ovaplant™, Syndel International) at approximately 15 µg/kg body weight.

Spermatozoan density was determined using a haemocytometer. Milt was first diluted in a 50-ml test tube by adding 10 µl of milt to either 30 ml (spermatocrit < 80%) or 40 ml (spermatocrit > 80%) of a non-activating solution (150 mM sucrose, 1.7 mM CaCl₂, 7 mM MgSO₄, 86 mM glycine and 30 mM Trisma buffered at pH 8.0), and then mixed on a vortex mixer. Duplicate dilutions were made for each milt sample and triplicate counts of 50 squares (0.00025 mm²/square each) were made on the haemocytometer for each dilution. The mean of the three counts was calculated for each of the two dilutions, and then the mean of these two values used to calculate the actual sperm density. Samples were left undisturbed on the haemocytometer for a few minutes prior to counting, to allow sperm cells to settle. Counts were conducted using an inverted microscope (Hund Wetzlar Wilovert, 320× magnification).

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Spermatocrit was determined using milt collected into microhaematocrit tubes (75 mm length, 1.1–1.2 mm inner diameter) and centrifuged at 5500×g. An initial experiment was conducted to determine the duration of centrifugation required to achieve stable readings, by measuring spermatocrit at 10-min intervals for up to 1 h of centrifugation. Duration of centrifugation had a significant effect on spermatocrit when considering all time intervals ($F = 11.1$, $p < 0.001$, $n = 36$), but there was no statistically significant difference among spermatocrits after centrifugation for 30 min (72%), 40 min (66%), 50 min (64%) or 60 min (61%) ($F = 2.3$, $p = 0.11$, $n = 24$). Based on these results, 40-min centrifugation was used for subsequent spermatocrit determinations, as this appeared to be the duration where the curves leveled out (Fig. 1). Spermatocrit for each milt sample was estimated based on the mean value of six tubes, with centrifugation starting within an hour of sample collection.

Sperm motility was determined by diluting 10 µl of milt in 1 ml non-activating solution, and then 1 µl of this diluted solution was pipetted onto a 1% (w/v) BSA-coated microscope slide. Activation was achieved by adding 25 µl of a standardized (33 ppt) saltwater solution prepared by adding Instant Ocean™ salt to seawater. Milt samples were stored on ice and the non-activating and seawater solutions stored at 7°C–8°C until needed. Once diluted, milt was held on ice in a cooler between preparations of replicate slides. The duration of sperm motility was subjectively
Fig. 1. The influence of centrifugation time (at 5500×g) on spermatocrit in Atlantic halibut. Each line represents data for milt collected from a different male, as mean values of six replicate samples per male at each time interval. The vertical line denotes the centrifugation time interval used for all subsequent experiments.

evaluated as the time elapsed from activation until < 5% of the spermatozoa maintained forward swimming activity. Sperm motility observations were done at room temperature (21°C–24°C), using six replicates per sample, within 2 h of milt collection. One person conducted all the sperm motility observations, in order to decrease the degree of variation.

Fertilization success was determined at sperm:egg volume ratios of 1:15.6 to 1:8000, equivalent to 4.6 × 10⁶ to 9.4 × 10⁵ spermatozoa/egg. (Numbers of spermatozoa per egg were calculated using the data in Fig. 2 to convert spermatocrit values to sperm

Fig. 2. The relationship between spermatocrit and sperm density for 36 milt samples collected from 17 male Atlantic halibut (● denotes samples obtained after GnRH(a) implantation; □ denotes no GnRH(a) implantation). For density measurements, each point represents the mean of two dilutions, with three counts per dilution. Plots with no bars indicate SD < 1.1 × 10⁻⁹.
densities, combined with egg counts of 5-10 ml samples from different batches of eggs 
(499 ± 38 eggs/sample.) Three trials were conducted (March 16, March 25 and April 
27, 1999) using different batches of eggs and milt for each trial. During each trial, 
triplicate samples from the same batch of eggs and milt were fertilized at each 
sperm:egg volume ratio. The intermediate ratio of 1:500 was used as a control, using 
fresh, undiluted milt. In all other cases, spermatocrit was adjusted to 58–60% prior to 
fertilization in order to obtain a standard sperm density; this was done by adding halibut 
seminal plasma that had previously been obtained through centrifugation and stored at 
−80°C for at least 2 days to inactivate any remaining spermatozoa (Stoss and 
Donaldson, 1982). Prior to dilution, spermatocrits of milt samples used for each 
fertilization trial were 68%, 80% and 93%, respectively.

Eggs were stripped into a 3-l plastic beaker and stored in a cooler on ice. Fertilization 
experiments were conducted in a cold room at 5.4°C–5.9°C. After adding the correct 
volume of milt to 25 ml eggs (ca. 1250 eggs) measured in a graduated cylinder, 25 ml of 
saltwater (33 ppt) was added and the mixture stirred for 20 s. After 10 min, the eggs 
were rinsed and transferred to beakers containing 500 ml saltwater (UV-treated and 
filtered through 5.0 and 0.35 µm cartridge filters) for incubation in the dark. At 18–22 h 
post-fertilization (at the 8- and 16-cell stages), samples of eggs were preserved in 
Stockard’s Solution (Bonnet, 1939) for fertilization assessment. Eggs were considered 
to have been fertilized if cell divisions were visible at 160 × magnification. A minimum of 
300 eggs was examined from each sample for the calculation of absolute fertilization 
rate ([number of eggs with cell divisions]/[total number of eggs in sample]) and relative 
fertilization rate ([absolute treatment fertilization rate]/[absolute control fertilization 
rate]). Egg viability ([live fertilized + unfertilized eggs]/([live fertilized + unfertilized 
+ dead eggs]) was calculated for each egg sample. An egg was defined as unfertilized if 
it was clear or had a well-defined blastodisc; eggs that did not fulfill these criteria were 
defined as dead. Sperm motility was assessed both for undiluted (control) samples and 
for diluted samples at the beginning, middle and end of each fertilization trial. Sperm 
motility after dilution ranged from 45 to 175 s, and was not affected by the amount of 
dilution or time of testing within a trial.

Linear regression analysis was used to determine whether spermatocrit could be used 
to estimate sperm density, and whether there was a relationship between sperm density 
and sperm motility. Fertilization rate data were analyzed by one-way analysis of 
variance (ANOVA) on rank (Kruskal-Wallis) (MINITAB version 11.1) (Zar, 1996).

3. Results

A significant positive relationship was found between spermatocrit and sperm density 
(Fig. 2; p < 0.001, r² = 0.89). Sperm density ranged from $1.95 \times 10^{11}$ to $6.13 \times 10^{11}$ 
spermatozoa/ml at 23% and 99% spermatocrit, respectively. Sperm density and sperma-
tocrit decreased when males were implanted with GnRH(a), but this did not affect the 
relationship between sperm density and spermatocrit (Fig. 2). The lowest spermatocrit 
value obtained in fish that were not treated with GnRH(a) was 52%.
Fig. 3. The relationship between spermatocrit and sperm motility (defined as the time elapsed until <5% of the cells maintained forward swimming activity) for 36 milt samples collected from 17 male Atlantic halibut (● denotes samples obtained after GnRH(a) implantation; □ denotes no GnRH(a) implantation). Each point represents the mean of six replicates. The bars indicate SD.

No relationship was observed between sperm density and sperm motility at the densities tested (Fig. 3; \( p = 0.35 \), \( r^2 = 0.03 \)). The mean duration of sperm motility for individual samples ranged from 63 to 155 s. GnRH(a) treatments had no apparent effect.

Fig. 4. The relationship between sperm motility (defined as the time elapsed from activation until <5% of the spermatozoa maintained forward swimming activity) and the percentage of spermatozoa that were immediately activated upon addition of water to 36 milt samples collected from 17 male Atlantic halibut (● denotes samples obtained after GnRH(a) implantation; □ denotes no GnRH(a) implantation). The bars indicate SD.
Fig. 5. Fertilization rates (relative to control) for Atlantic halibut eggs fertilized at varying sperm:egg volume ratios. For the control, undiluted milt was used at a 1:500 sperm:egg volume ratio. All test ratios used milt diluted to a spermatocrit of 58–60% with halibut seminal plasma. Values are means of three trials ± SD.

on the relationship between sperm density and sperm motility (Fig. 3). The duration of sperm motility was not influenced by the percentage of spermatozoa that were immediately activated upon addition of water to the samples (Fig. 4; $p = 0.76$, $r^2 = 0.003$). The proportion of motile spermatozoa decreased with time from activation.

There was no difference in the fertilization rate among sperm:egg volume ratios tested ($H = 4.6$, $p = 0.71$, $n = 18$) (Fig. 5) The viability of egg batches used for the three fertilization trials were 99%, 99% and 92%, respectively. The actual fertilization rates for the control were 96%, 76% and 73%. Sperm motility estimates prior to fertilization for the trials were 163, 202 and 66 s.

4. Discussion

The highly significant relationship found between spermatocrit and sperm density allows for the use of spermatocrit as a simple and rapid estimator of sperm density in Atlantic halibut. Although such a relationship has previously been reported for several non-flatfish teleost species (Bouck and Jacobson, 1976; Piironen, 1985; Ciereszko and Dabrowski, 1993; Rakitin et al., 1999), an earlier study with turbot failed to find such a relationship (Suquet et al., 1992b).

The observed sperm densities for halibut ($2 \times 10^{13}$ to $6 \times 10^{11}$ spermatozoa/ml) are 1–2 orders of magnitude higher than reported for other teleost species (Mounib, 1978; Aas et al., 1991; Suquet et al., 1992b; Poole and Dillane, 1998; Fauvel et al., 1999;...
Atlantic halibut also have high total ejaculate volumes per stripping (1–60 ml; Methven and Crim, 1991) compared to turbot (0.2–2.2 ml; Suquet et al., 1992a, 1994). Higher sperm densities in halibut are likely a reflection of their spawning location, fecundity and egg size. Atlantic halibut spawn at depths of 300–700 m (Devold, 1938), in total darkness. Fecundity is very high: a 1.95-m female can produce up to 7 million eggs per spawning season (Haug and Gulliksen, 1988). Spread among 10–15 batches at 3–4 day intervals (Norberg and Holm, 1995), with approximately 50,000 eggs/l (Mangor-Jensen et al., 1998), this is equivalent to 140-l eggs per season with 9–14 l eggs/batch. Although cod have similarly high fecundity (Powles, 1958), they spawn in shallower water (Scott and Scott, 1988). Halibut also have among the largest eggs of all marine teleosts (Russell, 1976), with an average diameter of 3.0–3.5 mm (Haug et al., 1984) compared to 1.2–1.6 mm for cod (Scott and Scott, 1988). This combination of producing large volumes of large eggs in a dark environment may require exceptionally high sperm densities to ensure reproductive success.

Spermatocrit values obtained prior to GnRH(a) implantation were similar to those previously reported for Atlantic halibut (Methven and Crim, 1991). In our study, implanting males with GnRH(a) resulted in reduced spermatocrit values but this did not affect sperm motility. Increased sperm fluidity following GnRH(a) implantation has been reported for plaice, Pleuronectes platessa (Vermeirssen et al., 1998), winter flounder, P. americanus (Shangguan and Crim, 1999) and Atlantic halibut (Martin-Robichaud et al., 2000; Vermeirssen et al., 2000a). GnRH(a) treatment was found to improve sperm motility in yellowtail flounder, P. ferrugineus (Clearwater and Crim, 1998), but not in winter flounder (Shangguan and Crim, 1999). However, in a study with Atlantic halibut (Vermeirssen et al., 2000a), motility was reported to be significantly higher in treated milt samples compared to controls 40 days after implantation. In our study, samples were collected 2–3 weeks after GnRH(a) implantation, which may explain why there were no differences among the samples.

Fertilization success was largely independent of sperm density within a range of $9 \times 10^5$ to $5 \times 10^8$ spermatozoa/egg. Thus, although this study failed to find a minimum sperm:egg volume ratio for ensuring good fertilization success, it did show that high fertilization success can be obtained over a wide range of sperm:egg volume ratios. Vermeirssen et al. (2000b) have recently shown that this ratio can be successfully lowered to $10^4$ spermatozoa/egg, but this is still higher than the sperm densities necessary for successful fertilization in other teleost species (Gwo et al., 1991; Suquet et al., 1995).

Acknowledgements

This research was funded through an NSERC Strategic Grant, with additional infrastructure support from Fisheries and Oceans Canada. We thank Drs. Michael Reith (NRC Institute for Marine Biosciences, Halifax) and Allen Curry (UNB Department of Biology, Fredericton) for their input into this study. In addition, we thank Dr. Jim Powell at Syndel International for providing the sGnRH(a).
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