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MASTER OF SCIENCE

IN

FISH BIOLOGY

"Is it important to consider males while measuring the reproductive potential of a fish stock?"

by

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PREFACE

This document presents a masters thesis in Fish Biology at the University of Bergen. The thesis was undertaken in cooperation with the research group Fisheries Ecology and Aquaculture (University of Bergen) and the Institute of Marine Research, Bergen. During my Master thesis research I contributed to an article which is due to be published in the Journal of Northwest Atlantic Fishery Science and is referred to as Nash *et al.* (in press) in the thesis. The article is the initial exploration of the paternal contribution to stock reproductive potential in Northeast Arctic cod. The thesis is the continuation of that quest.

First of all I want to thank my two main supervisors, Professor Audrey J. Geffen and Dr. Richard D. M. Nash. Thank you for supervising me and believing in me when I most certainly did not believe in myself. Thanks also to my co-supervisor, Professor Arne Johannessen, for giving me constructive feedback.

Assistant Professor Martin Blom and the IMR crew at the R/V Johan Hjort: Thank you all for helping me collecting the samples and for making the cruise with R/V Johan Hjort one of the coolest things I have ever experienced! Thanks to Dr. Jon Egil Skjæraasen for providing me with data of cod from the 'skipped spawning' project at IMR, and to Dr. Øystein Sæle for providing histological data of cod testis. Prof. Harald Kryvi and Senior Research Technician Nina Karin Ellingsen: Thank you for all the good advices and for making "teleost fixative" for me. Thank you, Senior Engineer Egil Severin Erichsen, for helping me to take pictures of the sperm cells in the field emission scanning electron microscope. And thanks to Dr. Einar Heegaard for comments on the statistics.

Dear family and friends. I made it!

Dad: Thanks for always pushing me and showing such an interest in everything I do. You made education important to me.
Mum: Thanks for calling me every Sunday for five years. Thanks for all the candy, supportive letters and for being the best mum ever!
Allan: THANK YOU for being such a marvellous oracle. What would I do without your nerdy skills?
Henning: Thanks for all the phone calls. I cannot count all the times you made my day.
Roy: O'Boy, it's time to catch up! Come visit me! =)
PK: Love you cutie pie! Thanks for waiting.

Thanks to Isabelle, for making the cool illustration on the front page. And thanks to all my old and new friends for being inspiring, supportive and talking me out of smashing my computer.

"Alt blir interessant, så lenge man lærer mye om det"
Dad

Heidi Finden

ABSTRACT

This study explored the paternal contribution to stock reproductive potential in Northeast Arctic (NEA) cod (*Gadus morhua* L.). An equation for estimating total annual viable sperm production (VSP) was parameterised for NEA cod based on new and historical data obtained at the Lofoten spawning grounds. A principal component analysis indicated that there were no obvious changes in the relationships between the variable male traits each year which supported the use of a single equation for fitting the historical data. VSP was estimated in the time period the stock has been assessed (1946-2005). The parameterised equation resulted in approximately tenfold higher VSP than the original equation, but followed the same temporal changes. The VSP from the parameterised equation was used to predict recruitment in a Beverton-Holt model, but resulted in similar recruitment predictions as the traditional spawning stock biomass or total egg productions methods. Male characteristics were evaluated as possible important traits affecting the reproductive potential, including condition, GSI, drumming muscles mass, sperm motility and sperm density. Sperm density was positively correlated with GSI in 2007, but negatively correlated to fish length. No correlations were found in 2008 between sperm density and any of the evaluated male traits. Sperm motility correlated positively to gonad weight, GSI and drumming muscle mass in 2008. Some of these results may indicate that sperm quality is related to fish size. Indications of sperm quality related to fish size may affect the reproductive potential in exploited fish stocks where size selective harvesting can cause a lower mean fish size and potentially a lower sperm quality. More detailed experiments, including direct assessment of fertilisation success in NEA cod, may improve the parameterised equation which might narrow the gap of knowledge of reproductive processes at the population level.

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1. INTRODUCTION

1.1 BACKGROUND

Northeast Arctic (NEA) cod (*Gadus morhua* L.) is one of the most productive cod stocks in the world and an economically important species spending most of its life in the Barents Sea (Mehl, 1991; Hjermann *et al.*, 2007). For hundreds of years there has been a fishery on this stock with the greatest catches at the spawning grounds in the Lofoten area in Norway (Pomeroy and Berkes, 1997). The International Council for the Exploration of the Sea (ICES) extensively use Virtual Population Analysis when estimating past values of stock sizes (Ulltang, 1977; Christensen, 1996), but according to ICES it has been difficult to estimate the recruitment of NEA cod due to underreporting of catches (ICES, 2007). In addition, there are difficulties associated with constructing robust equations for estimating the reproductive potential of fish stocks, and several attempts have been made to improve these estimates (Marteinsdottir and Thorarinsson, 1998; Trippel, 1999; Koster *et al.*, 2001; Tomkiewicz *et al.*, 2003; Trippel, 2003).

Reproductive potential is an estimation of a fish stock's ability to produce viable offspring that may recruit into the adult population (Trippel, 1999), hence it is an estimate of recruitment potential and an indicator of a stock's resilience to exploitation. Traditionally, the stock-recruitment relationships are based on the egg production (Ricker, 1954; Beverton and Holt, 1957). Spawning stock biomass (SSB) is assumed to be a reasonable proxy for egg production, hence SSB has been used instead of spawner egg production *per se* in recruit relationships (Tomkiewicz *et al.*, 2003). Problems associated within approached assumption are i.e. fish with a low condition that may skip spawning (Jørgensen *et al.*, 2006) and a low number of well-conditioned fish which are presumed to recruit equally according to the SSB. SSB is based on the biomass of mature fish and estimates the recruitment without reference to the condition, age or sex ratio of the fish (Marshall *et al.*, 1998; Marteinsdottir and Thorarinsson, 1998; Trippel, 1999).

The stock-recruitment relationship based on SSB has proven to be noisy in many cases and it often appears to be non-existent (Ajiad *et al.*, 1999; Trippel, 1999). It is important to develop methods to measure the reproductive potential of a fish stock that actually reflects the basis for recruitment in a realistic way. Trippel (1999) introduced the term Stock

Reproductive Potential (SRP) which was thought to be a more accurate representation of the recruitment with annual variations within a stock. The potential incorrectness with both SRP and SSB is the primary focus on females and fecundity (Tomkiewicz *et al.*, 2003; Trippel, 2003; Nash *et al.*, *in press*). The main reason that males have largely been ignored in these calculations is based on the assumption that there is an excess of sperm compared to the number of eggs available (Nash *et al.*, *in press*). Egg fertilisation rate is assumed to be very high since there are few examples of unfertilised eggs in the wild (see Howell *et al.*, 1991 for common sole, *Solea solea*).

Trippel (2003) found that using sperm production rather than SSB explains to some extent more of the variance in age three (age when maturity is attained) recruitment within Newfoundland and Labrador cod (*Gadus morhua*). He also suggests that there is merit in pursuing the concept of reproductive potential using the male portion of the population. There is a question of defining gamete quality in a meaningful way for both sexes, so as to determine the best predictors of fertilisation success and offspring survival for a population. What parameters to be measured for an accurate estimation of a stock's recruitment are still unclear. Trippel (2003) presents an equation converting data on the numbers and sizes of male fish in a stock to an estimate of viable sperm production. Many of the relationships such as the proportion of testes weight to total weight, spermatocrit and sperm fertilisation potential are available in the published literature. However, these relationships may be stock specific. There are currently no data to determine relationships specifically for NEA cod.

1.2 BIOLOGY AND BEHAVIOUR

NEA cod feed in the Barents Sea and in the beginning of December they start migrating towards the Lofoten spawning grounds where they reach a peak spawning during March-April (Dannevig, 1953; Hylen, 1964; Bergstad *et al.*, 1987). Males display aggressive behaviour during the spawning period and a hierarchy is established in the spawning area with large males dominating over the smaller ones (Hutchings *et al.*, 1999). Some studies have suggested that cod perform as promiscuous spawners, leaving few options for mate choice (Herbinger *et al.*, 1997; Morgan *et al.*, 1999). However, both new and old studies describe a lek spawning system where the females choose one or several males to mate with (Brawn, 1961b; Höglund and Alatalo, 1995; Hutchings *et al.*, 1999; Nordeide and Folstad,

2000; Rowe and Hutchings, 2003; Windle and Rose, 2007). Lek is a polygynous mating system where males aggregate in one certain area for displaying themselves for the females (Höglund and Alatalo, 1995). This gives the females an opportunity to choose between the males and whilst the females will leave the lek after mating the males will continue their display towards other females (Höglund and Alatalo, 1995).

NEA cod is a heavily exploited fish stock where size selective harvesting may have caused a decrease in mean length of males and females (Jorgensen, 1990; Nash *et al., in press*). Female NEA cod used to dominate the large length and older age classes, however no females with a mean length greater than 13 % of the male mean length have been observed since 1982 (Nash *et al.*, *in press*). This may affect the fertilisation rate. The removal of large, old fish of both sexes is giving a corresponding increase in mean condition factor (Nash *et al.*, 2005) which may again affect the gamete quality, behaviour and mating success among other attributes. As described in Brawn (1961b), male and female cod release their gametes during a ventral mount and Brawn (1961b) points out that it is important for cod to have a close alignment of the gonopores during the ventral mount to achieve a high fertilisation rate. Rakitin *et al.* (2001) found that males within 13% of the female length are able to sire entire batches of offspring, but they also mention that the small experimental tanks might prevent the males from performing as they would in the wild.

Male body size has an effect on mating success in a variety of fish species (Maekawa *et al.*, 1994; Warner *et al.*, 1995; Forsgren *et al.*, 1996). Within sand gobies (*Pomatoschistus minutus*) and bluehead wrasse (*Thalassoma bifasciatum*) the females tend to choose larger males during spawning (Warner *et al.*, 1995; Forsgren *et al.*, 1996). A large body size is advantageous when competing for females (Maekawa *et al.*, 1994) or nesting sites (Forsgren *et al.*, 1996), but even though mating success may lead to fertilisation success, these two terms may not necessarily correspond with each other. Fertilisation success depends on, among other factors, sperm density (cells per millilitres of milt) and sperm motility (Trippel, 2003). When it comes to sperm motility, there is a possibility that smaller males have a fair chance to fertilise eggs through sperm competition (Rakitin *et al.*, 2001; Vladic and Jarvi, 2001).

Northeast Arctic cod was the fish stock of interest with a focus on running males and therefore the primary samples were collected at the spawning grounds in the Lofoten area during the spawning season. Trippel's equation (2003) was the starting point for collecting the samples and choosing what sort of male reproductive traits to focus on, which included total fish length, fish weight, testes weight, age and milt samples. The purpose of the milt samples was to include them in Trippel's equation, but also to look at sperm quality measurements.

What exactly is sperm quality? As stated in Rurangwa *et al.* (2004) sperm quality is "a measure of the ability of sperm to successfully fertilise an egg". All sorts of different physical parameters that could affect the sperm cells ability to fertilise an egg can potentially be used to measure sperm quality (Rurangwa *et al.*, 2004). Sperm quality may depend on sperm density (Rakitin *et al.*, 1999a; Tvedt *et al.*, 2001; Trippel, 2003), sperm motility (Trippel, 2003; Gage *et al.*, 2004) and it may also be affected by sperm competition (Ball and Parker, 1996; Stockley *et al.*, 1997). In addition there are other male traits that may serve as indicators of sperm quality, such as the size of the drumming muscles (Engen and Folstad, 1999) or condition (Rakitin *et al.*, 1999a; Rowe *et al.*, 2007).

The genital tract has storage of spermatozoa in seminal fluid which remains immotile until activation upon contact with water (Rurangwa *et al.*, 2004). Spermatozoa density is one of the two most important criteria for male fertilisation potential according to Trippel (2003), hence it serves as an important quantitative measurement of sperm quality. Sperm density can be measured with two techniques; sperm counts of diluted milt samples and spermatocrit level (Rakitin *et al.*, 1999b; Trippel, 2003). Rakitin *et al.* (1999b) found that spermatocrit level correlates positively to sperm density in Atlantic cod (*Gadus morhua)*, but this has not been thoroughly studied in NEA cod. The second most important criteria for male fertilisation potential is sperm motility (Trippel, 2003), hence sperm motility was an interesting reproductive trait that was included in this study.

As mentioned previously, drumming muscles may be an individual trait whose size may be an indication of sperm quality (Engen and Folstad, 1999). Brawn (1961a) describes the

drumming muscles of the cod (*Gadus callarias* L.) as three flattened external muscles on each side of the swim bladder. Both male and females have the possibility to make sounds, but in cod these drumming muscles are sexually dimorphic with males having larger muscles than females (Northeast Arctic cod, Engen and Folstad, 1999; Atlantic cod, Rowe and Hutchings, 2004). During the spawning season drumming muscles of males increase in size (Rowe and Hutchings, 2004) and primarily males will produce sound during this period (Brawn, 1961a). The males produce sound with their drumming muscles for both aggressive and courtship behaviour (Brawn, 1961a). As described in Brawn (1961a) a female showed more and more interest in a male for each of his "grunts" during courtship behaviour. Rowe and Hutchings (2008) found that drumming muscle mass is the only male reproductive trait that can explain the variability in male mating success and they suggested "that acoustic communication is fundamentally important to reproduction in Atlantic cod".

1.4 OBJECTIVES OF THIS STUDY

Male reproductive traits have been studied in the context of aquaculture research for decades, but knowledge about male reproductive potential in natural populations of commercial species is almost non-existent (Trippel, 2003). By following up the male contribution to stock reproductive potential one might get a better understanding of the stock-recruitment relationship. Since fisheries management are based upon these estimates it is important to understand the stock-recruitment relationship to provide information about a population's past and future dynamics (Rothschild and Fogarty, 1989). A greater knowledge of cod sperm quality and its effect on the recruitment are not only needed for the wild populations of cod, but also for broodstock management (Bell *et al.*, 1996; Kime *et al.*, 2001). There has always been a great focus on eggs and larvae quality within the fish farming industry and milt from different males are "mixed" to ensure a high fertilisation rate. However, the milt is often inadequate in quantity and quality and does not ensure optimal fertilisation rate or healthy larvae (Rurangwa *et al.*, 2004).

This study attempts to assess the importance of considering males while measuring the reproductive potential of a fish stock with an aim to increase the knowledge of male reproductive traits for Northeast Arctic cod in a natural population. The questions addressed in this thesis include:

- ✓ Is it representative to estimate the reproductive potential of a fish stock based only on the egg production?
- ✓ Is there a relation between sperm quality and the condition of the fish?
- ✓ Should sperm quality be included in assessments of a fish stocks reproductive potential?

Furthermore, the objective was to use field and laboratory data to determine the parameters necessary to estimate the total annual viable sperm production of NEA cod. New data on wild NEA cod was integrated in Trippel's (2003) equation for estimating total viable sperm production for the stock together with historical data on NEA cod in the time period the stock has been assessed (from 1946-2005).

2. MATERIALS AND METHODS

2.1 SAMPLING

2.1.1 SOURCE OF FISH

The sexual characteristics of annually reproducing fish occur once a year and often over a relatively short period of time. In regard to both laboratory experimentation and field work there was a rather short time limit in which to undertake the experiments and collect the necessary data. Many of the protocols used in this study needed to be refined and tested before being applied in the field. A series of preliminary investigations were thus undertaken for development of methods. In regard to the field studies, these experiments were also necessary as preplanning was vital to ensure all equipment and protocols were in place since if equipment was missing or protocols inappropriate then the experimental design could have been compromised. The preliminary investigations are described under the section called "2.2 Method development".

Three field studies were undertaken and samples were taken of; 1) Pre-spawning cod at Andenes in 2007, 2) Cod at the spawning grounds in 2007 and 3) Cod at the spawning grounds in 2008. A total of 192 male cod were sampled where 70 pre-spawning males were collected at Andenes in 2007, 74 ripe males were collected at the spawning grounds in 2007 and 48 ripe males were collected at the spawning grounds in 2008. The samples of the 74 male NEA cod at the spawning grounds in 2007 are the main samples in this project. Standard data of fish length and weight, gonad weight and age were taken along with samples of milt that were fixed. The same type of data were obtained at the spawning grounds in 2008 of 48 male NEA cod, but at this survey there were taken additional samples of sperm motility measurements and drumming muscle weight. Historical data from the time period NEA cod has been assessed (1946 to 2005) were used when estimating the total annual viable sperm production for this fish stock. The processing of the samples and the data analysis were conducted in the time period from the 18th of January 2007 to the 24th of April 2008 at the High Technology Centre, University of Bergen, Norway.

2.1.2 SPERM QUALITY

The milt samples from cod at the spawning grounds in 2007 were fixed in 3.6 % buffered formaldehyde (Appendix A, Table A.1) while the milt samples from cod at the spawning grounds in 2008 were fixed in a glutaraldehyde-formaldehyde fixation solution (Grotmol *et al.*, 2003) (Appendix A, Table A.2) (from now on referred to as teleost fixative). The purpose of all the fixed milt samples was to estimate the sperm density for each male and correlate it to individual male traits such as body weight and length, gonad weight, drumming muscle weight and age. In addition, the formaldehyde fixed milt samples were used to find the mean volume of single spermatozoa (spermatozoon) and this again was used to estimate the total amount of sperm cells in one gonad when knowing the average volume of one gonad. The equation for the total amount of sperm cells in gonads was integrated in a parameterised version of Trippel's equation.

Sperm motility was measured in fresh milt samples from cod at the spawning grounds in 2008. The purpose of these milt samples was to get a measurement of sperm quality and correlate it to the same individual male traits as mentioned previously. In order to see if drumming muscle weight could be used as an indicator of sperm quality both wet and dry weight of the muscles were measured and compared with the motility and density of spermatozoa.

Figure 1 shows the studied areas of sperm quality of NEA cod with the spermatozoa density and motility as direct measurements of estimating sperm quality and male traits as possible indirect measurements. Male traits were tested using correlation against the direct measurements of sperm quality to see if these parameters could be used as an indirect and rapid measurement of sperm quality (marked in italic in Figure 1).

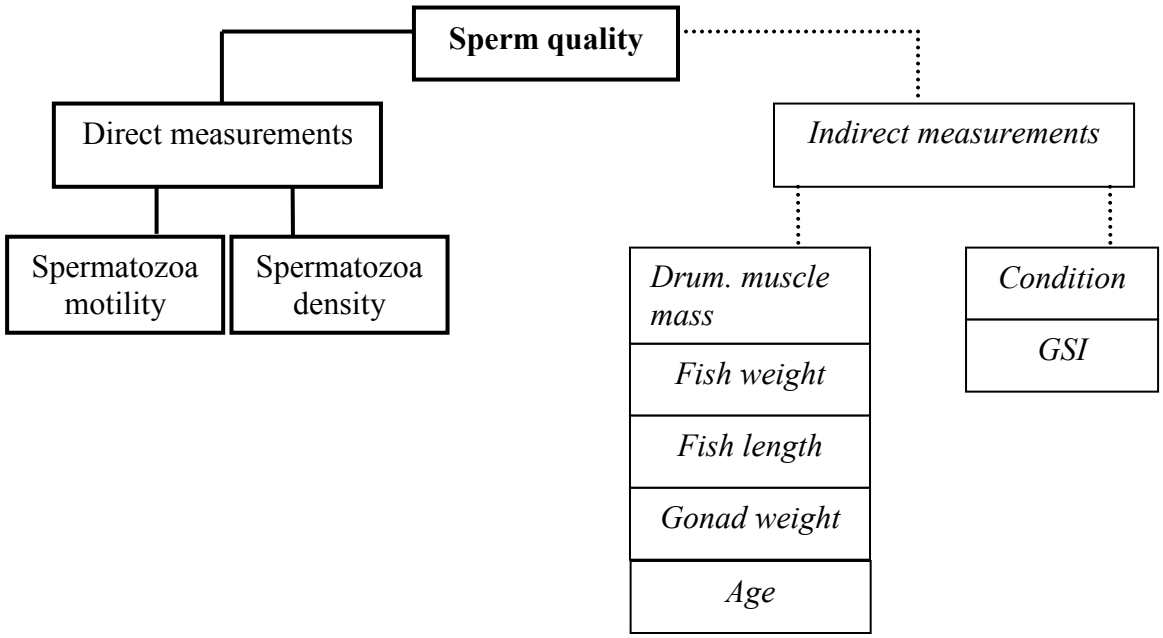


Figure 1: Flow diagram showing terms of interest with direct measurements of sperm quality and possible indirect measurements of sperm quality in male NEA cod.

2.2 METHOD DEVELOPMENT

2.2.1 CAPTIVE NORTHEAST ARCTIC COD

From the 18th to the 20th of January 2007 a total of 73 (51 males and 22 females) NEA cod that were related to a 'skipped spawning' project at the IMR, Bergen, were used for practise before sampling at the spawning grounds. These are the methods that were tested; 1) collection of milt samples for estimating spermatocrit and sperm density and 2) dissection and mass determination of drumming muscles from both sexes. Milt samples were taken from eight running males by gently pressing on each side of the abdomen to squeeze out the milt (Fig. 2). Paper towels were used to keep the area around the genital papilla clean and avoid contamination with faeces and blood. The milt samples were used to measure spermatocrit and sperm density. Haematocrit capillary tubes (75 mm length, 1.1–1.2 mm inner diameter) were filled to approximately 70 % with milt and centrifuged at 7500 rpm from 5 - 30 minutes with a Haematokrit 210 centrifuge (Hettich Zentrifugen). The spermatocrit (densely packed sperm cells) value was read as percentage of the total amount of milt. The sperm density was measured by counts of sperm cells in a modified Neubauer haemocytometer. The haemocytometer is a special microscope slide with a 0.1 mm deep

chamber in the middle. The chamber has a grid made of 400 small squares where each square is 0.05 x 0.05 mm. A fixed amount of milt was diluted with a fixed amount of freshwater, mixed thoroughly and transferred to the haemocytometer. Pictures were taken using the imaging software NIS Elements F (Nikon) with a Digital Sight D55M camera (Nikon) attached to a BX60 microscope (Olympus). The sperm cells in the pictures were counted by hand and the density of cells per millilitre was estimated. Two images were captured, one at 200 times magnification and one at 400 times magnification for each sample. Drumming muscles were dissected with a scalpel (Fig. 3) from all the 73 fish. Data of total fish length, fish weight and the wet and dry weights of the drumming muscles were provided by Dr. Jon Egil Skjæraasen (University of Bergen, *pers. comm*.)

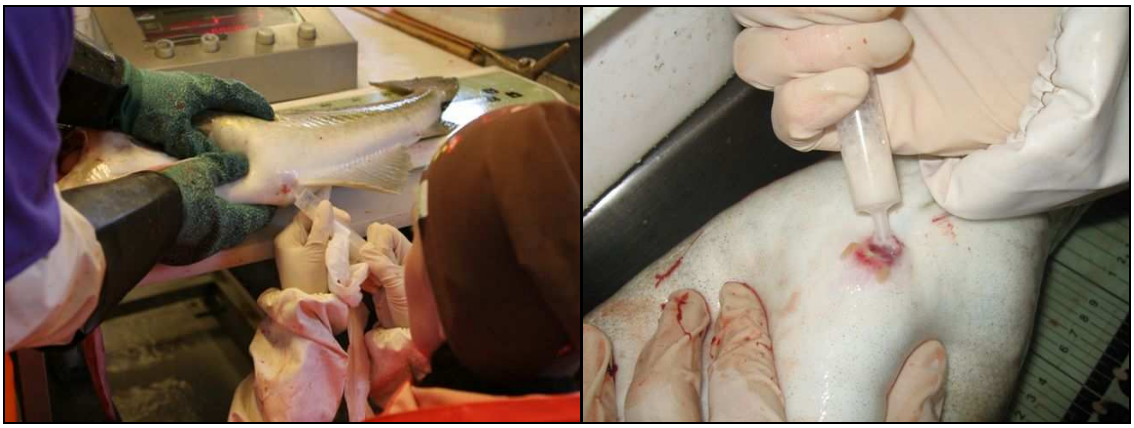
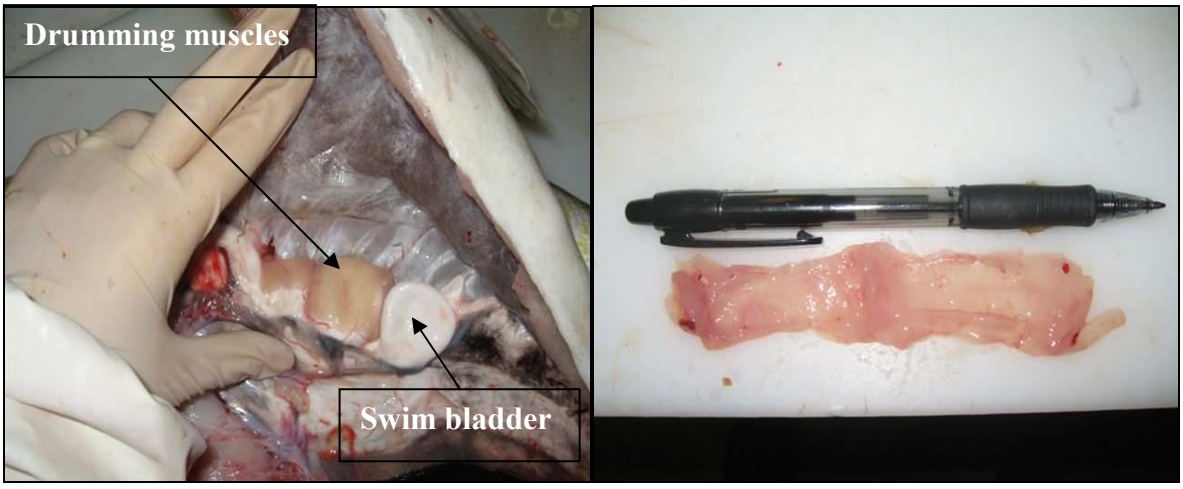


Figure 2: Collecting of milt samples.



A **B**

Figure 3: A) Picture of drumming muscles attached to the swim bladder and B) Picture of drumming muscles cut out from the fish.

2.2.2 CAPTIVE COASTAL COD

From the 14th to the 15th of February 2008 six males of captive coastal cod (*Gadus morhua*) were obtained from the IMR, Bergen. Drumming muscles and milt samples were only taken from five of the males, since one of them was not running. The milt samples were used for estimating sperm density, sperm motility and sperm weight. One millilitre of milt was fixed in ten millilitres of teleost fixative for later estimating the sperm density (same counting procedure as described in section 2.2.1). The sperm motility was tested to decide what type of motility measurements would suite best onboard the research vessel. Based on own unpublished experiments, Trippel (2003) considered cod milt to have a higher density than seawater (1.024 gram per mL at 25 ^{o}C) and this allocation was tested by weighing milt samples using two different volumes; 1 mL and 100 µl. A EW3000-2M scale (KERN) was used to measure the weight of one millilitre milt whilst a Genius ME235P scale (Sartorius) was used to measure the weight of 100 µl milt. Drumming muscles were dissected to get an idea of how time consuming the work would be, and deciding whether or not to use this method onboard the research vessel. Both wet and dry weight of the drumming muscles was measured on a Genius ME235P scale (Sartorius). The muscles were dried for 24 hours at 60 ^{o}C in a Termaks oven (Environmental Laboratory Equipment) before the dry weight was measured.

2.3 PRE-SPAWNING NEA COD AT ANDENES IN 2007

Northeast Arctic cod from the Lofoten spawning area were sampled at a fish processing plant at Andenes from 8th to the 10th of March, as part of regular sampling by IMR. Standard data of fish length, fish weight, gonad weight and age were taken of 70 pre-spawning males. No milt samples were taken.

2.4 COD AT THE SPAWNING GROUNDS IN 2007

Data were provided by Ass. Prof. Martin Blom, Aalesund University College (HiAls), and the Institute of Marine Research (IMR), Bergen. These samples were obtained at the spawning grounds in the Lofoten area from the 17th of March to the 7th of April 2007 (see Fig. 4). Milt samples were collected along with data of fish length, fish weight, gonad weight and age (Table I). The males were stripped and one millilitre of milt was transferred

to scintillation tubes with a fixed amount, 15 mL, of 3.6 % buffered formaldehyde. Also small samples, 3 x 3 cm, of all the gonads were frozen at - 20 ^{o}C.

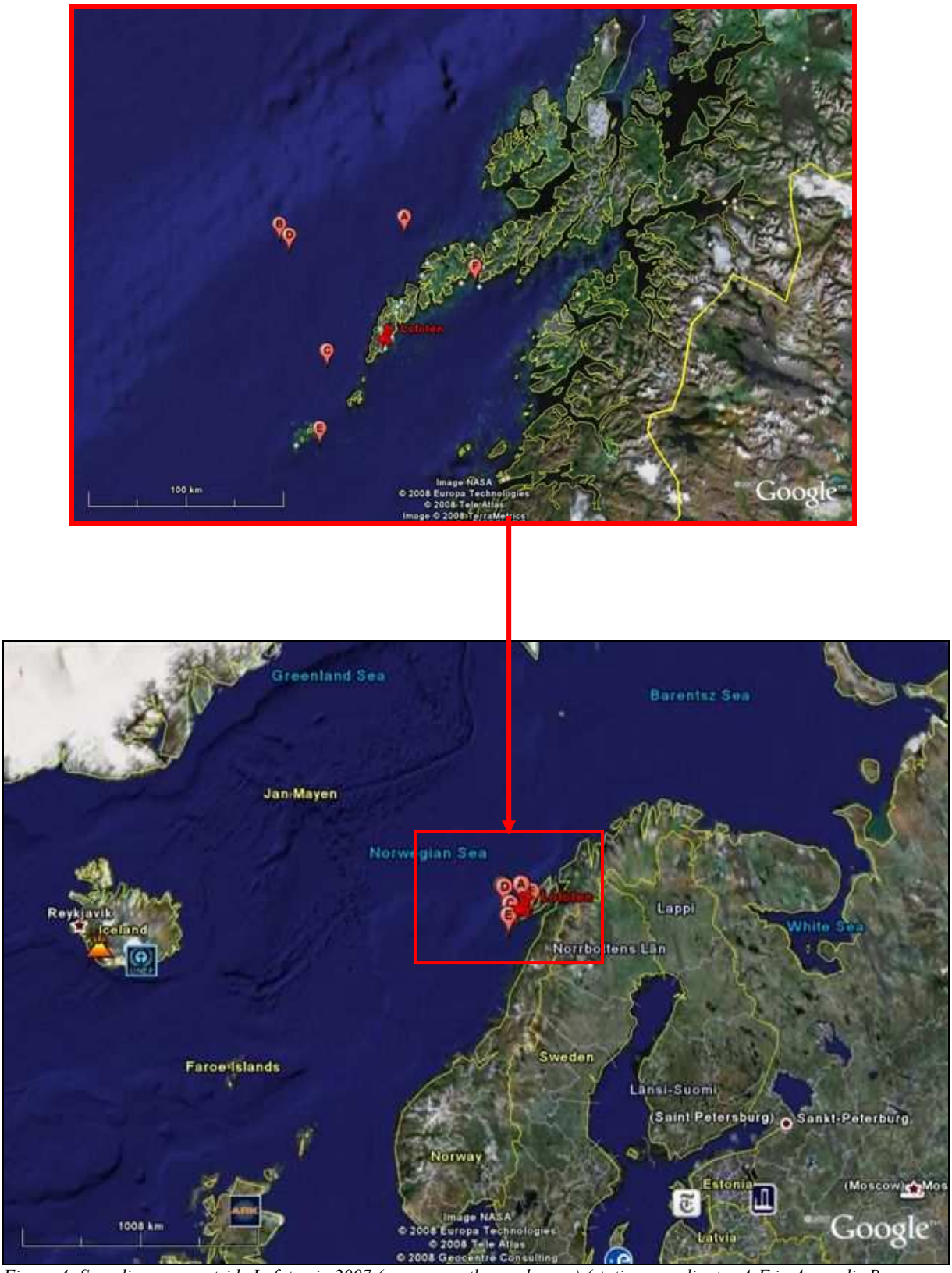


Figure 4: Sampling area outside Lofoten in 2007 (source: earth.google.com) (station coordinates A-F in Appendix B, Table B.1).

Table I: Samples and data collected at the spawning grounds in 2007

Sample	Number of samples
Milt in formaldehyde fixative	74
Samples of testes (3x3 cm)	72
Fish length	74
Fish weight	74
Testis weight	72
Age	74

2.4.1 SPERM DENSITY

As an important dimension of sperm quality the spermatozoa density was estimated. It was measured by counts of diluted milt samples (from the formaldehyde-milt samples) in a modified Neubauer haemocytometer (Fig. 5) (described in section 2.2.1). The containers with milt and formaldehyde were thoroughly mixed for approximately two minutes. A plastic pipette was used to transfer the mixture to the haemocytometer. Pictures were taken with a Digital Sight D55M camera (Nikon) attached to a BX60 microscope (Olympus). If the sample was too dense it was diluted with a fixed amount of freshwater. For each sample of the sperm-formaldehyde mixture four pictures were taken: two pictures at 100 times magnification and two at 200 times magnification, and for each sample this was replicated three times. In summary, a total of approximately 900 pictures of the 74 sperm-formaldehyde samples were taken for estimating sperm density. Approximately half of the pictures were used for sperm counts while the other half were just taken as a backup file (in case of bad quality in the first ones).

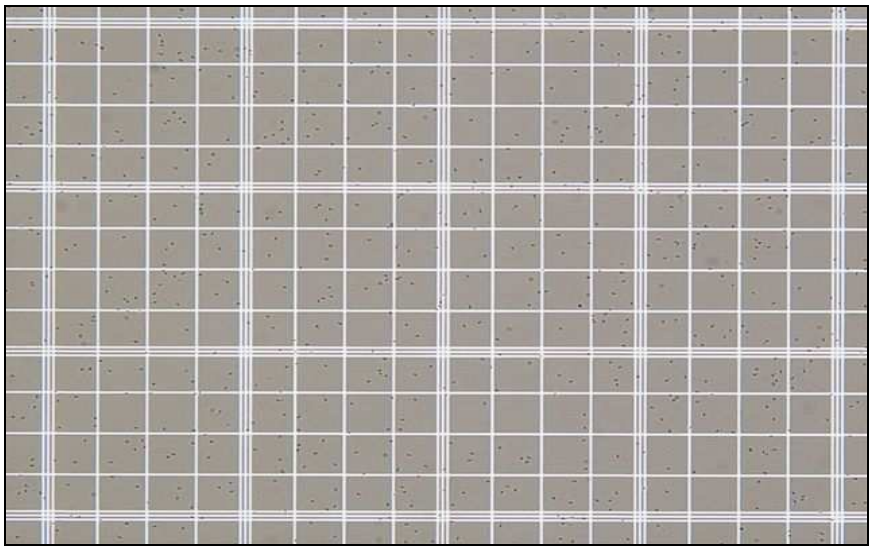


Figure 5: Picture of spermatozoa in the haemocytometer (each small square equals 0.0025 mm^2).

Thirty pictures (ten of good, ten of medium and ten of bad quality) were chosen to test the accuracy of the software program Image J version 1.37 (National Institutes of Health) for automated counting of sperm density. The cells in these pictures were counted by hand and compared with the automatic counting subroutine in Image J. Image J was then chosen for automated counting for the rest of the pictures. The image threshold level was set to mark only the heads of the sperm cells (Fig. 6). Then Image J counted all the marked dots within a selected area of 192 small squares (Fig. 7). The equation for estimating the realistic sperm density of a sample is given as:

$S_D = ((\tilde{N} * (400 \text{ squares}/192 \text{ squares})) * 10^4) / D_F$

S_D = sperm density (cells/mL)
$\tilde{N}$ = the average number of sperm cells in a sample counted in 192 small squares
10^4 = the volume conversion factor for one mm^2
D_F = the dilution factor as a combination of the dilution with both formaldehyde and water

→ D_F = dilution one * dilution two = $D_1 * D_2$

$D_1 = M / T$
where;

M = milt volume (mL)
T = total volume = milt volume (mL) + formaldehyde volume (mL)

$D_2 = M_2 / T_2$
where;

M_2 = mix volume (mL) = milt + formaldehyde volume (mL)
T_2 = total volume (mL) = mix volume (mL) + water

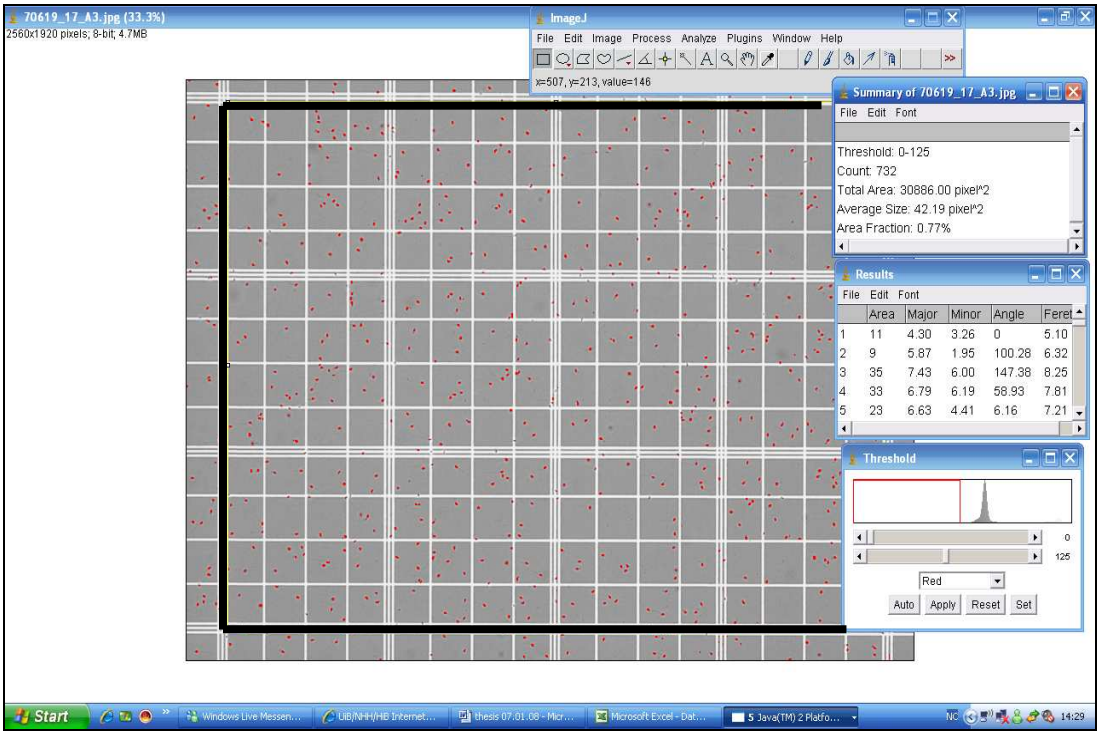


Figure 6: Automatic counting of sperm cells with Image J within a selected area (black box) (each small square equals 0.0025 mm^2).

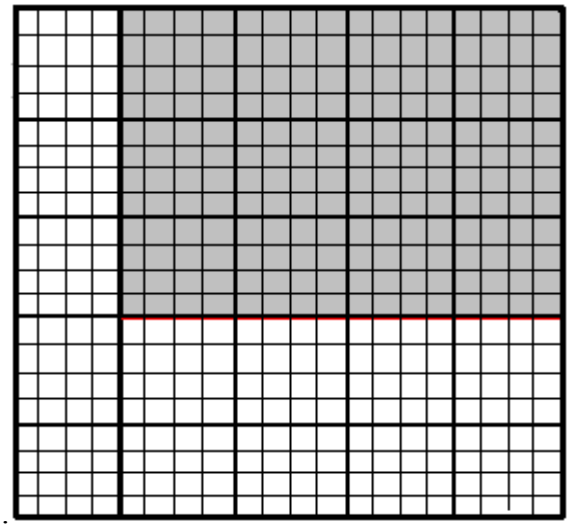


Figure 7: Haemocytometer with the dark grey field covering the selected area in Figure 6 (each small square equals 0.0025 mm^2).

2.4.2 SPERMATOZOA WEIGHT

Spermatozoa weight was measured to test if these estimations could be used to recalculate milt weight directly, which again could be used in Trippel's equation of viable sperm production. Twenty scintillation tubes with sperm/formaldehyde mixture were shaken for two minutes. One millilitre mixture from each scintillation tube was transferred with an automatic micropipette to pre-weighed 1.5 ml Eppendorf tubes (Eppendorf). Then the

Eppendorf tubes were centrifuged at 7500 rpm for 5-30 minutes producing a smear pellet of sperm cells and a supernatant of seminal fluid and formaldehyde. The supernatant was pipetted to 20 new pre-weighed 1.5 ml Eppendorf tubes and weighed. Some of the supernatant was checked under a microscope for sperm cell contamination. The tubes with the smear pellet were centrifuged one more time to press out more supernatant. Then they were dried in a desiccator for 24 hours and weighed. This procedure gave an estimated weight of just the sperm cells, without seminal fluid or water.

2.4.3 ANNUAL VIABLE SPERM PRODUCTION

The total annual viable sperm production was estimated for the NEA cod stock from 1946 to 2005. The general method for estimating the annual viable sperm production is laid out in Trippel (2003), but is reproduced here with minor variations, as given in Nash *et al*. (*in press*):

$$VSP = \sum_{i=I,l=L}^{I,L} n_{i,l} . p_{i,l} . t_{i,l} . v_{i,l} . s_{i,l} f_{i,l}$$

where:

VSP = Viable Sperm Production (In Trippel (2003) this is given as VSPP or Viable Sperm Produced by Population)

i = age in years

I = the oldest age class

l = 5 cm length class

L = largest length class

$n_{i,l}$ = number of males in each age and length class

$p_{i,l}$ = proportion of mature males in each age and length class

$t_{i,l}$ = mean testes weight (kg) of males in each age and length class

$v_{i,l}$ = mean volume (mL) of milt produced per kg testes in each age and length class

$s_{i,l}$ = mean number of spermatozoa per mL of milt for each age and length class

$f_{i,l}$ = sperm fertilisation potential as a function of Fulton's condition factor (K) see Nash *et al.* (*in press*) in each age and length class.

2.4.4 CALCULATIONS OF BODY DATA

Condition, K-factor

The equation for sperm fertilisation potential includes Fulton's condition factor (Trippel, 2003) and therefore Fulton's equation is given here (also see Nash *et al*., *in press*):

$$K = \frac{W}{L^3}.100$$

Where:

W = whole fish weight (g)

L = total fish length (cm)

The GonadoSomatic Index (GSI)

A relationship between age and GonadoSomatic Index (GSI) for NEA cod is given by Nash *et al*. (*in press*) (based on unpublished data of NEA cod from R. D. M. Nash and O. S. Kjesbu, IMR: *pers. comm*.) with zero percent applied for three years olds, six percent for four year olds with an increase by 1 - 13 % for 12 year olds and 13 % also applied to the fish over 12 years old. The equation for estimating GSI is given as:

$$GSI = \frac{T_w}{W}.100\%$$

Where:

T_w = testis weight (g)

W = whole fish weight (g)

2.4.5 TOTAL SPERM PER MALE

The total sperm per male was estimated to find the mean volume (millilitres) of milt produced per kilogram of testes and the mean number of spermatozoa per millilitres of milt, and the equations for estimating the total sperm per male are given in Nash *et al.* (*in press*). Three percent of the testes weight was removed, assuming it to be connective tissue, primary spermatogonia and residual unspawned sperm (Trippel and Morgan, 1994b). This

removal gave the weight of milt, which was converted to volume using a value of 1.04 (Trippel, 2003). All numbers were provided by R. D. M. Nash (IMR, Bergen, *pers. comm.*) and estimated as given in Nash *et al.* (*in press*):

$$M_w = TsW \cdot 0.97$$

where:

M_w = milt weight
TsW = testes weight

$$M_v = M_w \cdot 1.04$$

where:
M_v = volume of milt
M_w = weight of milt

The sperm density was estimated to be 1.16 x 10^{10} sperm per mL by using the formula of (Rakitin *et al.*, 1999b) and a spermatocrit level of 0.6 (see Trippel, 2003).

2.4.6 SPERM FERTILISATION POTENTIAL

The data of the sperm fertilisation potential for the stock of NEA cod from 1946 to 2005 was provided by R. D. M. Nash, IMR *(pers. comm*). The equation for estimating sperm fertilisation potential (*f*) in each length class (see Trippel, 2003 and Nash *et al.*, *in press*) is given as:

$$f_l = 1.544(K_l) - 1.028$$

Where*:* K_l = Fulton's condition factor for a specific length class (as described previously).

2.4.7 PARAMETERISED EQUATION FOR ESTIMATING THE VIABLE SPERM PRODUCTION

Based on the new data of NEA cod from the spawning grounds in 2007 a parameterised variation of Trippel's equation (2003) was formulated. An equation for estimating GSI based on fish length was produced as described in the following section ("2.4.8 Equation for estimating GSI based on fish length") and this equation sets the basis for the parameterised variant of Trippel's equation. The mean gonad volume per gram testis, the mean cell volume and the total sperm amount in the gonads was calculated as given in the chapter "2.4.9 Total sperm per male". Data of sperm fertilisation potential (*f*) and yearly mean fish weight (*Y*) was provided by R. D. M. Nash (IMR, Bergen, *pers. comm*.) and Nash *et al.* (*in press*).

$$VSP = \sum_{i=I,l=L}^{I,L} \frac{(G_{eq} \times Y_{i,l} \times G_{i,l})}{C_{i,l}} \times S_{i,l} \times N_{i,l} \times f_{i,l}$$

where:

VSP = Viable Sperm Production

G_{eq} = equation for estimating GSI based on fish length (see next section)

i = age in years

I = the oldest age class

l = 5 cm length class

L = largest length class

Y = yearly mean fish weight (g)

G = mean gonad volume per gram testis (m^3/g)

C = mean cell volume (m^3/cell)

S = sperm ratio in gonads (without connective tissue)

N = number of males

f = sperm fertilisation potential as a function of Fulton's condition factor (K) (Nash *et al.*, *in press*) in each age and length class.

2.4.8 EQUATION FOR ESTIMATING GSI BASED ON FISH LENGTH

GSI of the 72 males was plotted against the total length of these males and a trend line was fitted. By displaying the slope and the intercept an equation for estimating GSI based on fish length was created (Fig. 8, $R^2 = 0.028$). Figure 8 and its equation are introduced here rather than in the Results section since the equation needed to be included in the parameterised equation (parameterised equation, see section 2.4.7). Equation for estimating GSI based on fish length, G_{eq}:

$G_{eq} = (0.0004 \times T) + 0.0622 = \text{GSI}$

T = total fish length (cm)

The equation was used when estimating the annual viable sperm production (VSP).

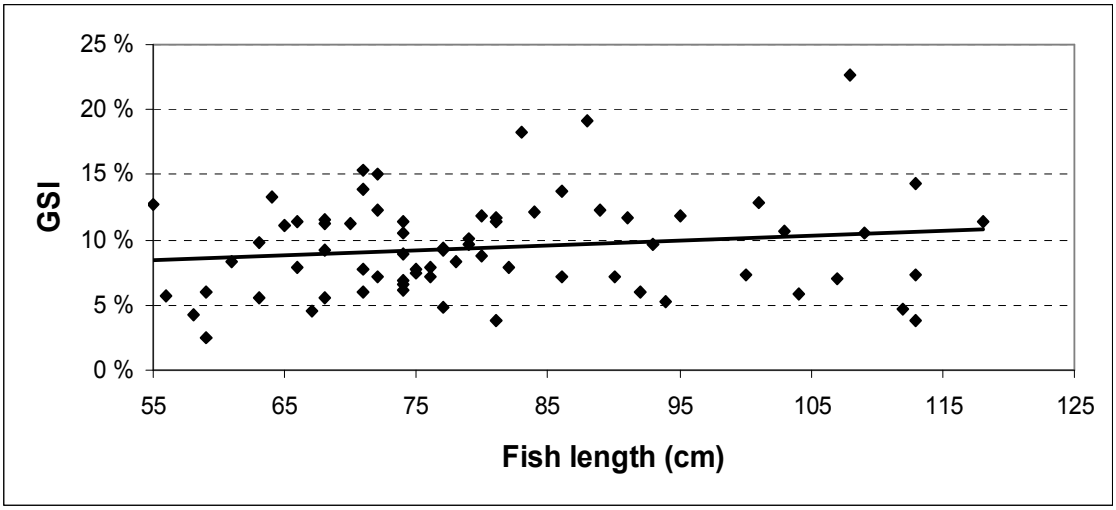


Figure 8: Plot of the GonadoSomatic Index (GSI) based on the total fish length.

2.4.9 TOTAL SPERM PER MALE

A mean spermatozoon volume was estimated based on light microscope and field emission scanning electron microscope pictures of the milt. Then the volume of testis was estimated. The total amount of sperm cells in each gonad was found by dividing the gonad volume by the mean spermatozoon volume.

2.4.10 MEAN CELL VOLUME

Pictures of sperm cells were taken with both a light microscope and a field emission scanning electron microscope. These pictures were used to estimate a mean spermatozoon volume, which again was used to estimate the total amount of spermatozoa in testes and thereby total sperm production per male.

Light microscope pictures and cell volume

A few drops from one of the sperm-formaldehyde samples were placed on a slide and a Vanox AHBT3 light microscope (Olympus) with phase contrast was used to get clear pictures of the cells. Pictures were taken at 400 times magnification with a ProgRes C14 camera (JenOptik). The pictures were used for estimating a mean cell volume. A line was drawn around single sperm cells and then Image J (version 1.37) calculated the width and length (minor and major) of the cells (Fig. 9). The cell volume was then calculated from this equation, as described in Geffen (2002):

$$C_V = 1.333\pi\ (0.5C_L)\ (0.5C_W)^2$$

Where:

C_V = cell volume

C_L = cell length (mm)

C_W = cell width (mm)

A mean cell volume was estimated: $\overline{C_V} = \frac{\sum C_V}{N}$, where: N = the number of specimens used to estimate the mean cell volume.

Field emission scanning electron microscope pictures and cell volume

Precipitate of sperm taken from one of the sperm-formaldehyde samples was transferred to an Eppendorf tube with 96 % ethanol. The Eppendorf tube was then centrifuged and some of the sperm precipitate was transferred to a plug of aluminium. The plug was placed under a hood until the ethanol evaporated. The sperm cells were then coated (to reflect electrons) in gold/palladium (Au/Pd) with a Polaron SC 502 Sputter Coater (Fisons Instruments) and pictures were taken in a Supra 55 field emission scanning electron microscope (Zeiss).

These pictures were also used to estimate a mean cell volume (see Fig. 10), as described previously (see “Light microscope”).

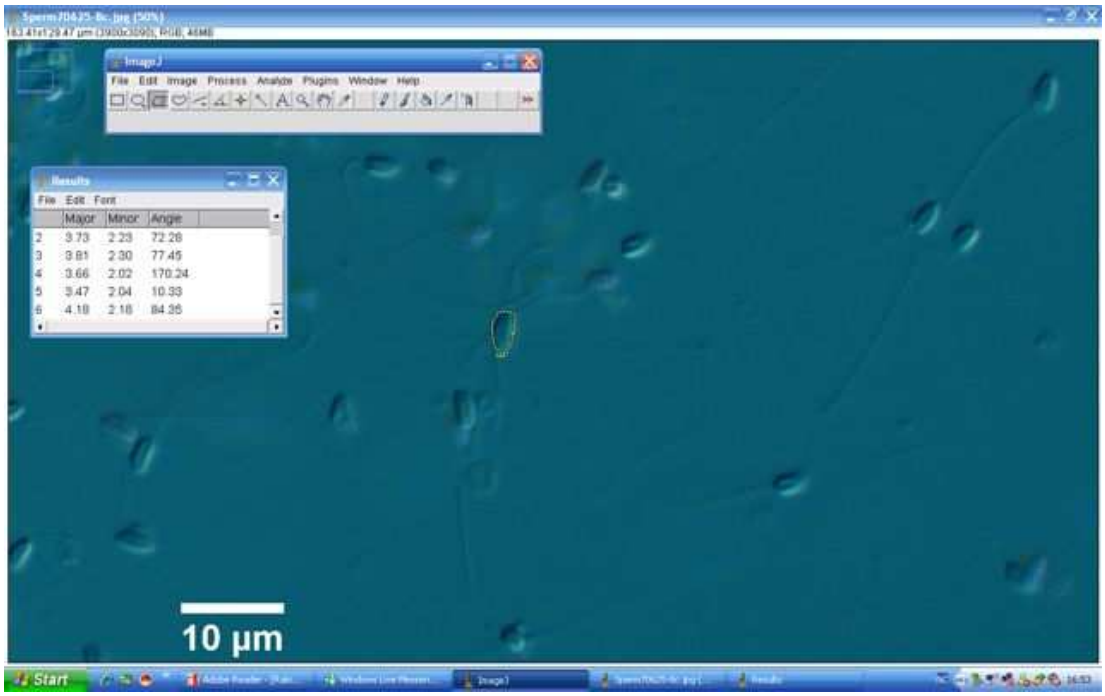


Figure 9: Length and width of sperm cells found by using Image J.

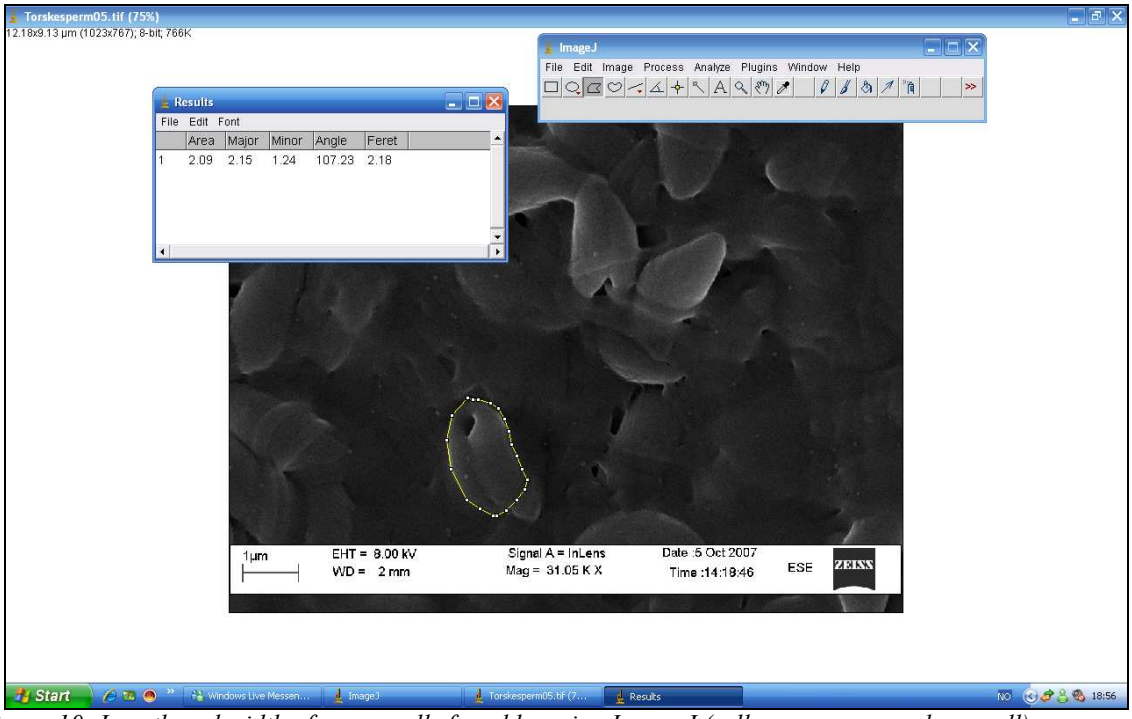


Figure 10: Length and width of sperm cells found by using Image J (yellow curve around one cell).

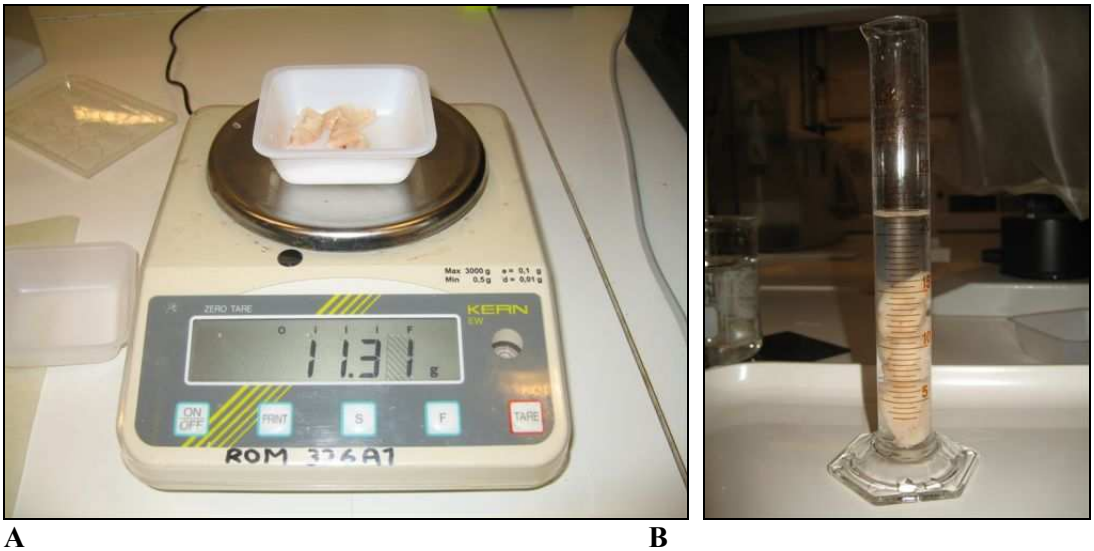
2.4.11 TESTIS VOLUME AND TOTAL AMOUNT OF SPERM CELLS

The 72 samples of testes were chopped into smaller pieces and weighed on a EW3000-2M (KERN) (Fig. 11A). A 25 mL glass cylinder was filled up with 15 mL of freshwater and the weighed pieces of testes were transferred to this cylinder. The volume of the chopped amount of testes was then noted as the displaced amount of water in the cylinder (Fig. 11B).

$T_v = D_w / T_p$

T_v = volume per gram testis (m^3/g)
D_w = displaced amount of water (mL)
T_p = weight of chopped testes (g)

A mean volume per gram testis, Tm, was estimated; $Tm = \frac{\sum Tv}{N}$, where: N = the number of specimens used to estimate the mean volume per gram testis.



A **B**

Figure 11: A) Weight of testis and B) Volume of testis due to displaced water in a cylinder.

To find the whole volume of each testis the mean volume per gram testis, T_m, was multiplied with the total weight of each testis:

$T_V = T_m * T_W$

Where:

T_V = testis volume (m^3)

T_W = total testis weight (gram)

T_m = mean volume per gram testis

The total amount of sperm cells in each testis was estimated by dividing the volume of each testis by the average volume of one sperm cell:

$T_S = T_V / C_A$

Where:

T_S = total amount of sperm cells

T_V = testis volume (m^3)

C_A = average volume of a sperm cell (m^3/cell)

Testes composition was analysed as a part of the NFR project "Produvann" (Meier *et al.*, 2007) and provided by Dr. Øystein Sæle (Nifes, *pers. comm*.). Based on these given histology data of coastal cod the content of connective tissue was set to 3.6 %, and this percentage was removed when estimating the total amount of sperm cells in the testes:

Realistic total amount of sperm cells = T_S * (1 – 0.036)

2.4.12 SEX RATIO PER STATION

For each station in the Lofoten area, fish lengths were measured for the whole catch of cod or a representative subsample of ten fish from each five cm length group. Sex and age determination were done for each length group. A frequency distribution of fish length per station was done in Excel (Microsoft Office 2003) and multiplied with the sex ratio for each length group per station.

From the 17th of March to the 5th of April 2008, 48 samples of male NEA cod were obtained in the spawning area around Lofoten (Fig. 12). Table II gives an overview of the collected samples at the spawning grounds. Milt was collected with a ten millilitres syringe and transferred to an empty container and then recollected with a one millilitre syringe and fixed in ten millilitres of teleost fixative in scintillation tubes. The rest of the milt in the container was stored on a box of ice in a refrigerator (3.5 ^{o}C) for later determination of motility. Ten of the one millilitre syringes were weighed on a M2000 scale (Marel) before and after the milt collection, to test if NEA cod milt was heavier than seawater.

Table II: Samples and data collected at the spawning grounds in 2008

Sample	Number of samples
Milt in teleost fixative	48
Motility measurements	48
Drumming muscle weight	48
Fish length	48
Fish weight	48
Testis weight	47
Milt weight	10
Age	48

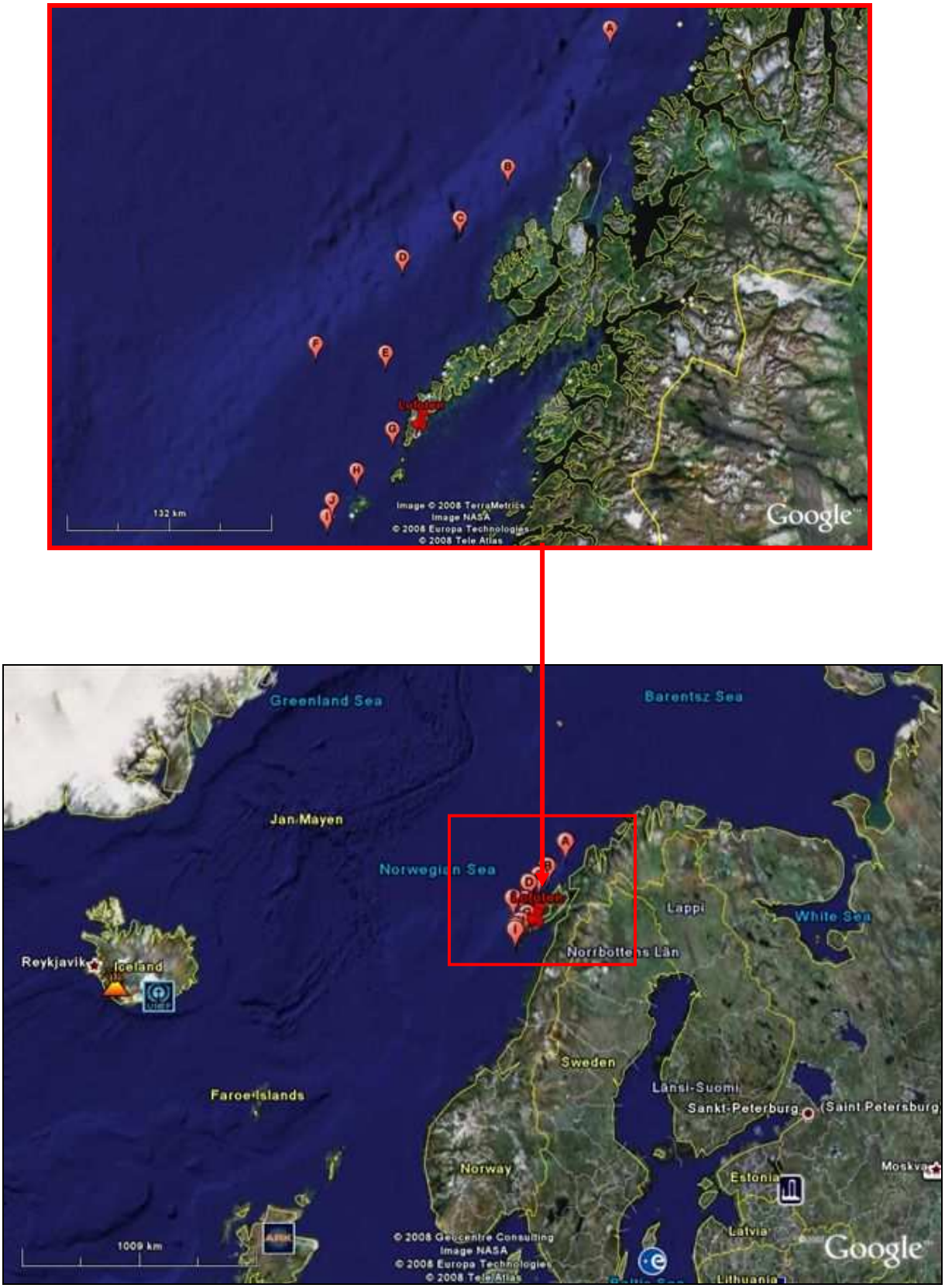


Figure 12: Sampling area outside Lofoten in 2008 (source: earth.google.com) (station coordinates A-J in Appendix B, Table B.2).

2.5.1 MOTILITY

The motility measurements were done onboard the research vessel with a S8APO binocular (Leica). Percentage of moving sperm cells (vibrating cells not included) was checked at 80 times magnification immediately after activation, after ten minutes and after one hour.

2.5.2 SPERM DENSITY

Sperm density of the samples fixed in teleost fixative was estimated in the laboratory at the High Technology Centre, Bergen, after the cruise (see 2.2.1 for details on the haemocytometer and section 2.4.1 for details on Image J (1.37v)). However, due to time limitations only two pictures were taken from one dilution per milt sample.

2.5.3 DRUMMING MUSCLES

Drumming muscles were cut out with a scalpel and the wet weight was measured onboard the vessel with a M2000 scale (Marel). Then the drumming muscles were frozen onboard the vessel for later determination of the dry weight on land. The drumming muscles were dried for 24 hours at 60^{o}C in a Termaks oven (Environmental laboratory equipment), and then the dry weight was measured on a Genius ME235P scale (Sartorius).

2.5.4 SEX RATIO PER STATION

The same method was applied as described in section 2.4.12.

2.6 STATISTICAL ANALYSIS

The software program R version 2.6.0 (R Development Core Team) was used for the majority of statistical analysis. All data were tested for a normal distribution with a Shapiro-Wilk test before any analysis was done. If the data were not normally distributed they were log transformed, and again tested for a normal distribution with a Shapiro-Wilk test.

A paired t-test was done in R (version 2.6.0) to test the null hypothesis; H_0: There is no difference in mean spermatozoa density when counted by hand versus counted by the software program Image J version 1.37 (National Institutes of Health). An ANCOVA test was done in R (version 2.6.0) to test if there was a significant difference in mean drumming muscle weight between males and females when body weight was adjusted for. To test if the sperm quality, in form of sperm density, varied through the different dates of sampling an ANOVA was done in R (2.6.0) to test the null hypothesis, H_0: There is no difference in mean sperm density collected at different dates. A Tukey Honestly Significantly Different (HSD) test was used to see if there was any significant difference between pair wise comparisons.

Pearson's product-moment correlation analysis between sperm density, total sperm amount, total fish length, total fish weight, gonad weight, GSI, condition, drumming muscle weight and motility were also done in R (2.6.0) to test if there was a significant linear relationship between the variables. The visualisation of Pearson's analysis was done in Statistica 7.0 software (StatSoft Inc, Tulsa, OK).

Principal components analysis was done in Statistica 7.0 software (StatSoft Inc, Tulsa, OK) to illustrate how similar or dissimilar the different variables of the male cod were when looking at the variances in an ordination, with the greatest variance by any projection of the data laying on the first coordinate (first principal component), the second greatest variance laying on the second coordinate and so on. Only the three first principal components (factor one, factor two and factor three) were used for the illustrations since these factors explain the majority of the variances.

Mean values are given with ± standard deviation.

3. RESULTS

3.1 METHOD DEVELOPMENT

3.1.1 SPERMATOCRIT

The r value indicated a tendency for a negative relationship between spermatocrit and the spermatozoa density in captive male NEA cod (Fig. 13: Pearson's product-moment correlation test; r = -0.596, p = 0.053, n = 8, raw data in Appendix C, Table C.1). However, the p value was not significant (p = 0.053).

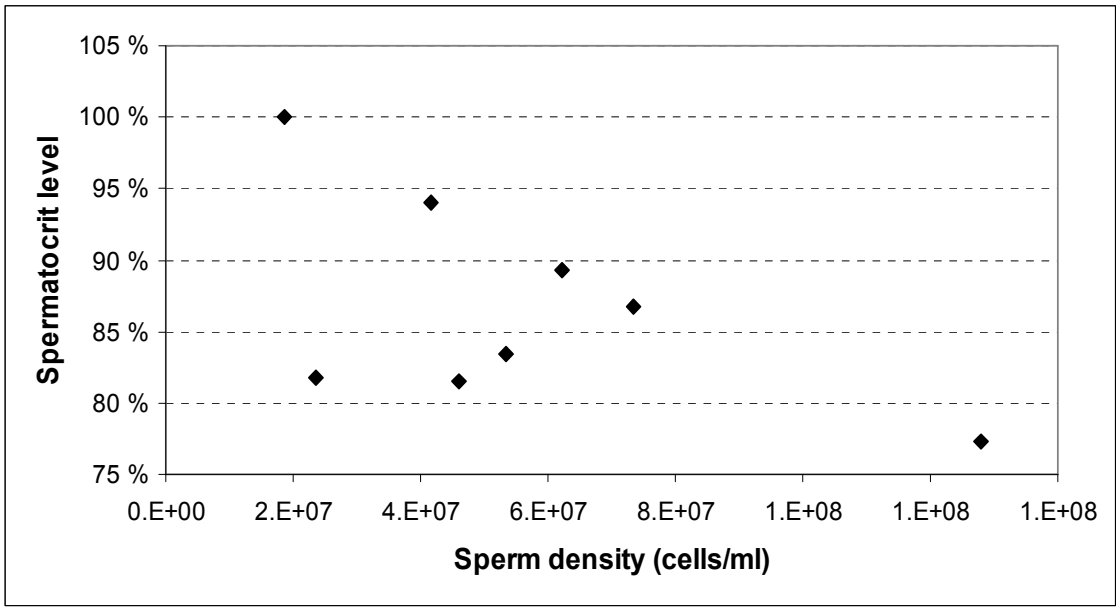


Figure 13: Relation between spermatozoa density and spermatocrit in captive NEA cod 2007.

3.1.2 DRUMMING MUSCLES

Drumming muscles were cut out of all the 73 fish related to the 'skipped spawning' project at IMR at the 18th to the 20th of January 2007. However, examination of the otoliths determined that 18 of the fish were coastal cod (O.S. Kjesbu, IMR, Bergen: *pers. comm.*), hence they were excluded from further calculations. Therefore, only 55 fish, with 17 females (Appendix C, Table C.2) and 38 males (Appendix C, Table C.3), were used for comparisons. A significant correlation was found between wet and dry weight of the drumming muscles (Paired t-test: $p < 2.2 \times 10^{-16}$, n = 55). Drumming muscle mass and sperm density were not correlated (Pearson's product-moment correlation: r = 0.125, p = 0.715, n = 11). The body weight of the fish was significantly correlated with both dry weight

(Pearson's product-moment correlation: r = 0.646, p = $9.9x10^{-8}$, n = 55) and wet weight (Pearson's product-moment correlation, r = 0.652, p = $6.7x10^{-8}$, n = 55) of the drumming muscles. Relative to body mass, males had larger drumming muscle wet weight than females (ANCOVA: F (2,52) = 23.07, p = 0.042), but this was not the case when using dry weight (ANCOVA: F (2,52) = 21.69, p = 0.066). The mean ratio of drumming muscle to the fish weight was estimated as 0.042 % (± 0.018 %) for the females and 0.061 % (± 0.036 %) for the males.

Drumming muscle samples were also taken from five captive cod males at the 14th of February 2008, as a preparation of methods before a cruise at the spawning grounds in 2008 (Appendix C, Table C.4). In these experiments there was also a significant relationship between the wet and dry weight of the drumming muscles in males (Paired t-test; p = $1.6x10^{-7}$, n = 8). Drumming muscle mass and sperm density did not correlate (Pearson's product-moment correlation: r = -0.164, p = 0.793, n = 8). The body weight of the fish was not significantly correlated to either dry weight (Pearson's product-moment correlation: r = 0.701, p = 0.188, n = 8) or wet weight (Pearson's product-moment correlation, r = 0.630, p = 0.254, n = 8) of the drumming muscles. Measurements of sperm motility were only done as a small practice of methods and measurements were not recorded or used in any correlations.

3.1.3 MILT WEIGHT

To investigate the potential importance of sample volume and if milt had a higher density than seawater, two sample volumes were utilised; 1 mL and 100 µl (Appendix C, Table C.5). The average weight of milt per millilitres from the one millilitre samples, measured on EW3000-2M scale (KERN), was 1.07 ± 0.041 g/mL. The average weight per millilitres of milt when measuring 100 µl milt, measured on a Genius ME235P scale (Sartorius), was 0.921 ± 0.085 g/mL. There was a significant difference between the two methods of weight measurements of the milt (One-way ANOVA: F (1,12) = 11.67, p = 0.005) with the highest mean weight estimated using a milt volume of one millilitre (Tukey HSD; p = 0.005).

3.2 PRE-SPAWNING NEA COD AT ANDENES IN 2007

A total of 70 males were sampled at Andenes in 2007. Unfortunately, the males were in a pre-spawning state and none of them were running. Syringes were used to get the sperm out of the gonads, but all methods applied were unsuccessful. Analyses of the otoliths determined that seven of the 70 males were coastal cod (O.S. Kjesbu, IMR, Bergen: *pers. comm*.), hence these were excluded from any further analyses.

The fish length of the 63 males ranged from 62 - 105 cm. The condition factor ranged from 0.72 to 1.18, and the GonadoSomatic Index (GSI) ranged from 3.2 – 16.4 %. A significant relationship was found between GSI and the weight of the fish (Pearson's product-moment correlation; r = 0.32, p = 0.01, n = 63) and between GSI and the length of the fish (Pearson's product-moment correlation; r = 0.27, p = 0.03, n = 63). These males were in a pre-spawning state with large and white gonads, but no running milt (Appendix C, Table C.6 and C.7).

3.3 COD AT THE SPAWNING GROUNDS IN 2007

Examination of the otoliths determined that 13 of the total catch of 74 male cod were coastal cod (O.S. Kjesbu, IMR, Bergen: *pers. comm*.), hence these fish were excluded from further calculations. In addition, gonad weight data were lacking from two of the males so comparisons were done with 59 male NEA cod (Appendix C, Table C.8 and C.9). The fish length ranged from 55 – 118 cm. The condition factor ranged from 0.76 – 1.07 and the GSI ranged from 5.2 – 19.1 %.

3.3.1 SPERMATOZOA DENSITY

There was no significant difference in mean spermatozoa density between counts of cells with Image J (1.37v) and manual counts (Paired t-test: p = 0.049), hence Image J was chosen for further estimations of sperm density (Appendix C, Table C.10). The density of sperm cells ranged from a minimum of 1.68×10^8 ($\pm 5.37 \times 10^7$) to a maximum of 1.15×10^{10} ($\pm 7.90 \times 10^8$) cells per mL. Milt collected at different dates had different means of spermatozoa density (Fig. 14: ANOVA; F (3,57) = 5.13, p = 0.003, n = 59). However, the

only significant difference was between samples collected at the 24th of March and the 31st of March 2008 (Fig 14: Tukey HSD: p = 0.001, n = 59).

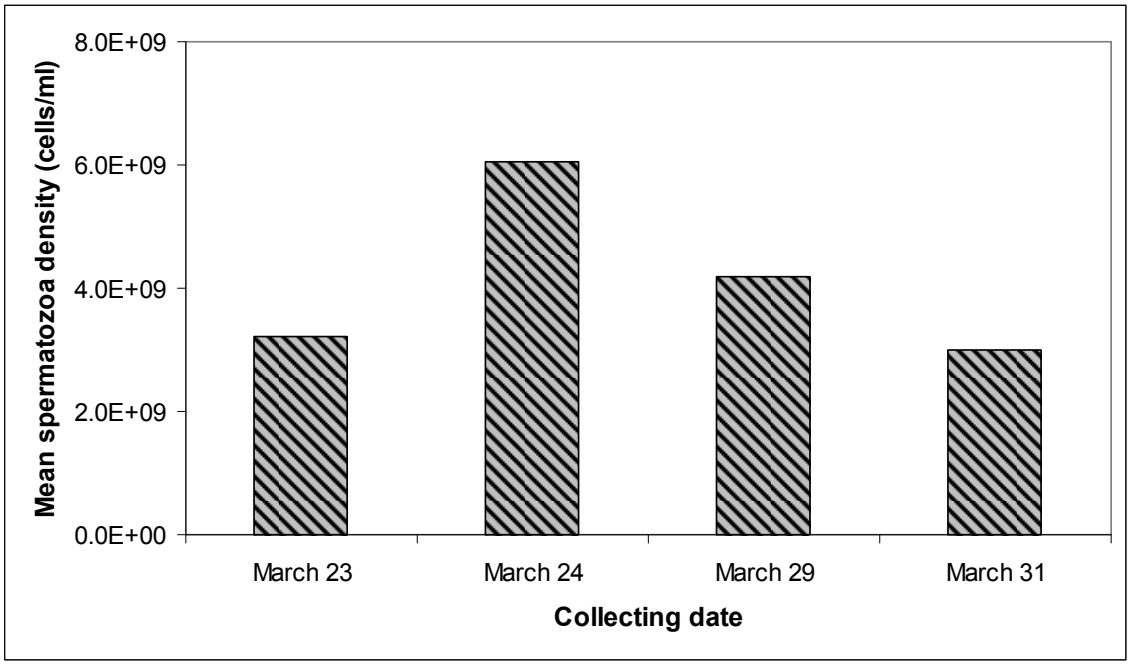
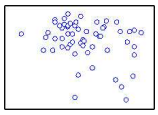
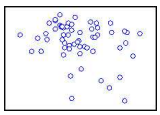
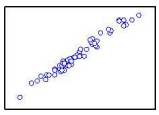
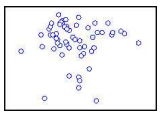
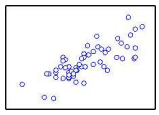
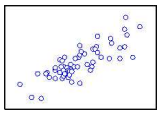
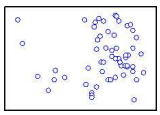
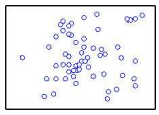
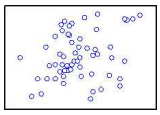
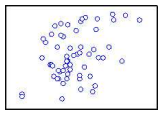
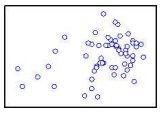
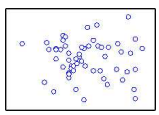
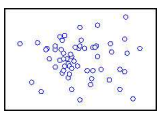
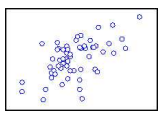
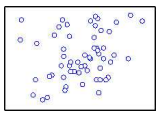


Figure 14: Mean spermatozoa densities from milt samples in NEA cod collected at different dates at the spawning grounds 2007.

3.3.2 CORRELATIONS OF MALE TRAITS IN 2007

Larger fish were heavier (Table III; n = 59, Fig. 15; n = 59) and there was a significant correlation between the condition and the weight of the fish (Table III; n = 59, Fig. 16; n = 59). No correlation was found between the sperm density and the gonad weight (Table III; n = 59). However, there was a significant, but weak, negative correlation between the sperm density and the total length of the fish (Table III; n = 59). Even so, this was offset by a significant correlation between GSI and density indicating that sperm density increases with GSI (Table III; n = 59). Significant correlations are in italic in Table III. Total sperm amount was not included in any of the correlations since it was estimated based on gonad weight and it gave the exact same output as correlations with gonad weight. No correlation was found between the age of the fish and any of the male traits. However, age has been excluded from the Results section due to the low variation in age, with the majority of the males having an age between seven or eight years.

Table III: Summary table of Pearson's product-moment correlation and plots (Statistica) between the different traits of 59 male cod from the Lofoten Survey 2007.

Traits	**Sperm density**	**Total fish length**	**Total fish weight**	**Gonad weight**	**K factor**	**GSI**
Sperm density		*r= -0.274* *p= 0.035*	r= -0.253 p= 0.053	r= -0.005 p= 0.969	r= 0.062 p= 0.639	*r= 0.346* *p= 0.007*
Total fish length			*r= 0.985* *p< 2.2e-16*	*r= 0.771* *p= 9.4e-13*	r= 0.140 p= 0.290	r= -0.069 p= 0.606
Total fish weight				*r= 0.810* *p= 8.2e-15*	*r= 0.308* *p= 0.018*	r= -0.023 p= 0.863
Gonad weight					*r= 0.399* *p= 0.002*	*r= 0.568* *p= 2.7e-6*
K factor						r= 0.247 p= 0.059
GSI						

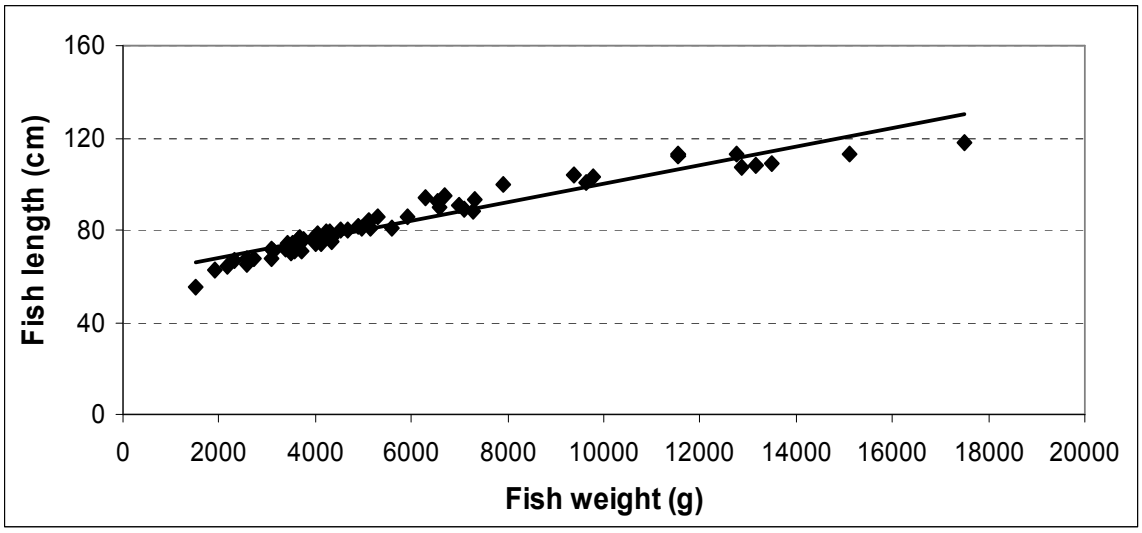


Figure 15: Relationship between fish weight and fish length in NEA cod at the spawning grounds in 2007.

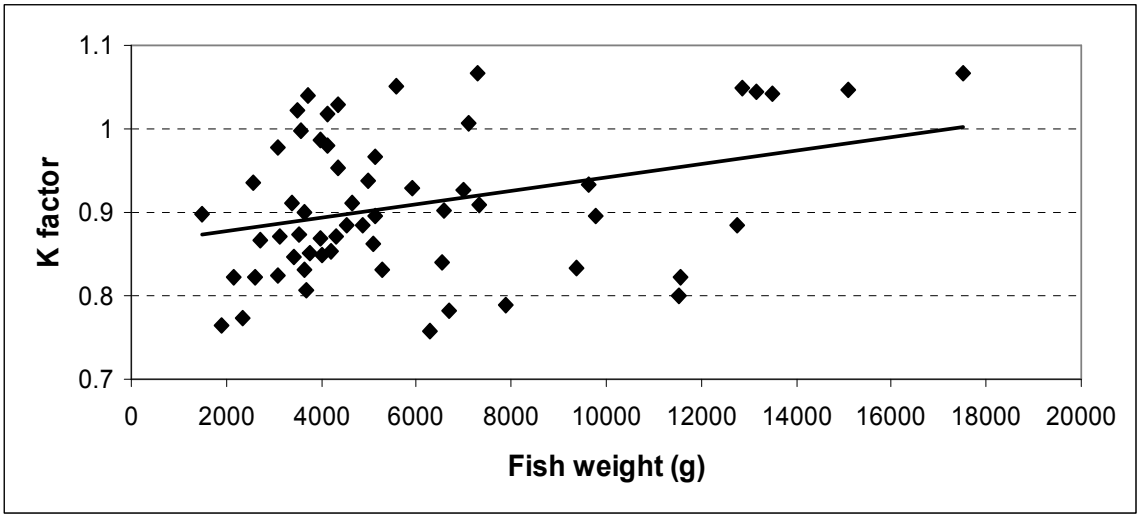


Figure 16: Relationship between fish condition and fish weight in NEA cod at the spawning grounds in 2007.

3.3.3 MILT WEIGHT

The spermatozoa cells spun down from the mixture of formalin and milt had an average weight of 0.125 g/mL. This represented the weight of just the spermatozoa cells, without water and seminal fluid. Unfortunately this result could not be used to calculate the original milt weight as the supernatant containing seminal fluid and formalin was contaminated with spermatozoa cells. Therefore, these data were not used in any equations.

3.3.4 SPERM CELL VOLUME

Light microscope (LM) and cell volume: The LM pictures were used to estimate the volume of single cells and the mean length and width of a cell was estimated to 3.46 ± 0.35 µm and 1.92 ± 0.14 µm, respectively (Appendix C, Table C.11). Average sperm cell volume (C_V) was, C_V = 6.75 μm^3/cell.

Field emission scanning electron (SEM) microscope and cell volume: The SEM pictures were also used to estimate the volume of single cells and the mean length and width of a cell was estimated as 2.47 ± 0.24 µm and 1.17 ± 0.07 µm, respectively (Appendix C, Table C.12). Average sperm cell volume (C_V) was, C_V = 1.77 μm^3/cell.

3.3.5 TESTIS VOLUME

The average testis volume per gram testes of 72 males, ranging in length from 52 to 118 cm, was estimated to be 0.996 ± 0.058 mL/gram (Appendix C, Table C.13).

3.3.6 PERCENTAGE OF SPERM CELLS IN TESTIS

Based on the given histology data of coastal cod testes (Meier *et al.*, 2007) the percentages of sperm cells in different stages of spermatogenesis (spermatozoa, elongated spermatids, round spermatids, spermatocytes and spermatogonia) were added together as a total amount of sperm cells (96.6 %), whilst the percentage of open space, blood vessels and lobula were considered as connective tissue (3.4 %) and removed from the estimated total amount of sperm cells in the gonads (Appendix C, Table C.14, Fig. C.1).

3.4 COD AT THE SPAWNING GROUNDS IN 2008

Examination of the otoliths determined that five of the total catch of 48 male cod were coastal cod (O.S. Kjesbu, IMR, Bergen: *pers. comm*.) and therefore these fish were excluded from further calculations. In addition, one of the NEA males was lacking data of gonad weight so only 42 of the male cod are presented in the results (Appendix C, Table C.15 and C.16).

The fish length ranged from 64 – 114 cm. The condition factor ranged from 0.70 – 1.21 while the GSI in 2008 ranged from 4.8 – 21.8 %. No significant difference was found in mean fish length (Fig. 17; One-way ANOVA: $F_{(2,161)} = 1.33$, $p = 0.268$), fish weight (One-way ANOVA; $F_{(2,161)} = 0.97$, $p = 0.380$), condition (Fig 18; One-way ANOVA: $F_{(2,161)} = 0.16$, $p = 0.851$) or GSI (Fig. 19; One-way ANOVA: $F_{(2,161)} = 1.24$, $p = 0.293$) between the samples of pre-spawning cod in 2007, cod at the spawning grounds in 2007 and cod at the spawning grounds in 2008.

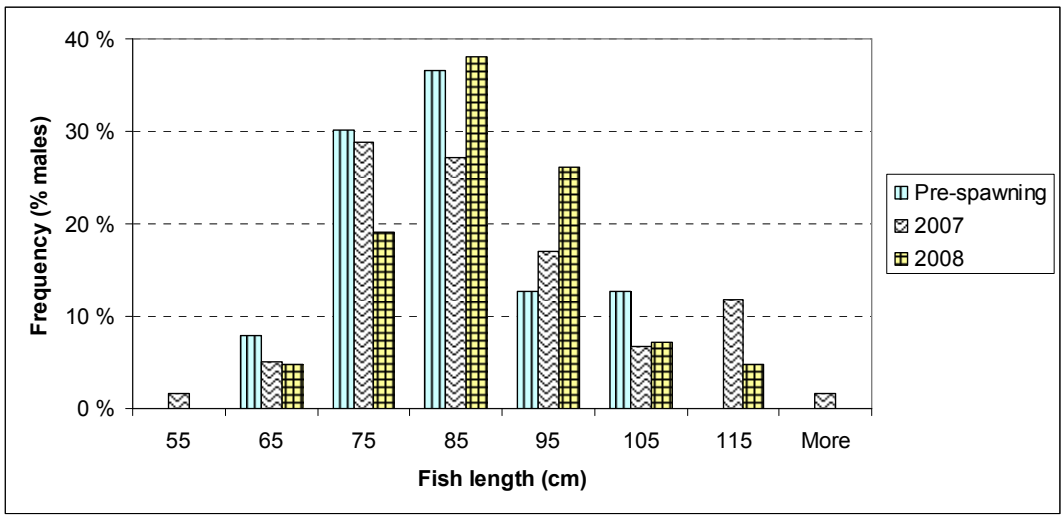


Figure 17: Distributions of fish length for pre-spawning cod in 2007 (n = 63), cod at spawning grounds in 2007 (n = 59) and cod at spawning grounds in 2008 (n = 42).

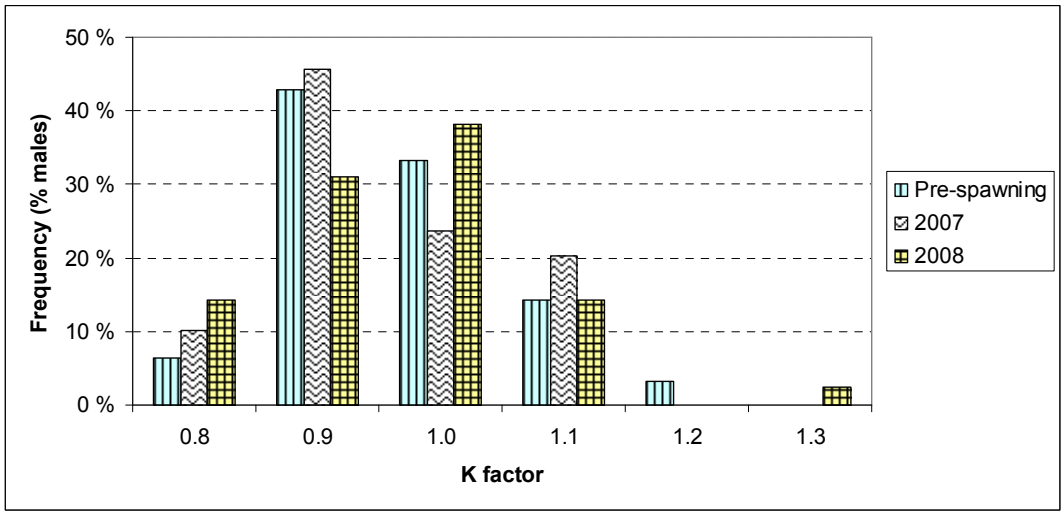


Figure 18: Distributions of condition factor for pre-spawning cod in 2007 (n = 63), cod at spawning grounds in 2007 (n = 59) and cod at spawning grounds in 2008 (n = 42).

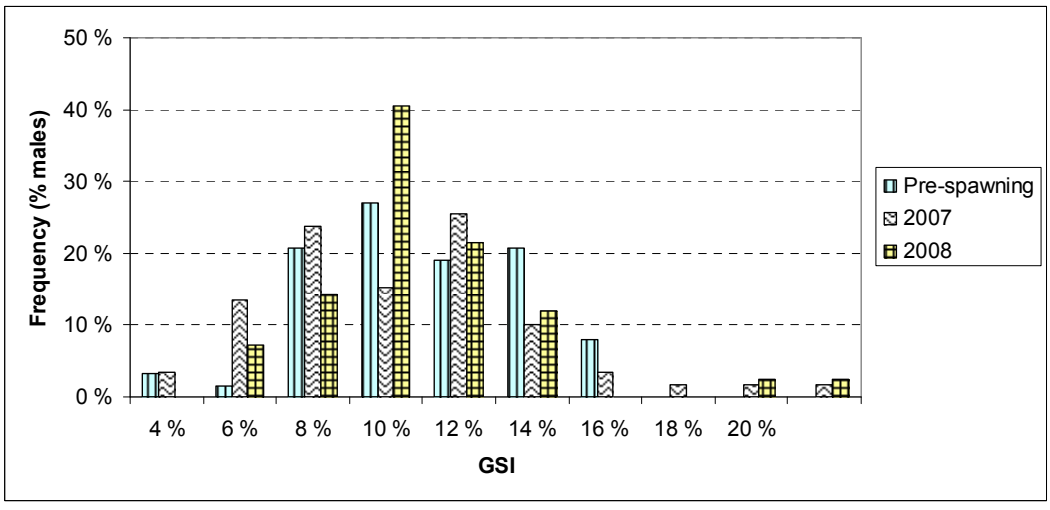


Figure 19: Distributions of GSI for pre-spawning cod in 2007 (n = 63), cod at spawning grounds in 2007 (n = 59) and cod at spawning grounds in 2008 (n = 42).

3.4.1 SPERMATOZOA DENSITY

The spermatozoa densities ranged from a minimum of $3.8x10^9$ to a maximum of $4.2x10^{10}$ cells per mL. There was a significant difference between mean spermatozoa densities in 2007 and 2008 (One-way ANOVA; F (1,99) = 38.39, $p = 1.3x10^{-8}$) with the highest mean densities estimated in 2008 (Tukey HSD; p = 0.0). However, no significant difference in mean sperm density were found between the different collection dates in 2008 (Fig. 20: One-way ANOVA; F (5,36) = 0.22, p = 0.954, n = 42).

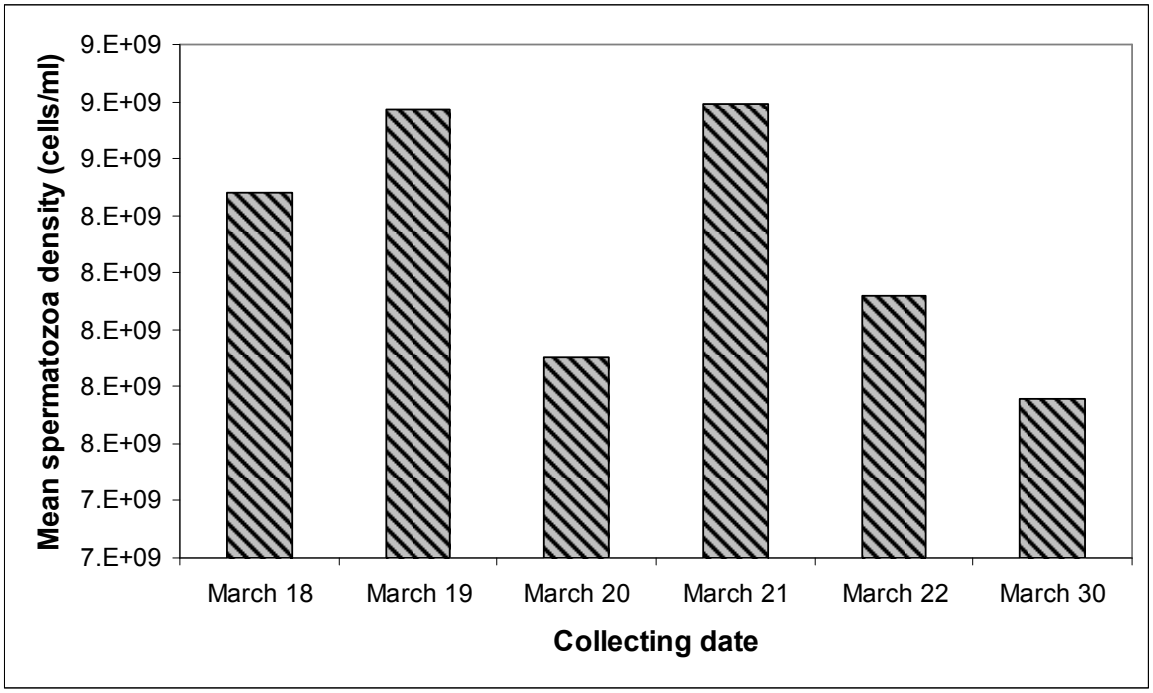


Figure 20: Mean spermatozoa densities of milt samples from NEA cod collected at different dates at the spawning grounds 2008.

3.4.2 MILT WEIGHT

NEA cod milt weight was measured to see if the density was higher than seawater. However, all of the milt samples that were measured onboard the research vessel weighed exactly one gram per millilitre milt when measured with a M2000 scale (Marel).

3.4.3 MOTILITY

The sperm motility was measured immediately after activation (time zero) and after ten and 60 minutes after activation (Fig. 21, n = 42) (see Appendix C, Table C.17 for raw data). The motility decreased rapidly from zero to ten minutes and almost no activity was

recorded after 60 minutes of exposure in seawater. One sample increased with 15 % in spermatozoa motility after ten minutes of exposure in seawater.

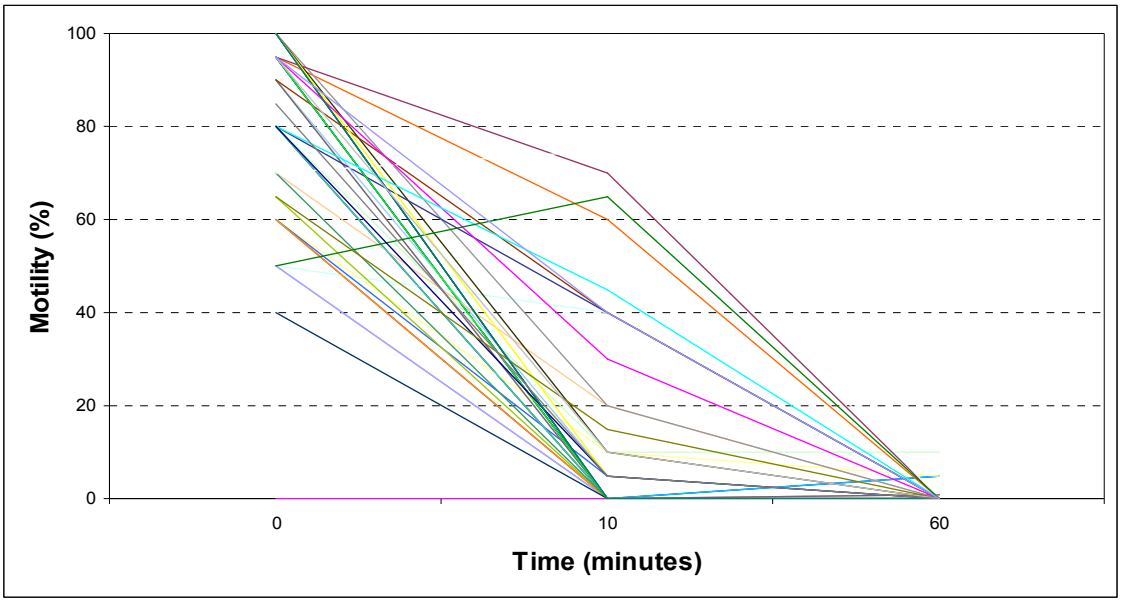


Figure 21: Sperm motility measured after 0, 10 and 60 minutes after activation in NEA cod at the spawning grounds in 2008.

3.4.4 DRUMMING MUSCLES

A large variation in drumming muscle mass was found in male NEA cod caught at the spawning grounds in 2008 (see Fig. 22 for qualitative comparison of dry weight between a small and a large drumming muscle). There was a significant correlation between the wet and the dry weight of the drumming muscles (Paired t-test: $p < 2.2x10^{-16}$, n = 42). The dry weight of the drumming muscles was also correlated with different variables of the male cod using Pearson's product-moment correlation (Table IV, n = 42). The dry weight of the drumming muscles was significantly correlated with 1) fish length (r = 0.509, p = 0.001), 2) fish weight (r = 0.531, p = 0.000), 3) gonad weight (r = 0.414, p = 0.006) and 4) motility measured after ten minutes (r = 372, p = 0.015) (Table IV, n = 42). These results showed that larger males have larger drumming muscle mass. No correlation was found between drumming muscle dry weight and sperm density (Table IV; r = 0.028, p = 0.858, n = 42). The mean ratio of drumming muscle to the fish weight was estimated to be 0.073 % (± 0.046 %).

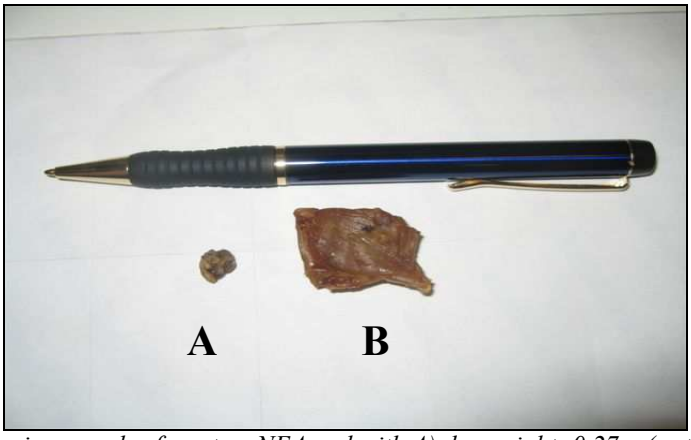
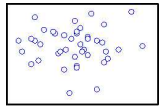
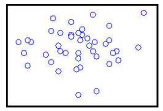
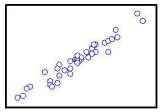
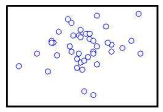
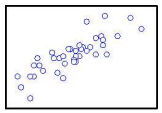
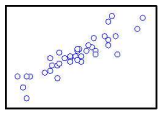
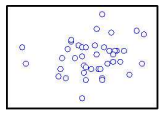
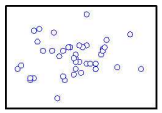
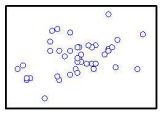
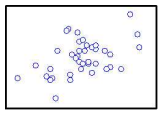
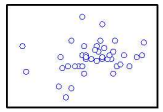
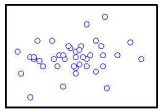
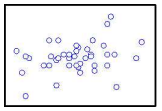
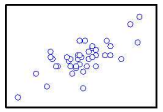
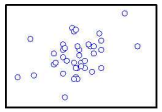
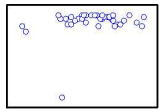
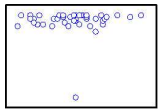
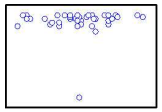
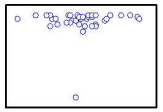
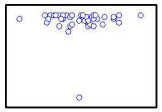
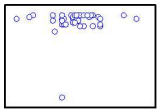
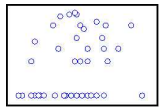
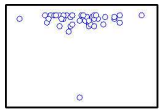
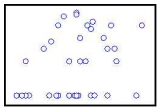
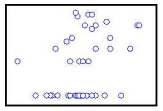
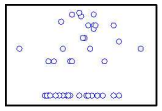
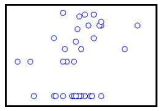
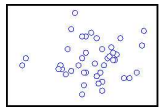
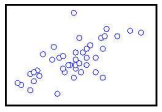
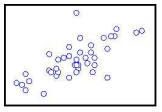
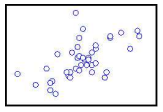
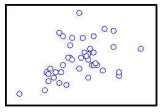
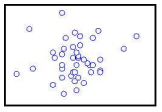
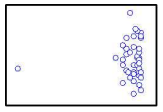
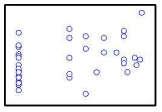


Figure 22: Dried drumming muscles from two NEA cod with A) dry weight; 0.27 g (wet weight; 1.60 g), B) dry weight; 4.16 g (wet weight; 13.00 g).

3.4.5 CORRELATIONS BETWEEN MALE TRAITS IN 2008

Larger fish were heavier (Table IV; n = 42, Fig. 23; n = 42) and there was a significant correlation between the condition and the weight of the fish (Table IV; n = 42, Fig. 24; n = 42). No correlation was found in 2008 between sperm density and fish length (Table IV; n = 42). However, no correlation was found between the sperm density and any of the variables. The dry weight of the drumming muscles was significantly correlated with the motility measured after ten minutes (Table IV; r = 0.372, p = 0.015, n = 42). Even so, the scatter plot does not reflect this correlation (Table IV, n = 42). There exists a positive and significant relationship between motility measured after ten minutes and GSI (Table IV; r = 0.310, p = 0.046, n = 42), and also with the same motility measurement and gonad weight (Table IV; r =0.371, p = 0.015, n = 42). By looking at the scatter plot of these two correlations, this relationship seems to be significant, when ignoring all the motility measurements that were measured as zero percent (straight line in scattered plots) (Table IV, n = 42). All significant correlations are marked in italic in Table IV (n = 42). Total sperm amount was not included in any of the correlations since it was estimated based on gonad weight and it gave the exact same output as correlations with gonad weight. No correlation was found between the age of the fish and any of the male traits. However, age has been excluded from the Results section due to the low variation in age, with the majority of the males having an age between seven and eight years.

Table IV: Summary table of Pearson's product-moment correlation and plots (Statistica) between the different variables in the 42 male NEA cod at the spawning grounds in 2008.

Traits	**Sperm density**	**Total fish length**	**Total fish weight**	**Gonad weight**	**K factor**	**GSI**	**Mot. at zero**	**Mot. at ten**	**Drum muscle dry**
Sperm density		r=0.003 p=0.985	r=0.049 p=0.756	r=0.155 p=0.327	r=0.185 p=0.240	r=0.244 p=0.119	r=0.226 p=0.150	r=0.039 p=0.806	r=0.028 p=0.858
Total fish length			*r=0.968* *p<2.2e-16*	*r=0.826* *p=1.6e-11*	r=0.139 p=0.381	r=0.211 p=0.179	r=0.040 p=0.799	r=0.262 p=0.094	*r=0.509* *p=0.001*
Total fish weight				*r=0.886* *p=6.4e-15*	*r=0.381* *p=0.013*	r=0.286 p=0.066	r=0.036 p=0.823	r=0.296 p=0.057	*r=0.531* *p=0.000*
Gonad weight					*r=0.455* *p=0.002*	*r=0.698* *p=2.8e-7*	r=0.077 p=0.630	*r=0.371* *p=0.015*	*r=0.414* *p=0.006*
K factor						*r=0.352* *p=0.022*	r=-0.009 p=0.957	r=0.205 p=0.192	r=0.222 p=0.158
GSI							r=0.103 p=0.516	*r=0.310* *p=0.046*	r=0.035 p=0.826
Mot. at zero								r=0.160 p=0.311	r=0.087 p=0.582
Mot. at ten									*r=0.372* *p=0.015*
Drum.muscle dry									

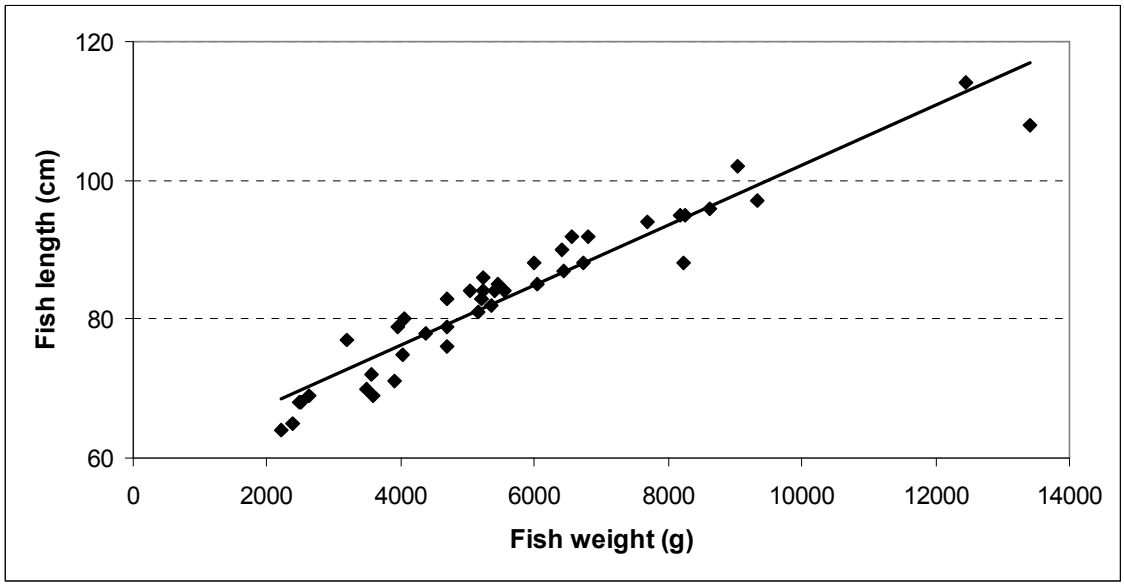


Figure 23: Relationship between fish weight and fish length in Northeast Arctic cod at the spawning grounds in 2008.

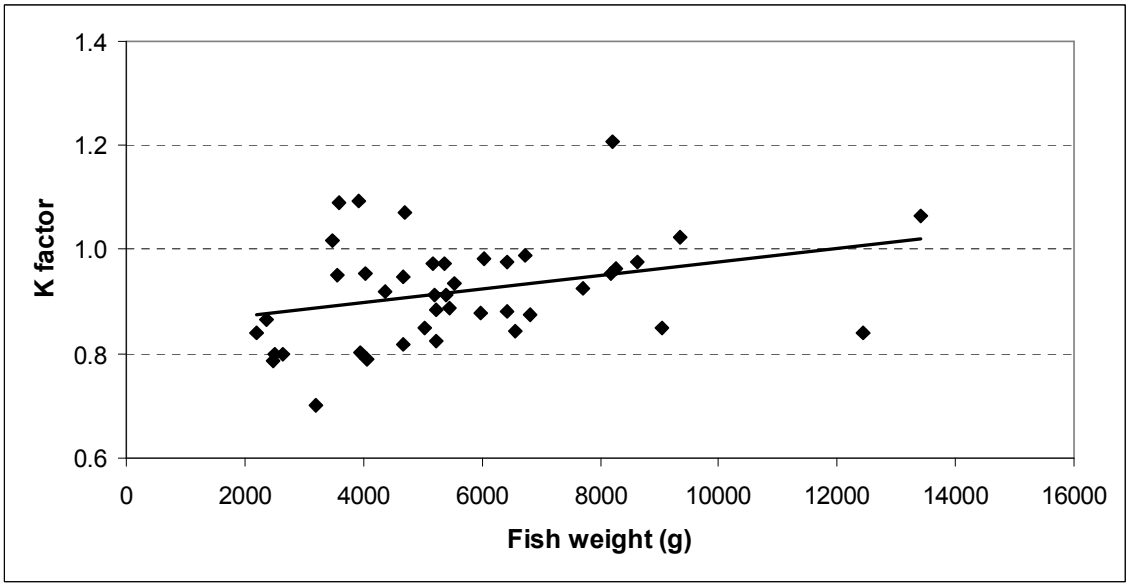


Figure 24: Relationship between fish condition and fish weight in NEA cod at the spawning grounds in 2008.

3.4.6 SEX RATIO PER STATION IN 2007 AND 2008

Both 2007 and 2008 showed a dominance of males in the selected stations at the spawning area in Lofoten, with 87.6 % males and 12.4 % females in 2007 (Appendix C, Table C.18, Fig. C.2) and 66.7 % males and 33.3 % females in 2008 (Appendix C, Table C.19, Fig. C.3).

The variances of the variables (Eigenvalues) describing male cod at the spawning grounds in 2007 and 2008 are given in Figure 25 (n = 59) and Figure 26 (n = 42), respectively. In 2007 factor one dominated with 50 % variance of the data, factor two contained 26 % while factor three contained 13 % of the variance (Fig. 25, n = 59). In 2008 factor one dominated with 44 % variance of the data, factor two contained 16 % while factor three contained 12 % of the variance (Fig. 26, n = 42). The variance contributors in male cod at spawning grounds in 2007 were fish length, fish weight and gonad weight in factor one, sperm density and GSI in factor two and condition in factor three (see factor loadings in Figure 27, n = 59). The variance contributors in male cod at spawning grounds in 2008 were fish length, fish weight and gonad weight in factor one, sperm density and GSI in factor two and condition and motility after ten minutes of activation in factor three (see factor loadings in Figure 28, n = 42). Total sperm amount was dropped from the principal components analysis since it is estimated based on the gonad weight, hence gave the exact same result as the gonad weight did.

Almost the same position of factor loadings were given when combining the variances from both years, except that the motility and drumming muscle mass contributors were excluded since none of these were measured in 2007 (Table V, n = 101, greatest factor contributors are in italic). A scatter plot of the two dominating factors (factor one and two) in a combination of variances from both years is shown in Figure 29 (n = 101). This plot shows a good mixture of the fish and indicates that no specific trait of the males differed between the spawning seasons. Only one group, marked with a red ring, show indications of differences from the rest of the group (Fig. 29). These fish were much smaller (in weight, length and gonad weight) and also had a lower sperm density, which excluded them from the general cluster of the rest of the variances.

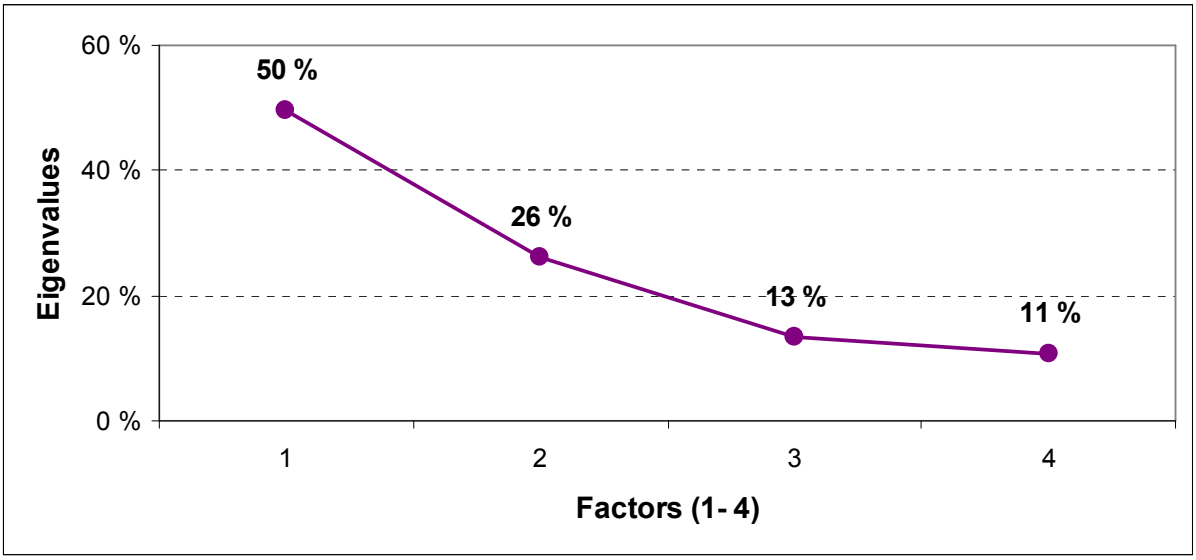


Figure 25: Eigenvalues from cod at spawning grounds in 2007.

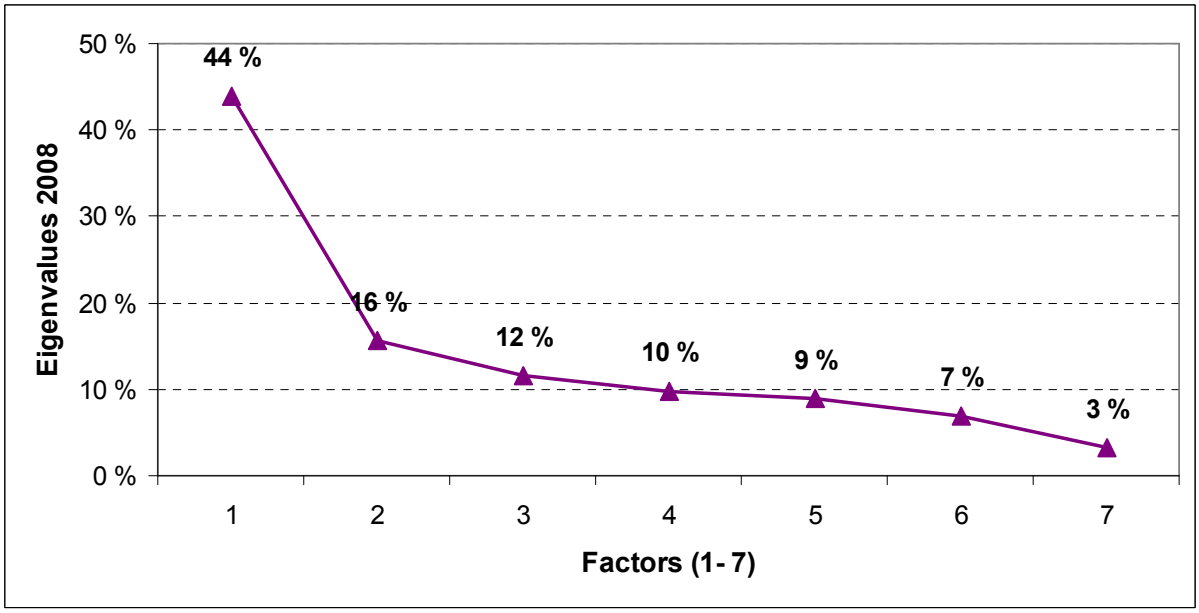


Figure 26: Eigenvalues for cod at spawning grounds in 2008.

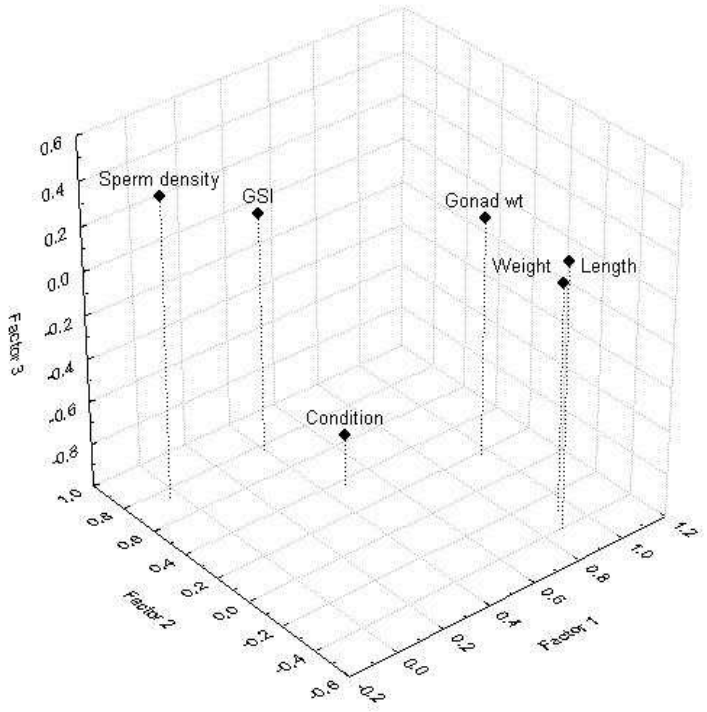


Figure 27: Factor loadings in cod at spawning grounds in 2007.

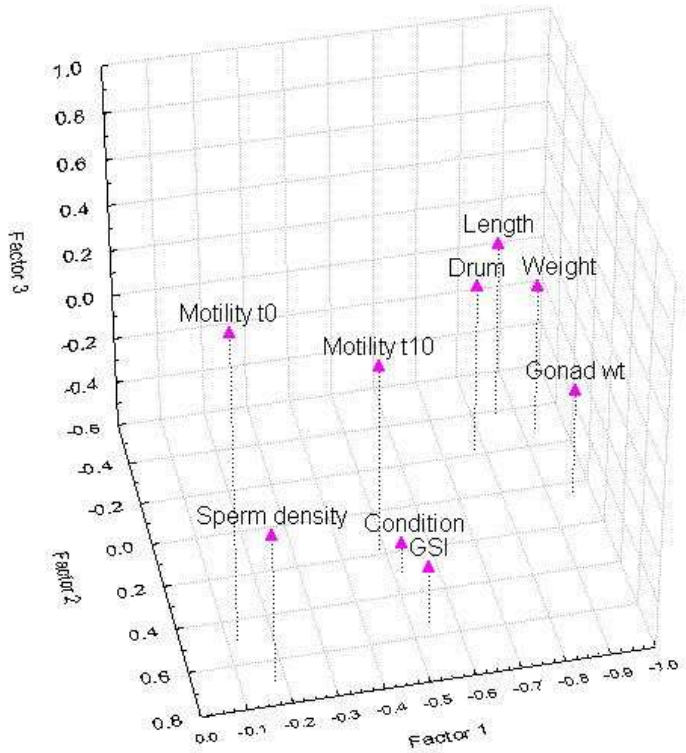


Figure 28: Factor loadings in cod at spawning grounds in 2008.

Table V: Factor contributions of variances in cod at spawning grounds in 2007 and 2008.

Contributor	Factor 1	Factor 2	Factor 3
Fish weight	*30 %*	6 %	0 %
Fish length	*26 %*	10 %	6 %
Gonad weight	*31 %*	2 %	1 %
Sperm density	0 %	*35 %*	18 %
GSI	5 %	*38 %*	1 %
Condition	8 %	9 %	*73 %*

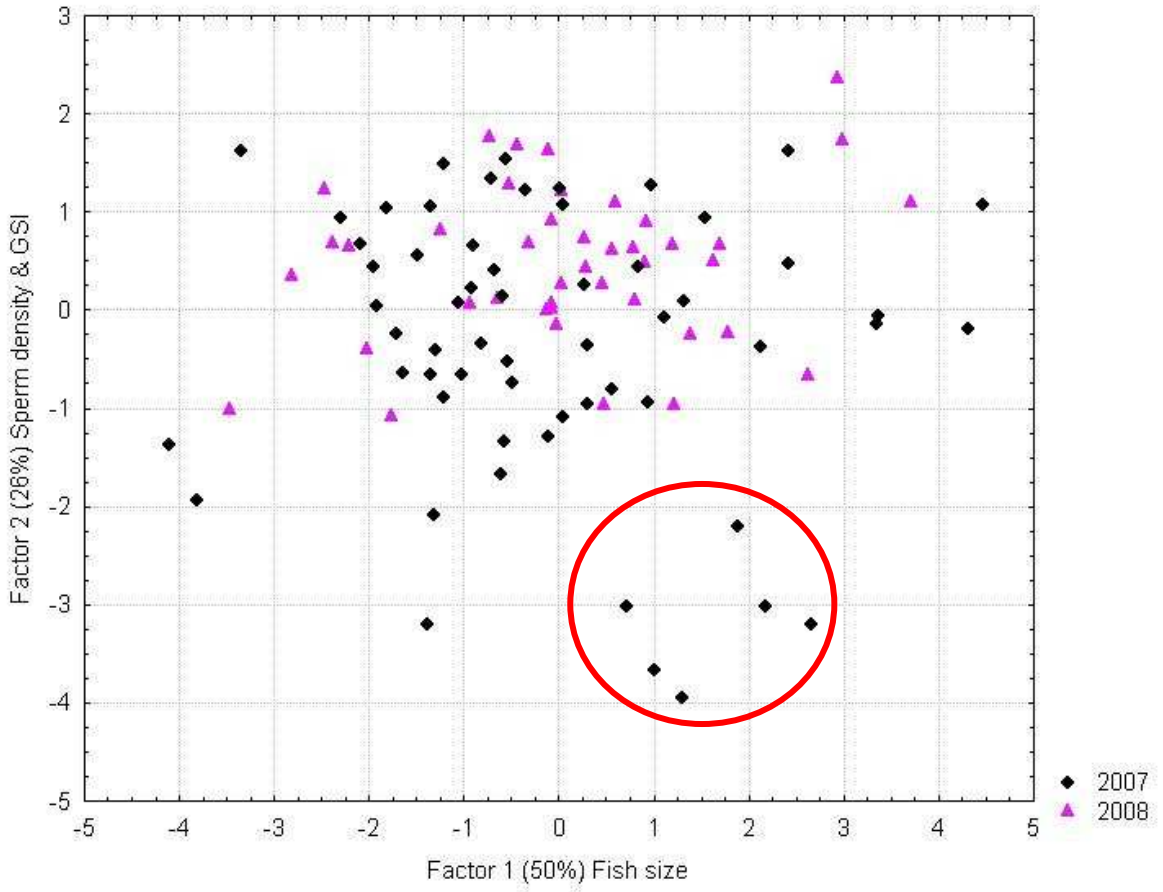


Figure 29: Scatter plot of factor 1 and factor 2 in cod at spawning grounds in 2007 and 2008. Red circle marks a separation of six fish from the mass of points.

The annual viable sperm production (VSP) was estimated with Trippel's equation (2003) (Fig. 30) and with the parameterised equation (Fig. 31) (Appendix C, Table C.20). The viable sperm production from the years 1981 to 1984 are stippled red in Figure 30 and Figure 31 since these years are only based on limited length and weight data (see Marshall et al. (2004), Figure 4, page 1908), consequently giving suspect results. There were no significant difference in the means between the annual viable sperm production estimated with the two equations (Fig. 32; Paired t-test: $p = 1.537 \times 10^{-10}$, $R^2 = 0.993$) (Appendix C, C.20). However, adding a trend line to the plot of the two equations gives an equation of; $Y = 0.08X + 2*10^{18}$, which gives a slope of 0.08 indicating that there is a scalar in the relationship (Fig. 32, $R^2 = 0.993$). This scalar indicates that the VSP estimated with the new equation always have higher values than the VSP estimated with Trippel's equation.

Figure 33 shows the temporal trend from 1946 to 2005 of SSB, VSP (estimated with the parameterised equation) and age three recruits, where SSB and VSP follows approximately the same trend except for a high peak in VSP around 1992 to 1995 (the data from 1981 to 1984 are removed from Figure 33 due to the poor data within these years, see Marshall *et al.*, 2004). The viable sperm production should increase with increasing spawning stock biomass. However, this was not always the case. Between 1992 and 1996 there was a vast increase in VSP (Fig. 33). The SSB and recruitment was low between 1992 and 1996. Even so, the fertilisation potential was high which means that the condition of the fish must have been high (Fig. 33) and this may affect the VSP. There were some interannual differences in the total sperm production, which were reflected in the plot of viable sperm production and spawning stock biomass (Fig. 34). Even so, there was a significant correlation between SSB and VSP (Pearson's product-moment correlation; $r = 0.745$, $p = 4.6 \times 10^{-11}$). A Beverton-Holt curve was fitted to the plot of viable sperm production versus age three recruits in Figure 35, showing a classical asymptotic relationship between a measure of population reproductive effort and subsequent recruitment.

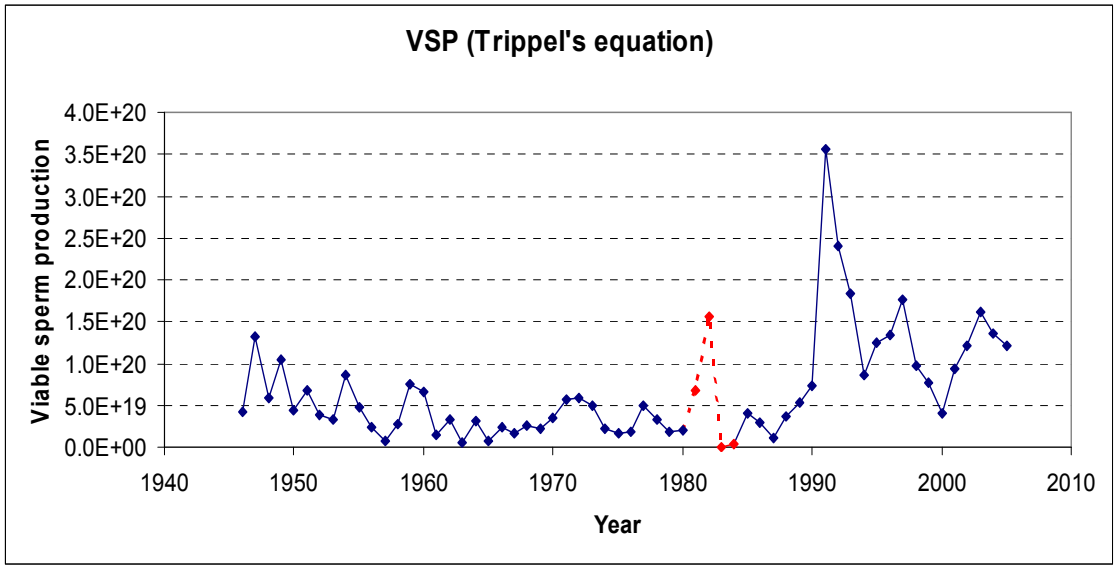


Figure 30: Annual viable sperm production for NEA cod (1946-2005) based on Trippel's (Trippel, 2003) equation.

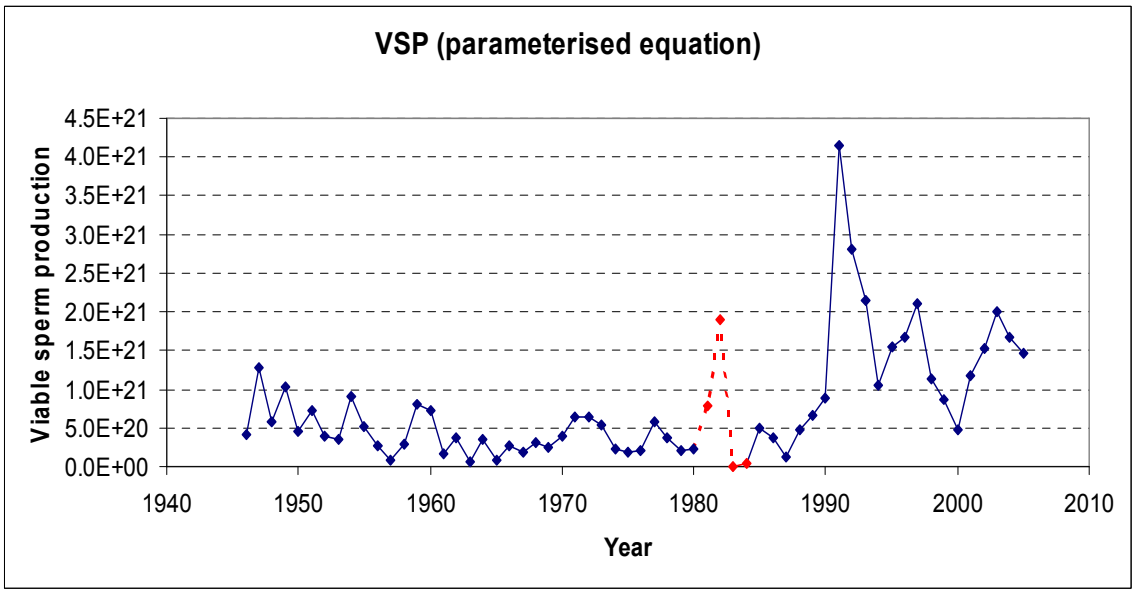


Figure 31: Annual viable sperm production for NEA cod (1946-2005) based on the parameterised equation.

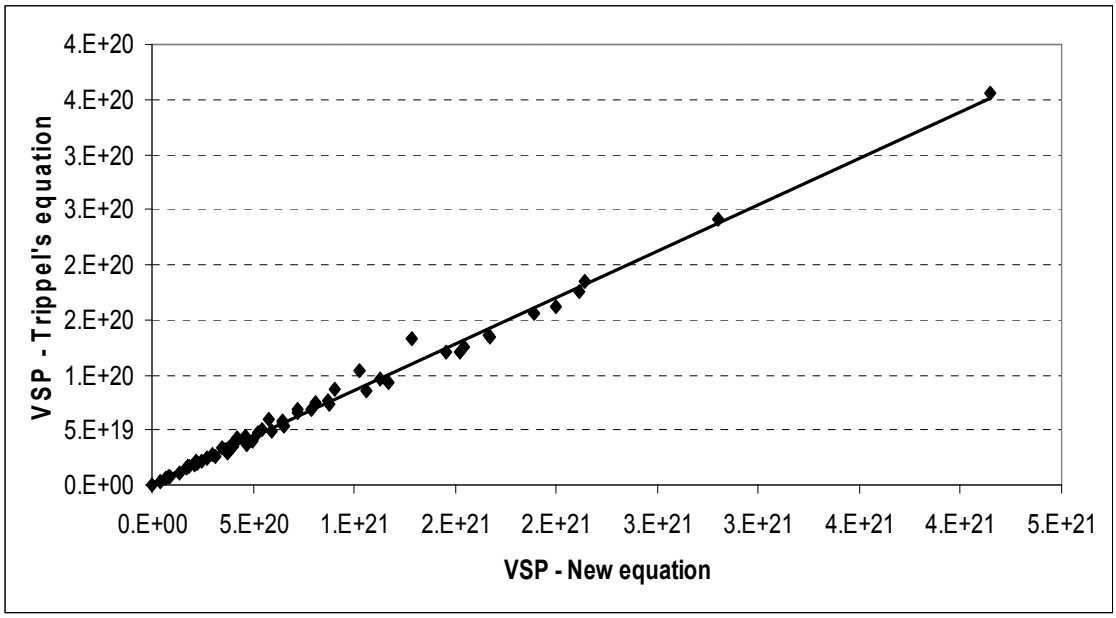


Figure 32: Comparing the annual viable sperm production from both equations.

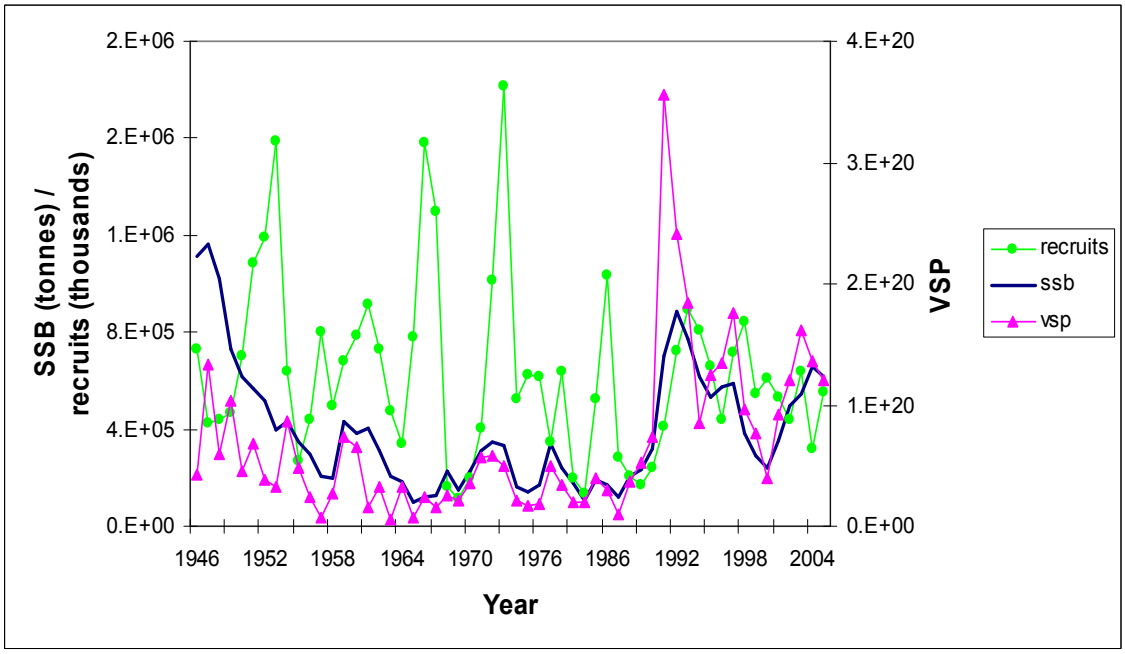


Figure 33: Spawning stock biomass (SSB), viable sperm production (VSP) and age three recruits (recruits) in the years 1946-2005 (1981-1984 are excluded).

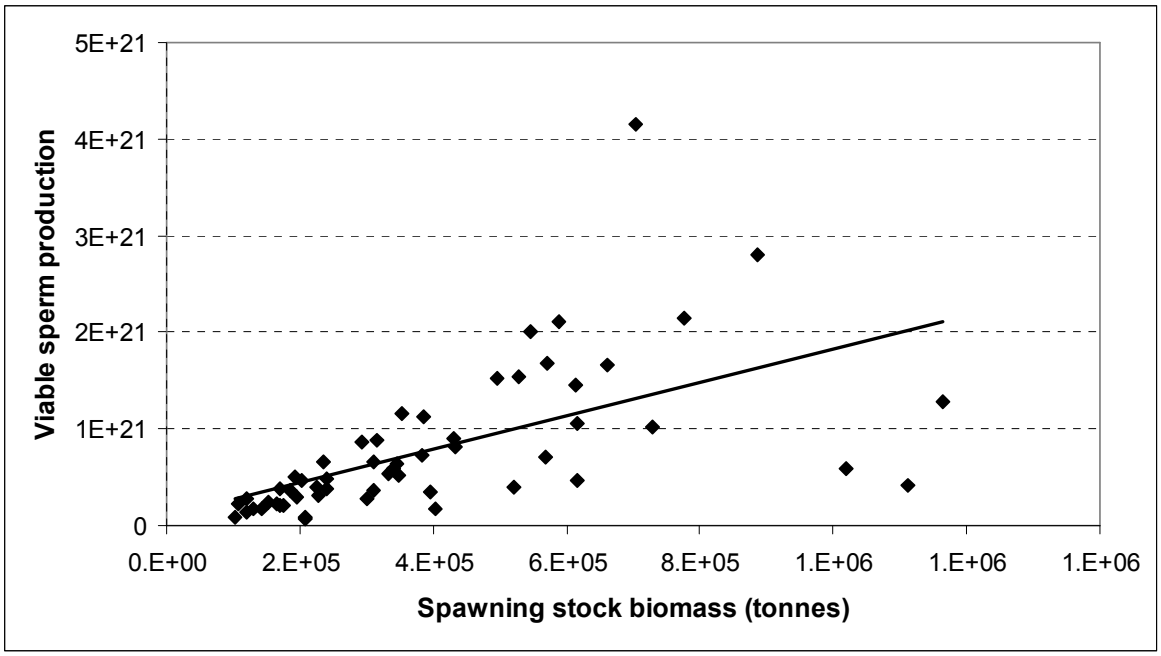


Figure 34: Relation between spawning stock biomass and total viable sperm production (parameterised equation) for NEA cod with a fitted linear curve.

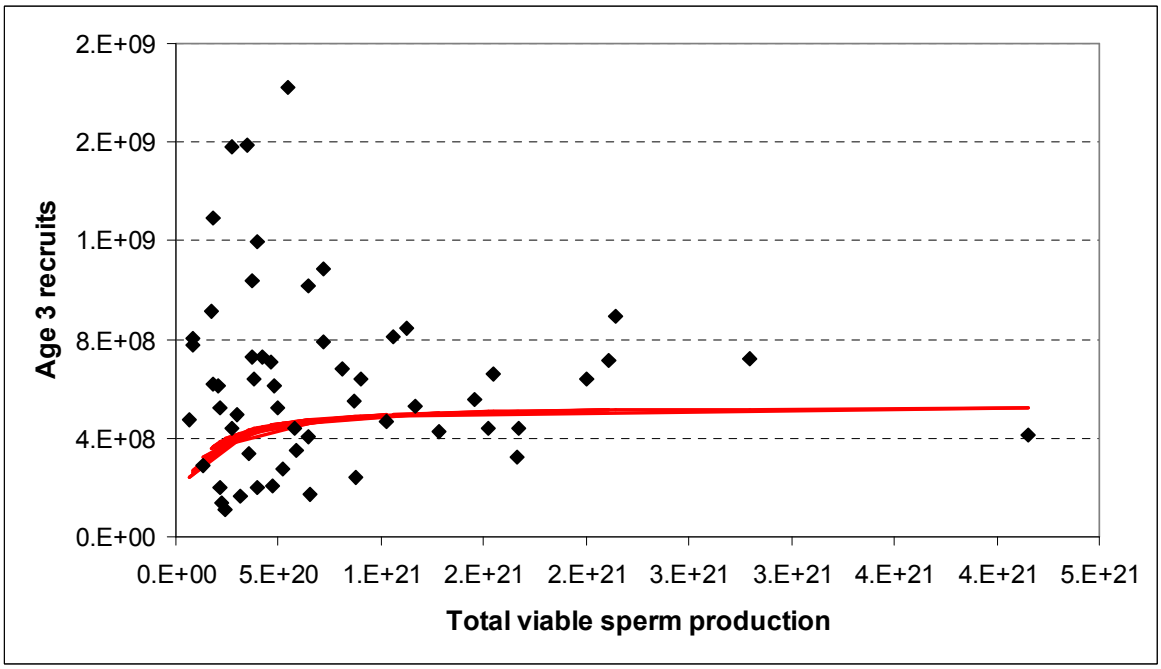


Figure 35: Relation between total viable sperm production and age three recruits for NEA cod with a fitted Beverton - Holt curve.

4. DISCUSSION

Sperm quality has been thoroughly studied in humans and it is well-known that it has a great influence on fertilisation success (Testart *et al.*, 1983; Bhattacharya *et al.*, 2001; Braude and Rowell, 2003), with only a fraction of the released spermatozoa having the ability to fertilise an egg (Chang and Pincus, 1951). In regard to fish and reproductive potential at both the individual and the population level the male contribution has been more or less ignored (Trippel, 2003). Fish with external fertilisation shed gametes in open water with males releasing an enormous amount of sperm cells compared to the release of eggs from females (Ball and Parker, 1996). One obvious question is why focus on the males when there is plenty of sperm to fertilise all eggs? Unlike mammal sperm, which are motile before ejaculation and remain motile for hours (Kime *et al.*, 2001), fish sperm activates and become motile in contact with water (Gilkey, 1981; Kime and Nash, 1999; Rurangwa *et al.*, 2004) and usually the motility only last for a couple of minutes (Kime *et al.*, 2001). However, cod sperm can achieve fertilisation of eggs after 60 minutes exposure in seawater (Trippel and Morgan, 1994b). Regardless of the long lasting motility of cod eggs the sperm cells have to be released relatively close to the eggs to ensure fertilisation before water movement disperses the gametes (Pennington, 1985; Howell *et al.*, 1991; Marconato *et al.*, 1997). For these reasons, there has been a slight shift of focus in the recent years towards the study of males and sperm production when trying to find a better explanation of the stock-recruitment relationships (Trippel, 2003).

To address the imbalance in the available information on female versus male contribution to the reproductive potential of a stock the present study examined a variety of traits in males and their sperm production with the intent of being able to estimate the total annual viable sperm production of the population. In addition, the population sperm production was explored as a potential contribution of explaining variability in recruitment. Data of male NEA cod was collected at the spawning grounds with the aim of increasing the knowledge of the male contribution to the stock dynamics. Data of fish weight, fish length, gonad weight, drumming muscle mass and milt (density and motility) were collected and processed to investigate possible relationships between male traits and sperm quality. Such relationships could influence the stock-recruitment relationships. Whilst Nash *et al.* (*in press*) raised the question of male contribution to stock reproductive potential in NEA cod there have not been

any specific studies to evaluate male traits of NEA cod and their potential contribution to recruitment. Trippel (2003) originally presented viable sperm production (VSP) as an alternative for egg production, with data derived from different cod stocks. In this study data specific to NEA cod have been obtained, thus parameterising Trippel's equation with relationships specific to this stock. Furthermore, different relationships were constructed where a more logical methodology was available. In Trippel's original formulation he was forced to use available data and relationships thus the formulation of the equation for estimating VSP was most probably not the best solution. In this study data were compiled to estimate the annual viable sperm production for NEA cod in the time period the stock has been assessed (1946 to 2005).

4.1 CRITIQUE OF METHODS

In Trippel (2003) the volume of milt is estimated from the weight of milt by multiplying with a factor of 1.04, considering milt is heavier than seawater (based on unpublished data by Trippel). To test if milt was actually heavier than seawater, milt from captive Atlantic cod in 2008 was weighed utilising two sample volumes (1 mL and 100 µl). The milt weighed both less and more than seawater depending on the volume utilised. The weight of the milt was significantly larger when weighing one millilitre of milt instead of 100 µl. This might be due to the scales that were used. The Sartorius scale is more sensitive than the scale from KERN and might measure the weight more correctly. However, the volume unit of 100 µl weighed on the Sartorius might be too small and artefacts such as air bubbles might have a large impact when utilising such small quantities. Also milt from NEA cod at the spawning grounds in 2008 was weighed to test if it was heavier than seawater. However, measurements taken onboard a vessel at sea, even using a motion compensating balance, were not as accurate as measurements undertaken on land and this is why all the milt samples from 2008 weighed exactly one gram per millilitre milt. The measurements of milt weight undertaken in this study were considered not to improve the estimate of Trippel (2003) and so a factor of 1.04 was used when estimating the VSP with Trippel's equation.

The equation for estimating GSI based on fish length was only based on 72 male cod that were collected at the spawning grounds in 2007. These cod may not be representative for the stock. It could be discussed whether the historical data of NEA cod should be used for estimating this equation or not. Even so, the historical database of the IMR does not include

data of male traits such as gonad weight or sperm characteristics, hence cod from the spawning grounds in 2007 were used since data of male traits were obtained from these fish. It was expected that larger males would invest significantly more in gonads than the smaller males. However, the equation revealed that the relative weight of gonads is approximately the same for a large and a small male.

Trippel (2003) assumes that three percent of the cod testes are connective tissue, primary spermatogonia and residual sperm based on findings in Trippel and Morgan (1994a). The percentage of testis that was assumed to be connective tissue and residual sperm in this study was set to 3.6 % based on the given histology data. The histology data were of coastal cod testis and might not be representative for NEA cod. This will then affect the estimated annual sperm production for the stock of NEA cod. New data of histology in testes of NEA cod are suggested for further investigations.

All the milt samples in this study that were taken for density estimations were fixed in 3.6 % buffered formaldehyde or teleost fixative (glutaraldehyde-formaldehyde fixation solution, see Grotmol *et al*., 2003) immediately after collection and counted within five months of sampling in 2007 and within one month of sampling in 2008. There were no possibilities of undertaking cell counts at sea, therefore preservation of sperm for later processing and estimation of sperm density was necessary. The milt samples from 2007 tended to dissolve in the formalin after approximately six months of storage, therefore the teleost fixative (Grotmol *et al.*, 2003) was chosen as a fixative for the milt samples in 2008 to ensure optimal preservation of the sperm cells. Due to time limitations sperm densities were estimated from a smaller number of pictures of milt from the cod at the spawning grounds in 2008 than from cod at the spawning grounds in 2007. The counts in 2008 were only based on two pictures taken from the same diluted sample of milt, whereas the density estimations from 2007 were based on five or six pictures from two dilutions of each sample. The estimations of spermatozoa density in 2008 should therefore be treated with some caution.

The scintillation tubes that were used for storage of fixed milt during the Lofoten survey 2007 were supposed to be filled with exactly 15 mL of buffered formaldehyde before the cruise, but unfortunately the amount of fixative was not accurate. This was not discovered until after the sampling, when 40 unused scintillation tubes had an unequal volume of buffered

formaldehyde. How this could occur is unclear. One reason may have been because 3.5 mL disposable plastic pipettes were used to transfer formaldehyde to the scintillation tubes instead of the automatic micropipettes that were used in 2008. Another explanation might be that the lids were not tight enough, allowing formaldehyde to evaporate, but this is not likely since the screw caps on the scintillations tubes are of good quality. The milt samples from 2007 were also collected with 3.5 mL disposable plastic pipettes before they were fixed in formaldehyde, and these pipettes may not be as accurate as the one millilitre syringes that were used during the Survey cruise in 2008. In addition, air bubbles were carefully removed to get an exact volume in 2008, but this was not done in 2007. These errors could greatly affect the sperm density and might be the reason why the mean spermatozoa density in 2008 was significantly higher than the mean density in 2007.

The estimated mean spermatozoa volume was based on the microscope pictures of milt from cod at spawning grounds in 2007. The mean cell volume estimated from the light microscope (LM) pictures was almost twice as large as the mean volume estimated from the field emission scanning electron microscope (SEM) pictures, even though the pictures were taken of the same milt sample. The reason for this dissimilarity in volume is most probably because the spermatozoa cells in the SEM pictures were dried in ethanol as a part of the sample preparation procedure, which may result in dehydration of the cells and cause shrinkage. The SEM pictures are best suited for estimating the diameter of a cell due to the great magnification and high resolution of the pictures, but the cell size was surprisingly small. For Atlantic cod mean spermatozoa diameter is found to range between 2.70 ± 0.29 and 3.70 ± 0.42 μm, with observed no seasonal change in size (Rakitin *et al.*, 1999b), so these values were more similar to the width and length of the cells estimated from the LM pictures (which ranged between 1.92 ± 0.14 and 3.46 ± 0.35 μm) than with the SEM pictures (which ranged between 1.17 ± 0.07 and 2.47 ± 0.24 μm). Even though the SEM pictures gives the best image of the spermatozoa the LM pictures were chosen for estimating the spermatozoon volume. The buffered formaldehyde probably caused some shrinkage to the cells in the LM pictures and this might be the reason why the mean size of the cells were slightly smaller than found in Rakitin *et al*. (1999b) The small cell size will also result in a larger amount of sperm when estimating the viable sperm production with the parameterised equation. However, even if the mean cell volume was twice as big, the total sperm production would still be higher when estimating with the new parameters than with Trippel's parameters. The recommendation is

that the diameter of NEA cod spermatozoa cells should be measured with a light microscope when samples are fresh to avoid any problems associated with fixatives, i.e. cell shrinkage.

4.2 MALE TRAITS AND SPERM QUALITY

Sperm motility is an important factor of sperm quality (Chauvaud *et al.*, 1995; Kime *et al.*, 1996; Trippel, 2003). Gage *et al*. (2004) found that male Atlantic salmon (*Salmo salar*) with rapid sperm motility obtain a higher fertilisation success. Rurangwa *et al*. (2001) found that there is a strong positive correlation between the hatching rate and sperm motility when fertilisation is obtained using a minimal sperm:egg ratio in African catfish (*Clarias gariepinus)*. Even though fish sperm cells generally are known to remain motile for a short period of time (Kime and Nash, 1999), especially among freshwater species (Christen *et al.*, 1987), Westin and Nissling (1991) found that a majority of sperm cells in Baltic cod (*Gadus morhua*) remains motile for approximately 16 minutes while Trippel and Morgan (1994b) found that approximately 50 % fertilisation rate can be achieved for Newfoundland cod (*Gadus morhua*) sperm after 60 minutes exposure in seawater. Sperm quality declines rapidly and experiments with sperm should begin a within few hours after stripping (Detweiler and Thomas, 1998; Trippel, 2003). The measurements of motility in this study occurred with some large time variations after collection (from one hour up to two days) due to occasional bad weather conditions, which made it difficult to estimate spermatozoa motility with a binocular microscope. Even so, milt samples stored on ice in a refrigerator (+ 3.5 ^{o}C) for more than three days still reached a motility of 60-80 % (personal observation). In addition, DeGraaf and Berlinsky (2004) found that cod sperm achieves > 80 % motility after two days of storage and 11 % motility after ten days of storage in a refrigerator (+ 3^{o}C).

Spermatozoa motility is significantly enhanced in the presence of ovarian fluid and eggs (Atlantic cod, Litvak and Trippel, 1998; Arctic charr, *Salvelinus alpinus*, Turner and Montgomerie, 2002). When cod spermatozoa cells are activated in the presence of eggs the spermatozoa cells will swim towards the eggs as long as the eggs are unfertilised (V. Makhotin, Institute of Ichthyology, Moscow State University, Russia: *pers. comm.*). No eggs were added to the motility measurements in this study, which may negatively affect the motility. However, the seawater that was used for activation of the sperm cells was taken from the spawning grounds, hence there may have been a presence of ovarian fluid which again may have a positive effect on the motility. In addition, seawater from the spawning

grounds was used to obtain the proper salinity when activating the sperm cells, since spermatozoa motility is proven to be greatly affected by salinity in Atlantic cod (Litvak and Trippel, 1998).

In a study by Rakitin *et al.* (1999b) spermatocrit correlates with sperm density in Atlantic cod when sperm density is estimated with a Coulter counter, but no correlation is found when density is estimated using a haemocytometer. One of the reasons why Rakitin *et al*. (1999b) rejects the sperm counts with the haemocytometer is due to the high coefficient of variation (27.7 %) among replicated diluted sperm counts in a haemocytometer. The density of the milt samples in this study was estimated based on replicated diluted sperm counts in a haemocytometer and gave an average coefficient of 15.3 % of milt samples from cod at the spawning grounds in 2007 and 3.4 % in milt samples from cod at the spawning grounds in 2008, which indicated that using a haemocytometer and automatic counting of sperm cells with Image J may be considered a reliable method for estimating sperm density. Estimating spermatozoa density with a haemocytometer is time consuming and therefore the Coulter counter would have been a good alternative in reducing sample processing time in this study. However, the Coulter counter that was available had a 100 µm nozzle and a noise threshold of 4.0 µm which required a minimum cell volume of 30 μm^3. The spermatozoa were too small (6.75 μm^3) to be measured with the Coulter counter. The estimated spermatozoa density in Atlantic cod reported by Rakitin *et al.* (1999b) ranged between 3.92 and 11.76 x 10^9 cells per mL when using a haemocytometer and between 7.33 and 20.25 x 10^9 cells per mL when using a Coulter counter. The estimated spermatozoa density in this study based on counts in a haemocytometer ranged between 1.68 and 115.44 x 10^8 cells per mL in 2007 and between 3.85 and 13.93 x 10^9 cells per mL in 2008. The large variance in sperm density in this study could be due to differences in the spawning state of the males.

When comparing spermatozoa densities of milt samples collected at different dates in 2007, a significant difference was found in sperm density between the dates. This may be because the males are at the peak of their spawning in the beginning of the sampling period and get more or less spent in the end, eight days later. No significant difference was found between sperm density and day of collection in 2008, which was probably due to the relatively small sample sizes on the different collection dates. However, even if there was no significant difference in sperm densities on the different collecting dates in 2008, the cod at the spawning grounds

were very ripe in the beginning of the cruise and getting into a post-spawning state at the end of the cruise. This could make a considerable impact when correlating spermatozoa densities to the variable traits of the male, without taking account for the different dates of collection. The variability in the timing of the samples in relation to the progression of the spawning period may influence the perception of the dynamics of the sperm densities, hence giving a misleading result of i.e. a large post-spawning male with a low spermatozoa density or a small ripe male with a high spermatozoa density.

A variety of studies in different fish species have shown a positive significant relationship between spermatocrit and spermatozoa density (Atlantic halibut, *Hippoglossus hippoglossus*, Tvedt *et al*., 2001; haddock, *Melanogrammus aeglefinus*, Rideout *et al*., 2004b; Atlantic cod, Rakitin *et al*., 1999b). Some base their studies on the assumption that there exists a relationship between spermatozoa density and spermatocrit in cod, hence they only measure spermatocrit level (Engen and Folstad, 1999; Rudolfsen *et al.*, 2005). However, no correlation between spermatozoa density and spermatocrit in captive NEA cod was found in the preliminary investigations. This result is not intuitively correct since spermatocrit is the amount of spermatozoa in milt and hence the level should increase when density increases. There are some important points that should be highlighted when evaluating the results of the spermatocrit-sperm density relationship in captive cod; 1) the sample size should have been larger, 2) the cod that were used were involved in a 'skipped spawning' project at the IMR and hence had been starved for several months, 3) the cod were kept captive for several months which might have affected their natural behaviour. However, earlier (unpublished) studies from 2005 and 2006 show no correlation between spermatocrit and sperm density in Atlantic cod (Audrey J. Geffen, University of Bergen: *pers. comm*.). Unfortunately, it was not possible to obtain spermatocrit data during the surveys on the spawning grounds to obtain information on wild cod.

Even though the captive NEA cod from the 'skipped spawning project' had been exposed to periods of poor feeding conditions they were not in particularly poor condition at the time of spawning (O.S. Kjesbu, IMR, Bergen, unpublished data: *pers. comm*.). However, the nutritional regime may have affected the mass of the drumming muscles as well as the milt characteristics. Engen and Folstad (1999) found that drumming muscle mass was sexually dimorphic in NEA cod with males having larger muscle mass than females. This was further

supported by Rowe and Hutchings (2004) who found that males have larger drumming muscle mass than females in Atlantic cod, and that the drumming muscles increase in mass prior to spawning with a decline thereafter. In the captive NEA cod in this study there was found a significant, but weak, tendency of males having larger drumming muscle mass than females when correlating with the wet weight of the muscles. On the contrary, males did not have larger drumming muscle mass than females when correlating with the dry weight. However, the p values for males having relatively larger drumming muscle mass than females were just above and below a value of 0.05 when correlating to wet and dry drumming muscle mass, respectively. The mean ratio of the drumming muscle weight to the fish weight was higher in male cod at the spawning grounds in 2008 than in the captive male cod from the preliminary investigations in 2007. Again, this may be a result of starvation of the cod from the 'skipped spawning' project at the IMR. The only correlation that was found between the variables of drumming muscle mass, sperm density and fish size in captive coastal cod in 2007 was wet and dry weight of drumming muscles. Moreover, five specimens are likely to be a sampling size too small for valid comparisons.

Engen and Folstad (1999) reports that males with larger drumming muscles have lower spermatocrit levels which they suggest can be explained by e.g. frequent ejaculate characteristics. In addition, Rowe and Hutchings (2008) found a significant relationship between drumming muscle mass and mating success in Atlantic cod. In this study there was no direct assessment of mating success so the relationship of mating success to drumming muscle size could not be assessed. The drumming muscle mass was not significantly correlated to sperm density or to spermatocrit level in captive NEA cod from the preliminary investigations of this study. Neither was drumming muscle mass and sperm density correlated in cod at spawning grounds in 2008. It was expected that larger males would invest more energy in drumming muscle mass, as described in Engen and Folstad (1999), and as expected larger fish at the spawning grounds had larger drumming muscles mass. Gonad weight was also significantly related to the weight of the drumming muscles. Drumming muscle mass correlated positively with sperm motility measured after ten minutes of activation. In addition, the motility measured after ten minutes of activation was correlated positively with GSI and gonad weight. This may indicate that large males, who have large gonads and large drumming muscles, have better sperm quality in form of longer lasting spermatozoa motility. Even so,

the plot of the variables does not indicate a clear relationship and is not substantial enough as a predictive tool.

Trippel (2003) suggests that gonad weight data of mature, but not running, gonads should be recorded each year to evaluate the annual amount of energy spent on reproduction. The data of the pre-spawning cod in this study were from fully mature, but not running, fish. It was expected that the GSI of pre-spawning cod at Andenes, north-western Norway, would be significantly larger than gonads in cod at the spawning grounds in 2007 and 2008, since the pre-spawning males had not spawned any milt. However, no significant differences were found. The distributions of fish weight, fish length, condition and GSI from both 2007 and 2008 seems to be fairly normal distributed when looking at the histograms, hence a representative assortment of NEA cod. Even so, none of the data were normal distributed when testing with Shapiro-Wilk test for normality. This may be due to the relatively small sampling sizes or that the stock may consist of a non normal distribution of males.

A number of the fish parameters in the correlation analysis were consistent between years, namely larger fish were heavier and condition increased with fish weight. A significant correlation was found between sperm density and GSI in 2007 which indicated that males with large GSI have more dense sperm. However, a negative, but weak, correlation between sperm density and fish length in cod at spawning grounds in 2007 indicated that larger fish have less dense sperm than smaller fish. Bekkevold *et al*. (2002) found that larger males sire larger batches of eggs than smaller male Atlantic cod. On the contrary, Rakitin *et al*. (2001) found that smaller male Atlantic cod have a higher reproductive success than males who are larger than females. In 2008, no correlation was found between sperm density and any of the traits. This may be due to the fact that density does not correlate to any traits or it can be due to errors in methods, for example that the sperm density was only measured based on two pictures from the same dilution of milt, as mentioned previously.

The mean size of male and female NEA cod has decreased in the recent years and while females used to dominate the large length and older age classes no females with a mean length greater than 13 % of the male mean length have been observed since 1982 (Nash *et al.*, *in press*). Brawn (1961b) pointed out that it is important to have a close alignment of the gonopores during the ventral mount to achieve fertilisation and based on this statement it is

reasonable to think that a shift towards similar size between the sexes will give an even closer alignment between the gonopores. A large male body size might be advantageous when competing for females (Maekawa *et al*., 1994; Warner *et al*., 1995; Forsgren *et al*., 1996), and so a decrease in mean size of NEA cod might induce a higher competition for females among a larger amount of similar sized and smaller males. However, if large male size indicates high sperm quality then the fertilisation potential of NEA cod might have decreased in the latter years due to the decrease in size.

When estimating the stock reproductive potential based on the biomass of the stock the sex ratio should be included in the calculations, however males are often assumed to represent approximately 50 % of the population (Trippel, 2003). There was a clear dominance of male NEA cod on all the selected stations in both 2007 and 2008. Morgan and Trippel (1996) found indications that the skewed sex ratio within spawning shoals of Atlantic cod remains skewed for most of the spawning season. The dominance of males at the selected stations of this study might be a consequence of the lek mating system in cod (as described in Höglund and Alatalo, 1995), with females leaving the spawning grounds immediately after mating while males continue their display towards other females. It is important to point out that these stations are only the stations of interest in this study, but more samples of NEA cod were obtained at other stations which form the larger database of the IMR, Bergen.

The results from the principal components analysis showed that the highest variance (approximately 50 %) in variables from cod at the spawning grounds from both years was related to fish size. Sperm traits dominate factor two and condition dominates factor three. Variances of variables that are positioned at almost the exact same place in the ordination, like fish weight and fish length, may indicate that one of these variables could be excluded from further analysis. This was also consistent for wet and dry weight of drumming muscles, which is why it is not necessary to use more than one of these variables for correlation analysis. Even if there was a diverse distribution of the importance of the variances from the different variables of the male cod, there was no clear division of groups when looking at the variances from both years in the scatter plot. Six males were separated from the general cluster, and these male differed from the others due to their small size and somewhat low spermatozoa density. The fact that there was no clear effect of the variances indicates that there was no major differences between males from one spawning season to the next, which

was an appreciated result for undertaking estimates using historical record of NEA cod (1946 to 2005) since it appears there was no obvious trends between the variables each year.

4.3 ANNUAL VIABLE SPERM PRODUCTION

Trippel's equation (2003) is explained in detail in this study to highlight what parameters to focus on when estimating annual viable sperm production. The equation of Trippel (2003) utilises population data of Newfoundland and Labrador cod and data based on experimental findings on captive cod. He mentions that the estimated viable sperm production is a preliminary investigation where several assumptions had to be made since there are gaps in the available data (Trippel, 2003). In this study, Trippel's equation was fitted to population data of NEA cod while the parameterised equation was fitted to the same population data together with the new data of male cod from the spawning grounds in 2007. The VSP from the parameterised equation in this study were almost tenfold higher than the VSP estimated from Trippel's equation but the relation between them was positively and significantly correlated. However, a slope of relationships between VSPs was 0.08 when adding a linear regression trend line to the plot, indicating that there was a scalar in this relationship which means that the VSP estimated with the parameterised equation was always higher than the VSP estimated with Trippel's equation. The linear plot of SSB and VSPs showed that the parameterised equation had a relatively larger output of VSP in the recent years (1992-2005), while it had a relatively smaller VSP in the latter years (1946-1990) when comparing to the VSP estimated with Trippel's equation. This might be caused by the fact that the sperm numbers from the parameterised equation were based on volume estimations from microscope pictures of spermatozoa cells instead of spermatocrit and sperm weight as in Trippel's equation (2003).

As shown in Marshall *et al*. (2004) the weight-length data of NEA cod in 1981-1984 are only based on Russian surveys, since there were no samplings from Norwegian vessels these years, hence the data are very limited and may be less reliable. The reason for estimating the VSP only to 2005 and not to the present (2008) is that the VSP is set up as a predictor of age three recruits which means that these fish are not estimated until they appear in the fishery, i.e. the 2006 year class appears in 2009, and the Virtual Population Analysis (VPA) is generally unreliable in the most recent years (Kell and Bromley, 2004).

A significant, positive linear relationship was found between SSB and VSP. There were some interannual differences between these two predictors of recruitment, however the significant relationship indicated that it would not make a large difference with using SSB compared to using VSP when explaining the age three recruitment. When comparing the plot of age three recruits, SSB and VSP, the recruitment seemed to follow the sperm production some years and the spawning stock biomass some other years, or both years together. However, neither SSB nor VSP predicted the recruitment very well. There was a peak in VSP between 1992 and 1995 that did not correlate to any of the other temporal trends, and the recruitment was also remarkably low in these years. The reason for this peak was due to the high fertilisation rate within these years, which again was due to high male condition.

A Beverton-Holt curve was fitted to the age three recruits and VSP data, which is a density-dependent curve that tends toward a finite recruitment at a given amount of viable sperm (or spawning stock biomass) (Beverton and Holt, 1957). This was not a particularly good fit but did indicate that the numbers of age three recruits increase rapidly with an increasing amount of viable sperm until it reaches equilibrium maximum. As mentioned in Trippel (2003) the SSB (and VSP) may not be suitable for estimating recruitment levels in the form of age three recruits, however it might be more suitable for describing the abundance of larvae. Chambers and Leggett (1992) found that maternal effects rather than paternal effects explains the variation in metamorphosis from the larval to the juvenile stage in winter flounder (*Pseudopleuronectes americanus)*, and based on this study combined with newer results, Chambers and Leggett (1996) concluded that early life size variations within marine fishes are due to maternal influences. However, other studies have concluded that males do also have an effect on early life traits in fishes, i.e. Evans and Geffen (1998) who found that larval length tends to increase with motility duration in Atlantic herring (*Clupea harrengus*) and Rideout *et al.* (2004a) who found paternal effects to have a significant influence on larval morphology, especially on larval length, in haddock. Several other studies have also found a significant paternal effect on early life traits (Atlantic herring, Bang *et al.*, 2006; Atlantic halibut, Ottesen and Babiak, 2007; Atlantic cod, Trippel and Neilson, 1992). However, all of these studies also include the importance of maternal effects on early life history traits. This indicates that the stock-recruitment relationship cannot be explained by the traits of only one of the sexes, and that the relationship is a complex composition of both sexes and their influence on early life traits.

4.4 CONCLUDING REMARKS

Three questions were raised in the introduction; 1) Is it representative to estimate the reproductive potential of a fish stock based only on the egg production? 2) Is there a relation between sperm quality and the condition of the fish? 3) Should sperm quality be included in assessments of a fish stocks reproductive potential? The answers to these questions are as followed:

The egg production *per se* is not a good descriptor of the reproductive potential of a fish stock. Annual viable sperm production can be used as a predictor of recruitment, but it does not describe the recruitment better than spawning stock biomass or even the egg production.

Condition factor did not correlate with sperm quality, but indications were found of larger males having higher sperm quality. Spermatozoa motility correlated positively to gonad weight, GSI and drumming muscle mass. In addition, the positive relationship between GSI and sperm density in cod at spawning grounds in 2007 indicated that males with a high GSI have high sperm quality. However, also a negative, but weak, correlation was found between sperm density and fish length.

Some indications of larger males having high sperm quality were found. Variation in sperm quality related to fish size could therefore be important for reproductive potential in exploited fish stocks where size selective harvesting can cause a lower mean fish size and potentially a lower sperm quality. More knowledge concerning paternal traits in wild and captive cod, including direct assessment of fertilisation success, should also be considered as a subject for further research. The stock-recruitment relationships are complex and a combination of both female and male traits might be the key to the door of recruitment mystery.

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6. APPENDICES

APPENDIX A: FIXATIVES

MILT FIXATIVES

Table A.1: Recipe for one litre of 3.6 % buffered formaldehyde (provided by IMR, Bergen, Norway)

Chemicals	Molecular weight	Final concentration (M)	Mol	Amount needed (g)
$NaH_2PO_4*H_2O$	137.99	0.02948	0.02948	4.068
$Na_2HPO_4*2H_2O$	177.99	0.04601	0.04601	8.189
Formaldehyde	Stock solution	Final concentration (M)		Amount needed (litres)
	37	3.6		0.097

Table A.2: Recepy of glutaraldehyde-formaldehyde fixation solution (teleost fixative), as given in Grotmol *et al.* (2003):

Chemicals	Amount needed
PBS (adjusted pH 7.35)	600 mL
0.2 M cacodylate buffer	200 mL
25 % glutaraldehyde	100 mL
10 % formaldehyde (from paraformaldehyde)	100 mL

STATION COORDINATES ON SPAWNING GROUNDS

Table B.1: Coordinates for sampling stations at the spawning grounds in 2007

Station	Latitude	Longitude
A	68.40	13.31
B	68.38	11.77
C	67.80	12.32
D	68.33	11.89
E	67.45	12.21
F	68.16	14.15

Table B.2: Coordinates for sampling stations at the spawning grounds in 2008

Station	Latitude	Longitude
A	69.93	16.78
B	69.23	14.82
C	68.97	13.97
D	68.79	13.01
E	68.26	12.62
F	68.34	11.56
G	67.82	12.62
H	67.60	12.03
I	67.35	11.54
J	67.45	11.63

CAPTIVE NORTHEAST ARCTIC COD 2007

Table C.1: Sperm density and spermatocrit level in eight captive male NEA cod

Fish number	Sperm density (cells/mL)	Spermatocrit level
19	4.2E+07	94.1 %
25	5.3E+07	83.4 %
18	1.3E+08	77.3 %
42	6.2E+07	89.4 %
29	4.6E+07	81.5 %
26	7.3E+07	86.7 %
23	1.9E+07	100.0 %
27	2.4E+07	81.8 %

Table C.2: Data of fish weight, fish length, drumming muscle dry and wet weight in female captive NEA cod in 2007, with fish number 1 to 17.

Fish number	Sex	Fish weight (g)	Fish length (cm)	Drum. wet weight (g)	Drum. dry weigth (g)
1	Female	4575	96	1.85	0.27
2	Female	7101	95	3.67	0.66
3	Female	7785	91	3.57	0.69
4	Female	6748	91	1.94	0.34
5	Female	7518	88	3.01	0.59
6	Female	5872	87	3.60	0.68
7	Female	3757	86	0.78	0.10
8	Female	5078	85	2.03	0.33
9	Female	5232	84	1.85	0.31
10	Female	4297	83	2.06	0.34
11	Female	3898	82	1.29	0.20
12	Female	4510	80	3.69	0.69
13	Female	2070	78	0.36	0.05
14	Female	3450	74	2.38	0.42
15	Female	3354	71	1.98	0.35
16	Female	1710	64	0.38	0.05
17	Female	1635	60	0.43	0.08

CAPTIVE NORTHEAST ARCTIC COD 2007

Table C.3: Data of fish weight, fish length, drumming muscle dry and wet weight in male captive NEA cod in 2007, with fish number 18 to 55.

Fish number	Sex	Fish weight (g)	Fish length (cm)	Drum. wet weight (g)	Drum. dry weigth (g)
18	Male	6390	98	2.32	0.38
19	Male	8251	93	5.35	1.00
20	Male	7555	90	2.58	0.46
21	Male	4727	88	1.09	0.17
22	Male	3687	86	1.66	0.25
23	Male	3925	86	1.83	0.28
24	Male	5489	84	3.93	0.74
25	Male	4077	83	0.75	0.11
26	Male	4023	82	2.69	0.47
27	Male	4632	82	1.95	0.35
28	Male	5105	81	1.52	0.28
29	Male	4890	81	3.43	0.66
30	Male	2742	80	1.31	0.16
31	Male	3357	80	1.29	0.20
32	Male	4257	78	4.18	0.74
33	Male	2416	78	0.93	0.13
34	Male	3989	78	2.70	0.43
35	Male	2760	77	1.16	0.17
36	Male	3451	77	2.39	0.39
37	Male	3529	76	1.45	0.25
38	Male	4096	76	1.29	0.20
39	Male	5084	75	5.06	1.00
40	Male	3020	75	3.16	0.57
41	Male	4175	74	1.27	0.22
42	Male	3032	72	1.70	0.30
43	Male	2257	72	0.60	0.09
44	Male	3030	71	5.87	1.14
45	Male	2150	69	1.16	0.17
46	Male	2490	68	3.73	0.68
47	Male	1930	67	0.69	0.11
48	Male	2065	66	0.83	0.13
49	Male	1780	66	1.04	0.15
50	Male	2075	62	1.59	0.26
51	Male	1975	61	1.13	0.19
52	Male	1720	59	1.35	0.23
53	Male	1810	59	2.34	0.41
54	Male	1505	58	0.97	0.15
55	Male	1530	58	0.54	0.08

CAPTIVE COASTAL COD 2008

Table C.4: Data of fish weight, fish length, gonad weight, drumming muscle wet and dry weight and spermatozoa density in five male captive coastal cod in 2008.

Fish number	Fish weight (g)	Fish length (cm)	Gonad weight (g)	Drum wet weight (g)	Drum dry weight (g)	Sperm density (cells/mL)
1	6570	77	796	6.59	1.37	9.9E+09
2	3640	63	489	3.83	0.73	8.5E+09
3	3402	56	427	4.64	0.85	1.4E+10
4	2988	65	327	3.70	0.70	3.6E+09
6	3686	63	418	2.28	0.44	1.7E+10

Table C.5: Milt weight in captive coastal cod estimated using different milt volumes, using 100 µl or 1 mL.

Milt volume	Weight (g/mL milt)
100 µl	0.989
100 µl	0.956
100 µl	0.963
100 µl	0.988
100 µl	0.879
100 µl	0.980
100 µl	0.964
100 µl	0.805
100 µl	0.761
1 mL	1.083
1 mL	1.060
1 mL	1.020
1 mL	1.133
1 mL	1.070

PRE-SPAWNING NEA COD IN 2007

Table C.6: Data of fish length, fish weight, gonad weight, GSI and condition in pre-spawning male NEA cod in 2007, fish number 1 to 30.

Fish number	Fish length (cm)	Fish weight (g)	Gonad weight (g)	GSI	K factor
1	69	2940	95	3.2 %	0.89
2	94	7080	813	11.5 %	0.85
3	76	4020	292	7.3 %	0.92
4	75	3660	293	8.0 %	0.87
5	97	6580	884	13.4 %	0.72
6	76	4120	516	12.5 %	0.94
7	77	4220	368	8.7 %	0.92
8	72	3260	246	7.5 %	0.87
9	65	2500	250	10.0 %	0.91
10	73	3140	417	13.3 %	0.81
11	80	4600	572	12.4 %	0.90
12	65	2140	109	5.1 %	0.78
13	81	4740	401	8.5 %	0.89
14	98	7400	772	10.4 %	0.79
15	72	3160	308	9.7 %	0.85
16	71	3220	420	13.0 %	0.90
17	79	3900	305	7.8 %	0.79
18	81	4540	401	8.8 %	0.85
19	79	4180	432	10.3 %	0.85
20	74	3380	242	7.2 %	0.83
21	72	3400	393	11.6 %	0.91
22	98	10020	822	8.2 %	1.06
23	100	10080	1236	12.3 %	1.01
24	84	4800	203	4.2 %	0.81
25	74	3800	478	12.6 %	0.94
26	101	9660	932	9.6 %	0.94
27	72	3380	263	7.8 %	0.91
28	92	7660	1176	15.3 %	0.98
29	101	8720	750	8.6 %	0.85
30	85	5220	360	6.9 %	0.85

PRE-SPAWNING NEA COD IN 2007

Table C.7: Data of fish length, fish weight, gonad weight, GSI and condition in pre-spawning male NEA cod in 2007, fish number 31 to 63.

Fish number	Fish length (cm)	Fish weight (g)	Gonad weight (g)	GSI	K factor
31	85	5240	807	15.4 %	0.85
32	95	7300	1086	14.9 %	0.85
33	85	6120	676	11.0 %	1.00
34	88	7740	870	11.2 %	1.14
35	79	4200	366	8.7 %	0.85
36	79	4440	563	12.7 %	0.90
37	80	4820	334	6.9 %	0.94
38	82	5160	387	7.5 %	0.94
39	72	3900	498	12.8 %	1.04
40	94	9120	1148	12.6 %	1.10
41	72	3500	470	13.4 %	0.94
42	82	5480	497	9.1 %	0.99
43	71	3460	476	13.8 %	0.97
44	75	3740	355	9.5 %	0.89
45	76	4440	505	11.4 %	1.01
46	65	2320	166	7.1 %	0.84
47	78	3880	341	8.8 %	0.82
48	62	2420	243	10.0 %	1.02
49	70	3020	288	9.5 %	0.88
50	96	9620	1496	15.6 %	1.09
51	84	5460	604	11.1 %	0.92
52	94	7520	897	11.9 %	0.91
53	90	8600	1189	13.8 %	1.18
54	90	5960	593	9.9 %	0.82
55	105	12080	1661	13.7 %	1.04
56	81	5400	433	8.0 %	1.02
57	77	4180	684	16.4 %	0.92
58	64	2420	222	9.2 %	0.92
59	83	5340	681	12.7 %	0.93
60	70	3140	321	10.2 %	0.92
61	67	2660	313	11.8 %	0.88
62	71	3040	327	10.7 %	0.85
63	67	2500	276	11.0 %	0.83

Table C.8: Data of spermatozoa density, fish length, fish weight, gonad weight, GSI and condition in male NEA cod at the spawning grounds in 2007, from fish number 1 to 30.

Fish number	Sperm density (cells/mL)	Fish length (cm)	Fish weight (g)	Gonad weight (g)	GSI	K factor
1	4.6E+09	65	2570	287	11.2 %	0.9
2	8.0E+09	78	4030	338	8.4 %	0.8
3	8.0E+09	68	2725	250	9.2 %	0.9
4	4.4E+09	74	3995	247	6.2 %	1.0
5	2.2E+09	74	4125	273	6.6 %	1.0
6	2.3E+09	71	3720	572	15.4 %	1.0
7	2.6E+09	81	5590	652	11.7 %	1.1
8	4.8E+09	83	5115	938	18.3 %	0.9
9	6.8E+09	72	3400	510	15.0 %	0.9
10	8.0E+09	101	9620	1239	12.9 %	0.9
11	2.0E+09	95	6705	797	11.9 %	0.8
12	4.5E+09	88	7270	1392	19.1 %	1.1
13	5.1E+09	103	9790	1046	10.7 %	0.9
14	4.0E+09	90	6580	467	7.1 %	0.9
15	8.1E+09	89	7090	870	12.3 %	1.0
16	3.6E+09	68	2585	298	11.5 %	0.8
17	6.3E+09	74	3650	383	10.5 %	0.9
18	3.3E+09	81	4980	571	11.5 %	0.9
19	9.1E+09	68	3075	344	11.2 %	1.0
20	9.2E+09	72	3075	376	12.2 %	0.8
21	6.1E+09	71	3115	241	7.7 %	0.9
22	6.7E+09	70	3505	395	11.3 %	1.0
23	4.5E+09	55	1493	190	12.7 %	0.9
24	1.2E+10	74	3430	304	8.9 %	0.8
25	6.5E+09	77	3965	366	9.2 %	0.9
26	5.5E+09	79	4300	416	9.7 %	0.9
27	2.0E+09	67	2324	106	4.6 %	0.8
28	2.7E+09	80	4530	396	8.7 %	0.9
29	6.6E+09	74	3535	243	6.9 %	0.9
30	1.8E+09	84	5110	621	12.2 %	0.9

Table C.9: Data of spermatozoa density, fish length, fish weight, gonad weight, GSI and condition in male NEA cod at the spawning grounds in 2007, from fish number 31 to 59.

Fish number	Sperm density (cells/mL)	Fish length (cm)	Fish weight (g)	Gonad weight (g)	GSI	K factor
31	2.0E+09	63	1910	107	5.6 %	0.8
32	2.3E+09	113	11530	847	7.3 %	0.8
33	3.1E+09	82	4875	383	7.9 %	0.9
34	5.9E+08	79	4210	424	10.1 %	0.9
35	8.1E+08	86	5290	730	13.8 %	0.8
36	2.6E+09	75	4135	323	7.8 %	1.0
37	6.4E+09	93	7315	711	9.7 %	0.9
38	5.6E+08	112	11550	548	4.7 %	0.8
39	5.9E+09	92	6540	392	6.0 %	0.8
40	1.1E+10	80	4665	555	11.9 %	0.9
41	3.0E+09	108	13150	2985	22.7 %	1.0
42	8.4E+09	94	6300	329	5.2 %	0.8
43	7.3E+09	75	4345	324	7.5 %	1.0
44	1.7E+09	77	3685	343	9.3 %	0.8
45	4.4E+09	76	3645	261	7.2 %	0.8
46	2.0E+08	77	4355	210	4.8 %	1.0
47	2.9E+09	76	3740	296	7.9 %	0.9
48	5.0E+09	109	13490	1422	10.5 %	1.0
49	1.6E+09	113	15110	585	3.9 %	1.0
50	4.6E+08	100	7890	573	7.3 %	0.8
51	1.7E+08	107	12860	898	7.0 %	1.0
52	3.9E+09	91	6990	822	11.8 %	0.9
53	3.8E+09	64	2156	286	13.3 %	0.8
54	3.3E+08	104	9370	546	5.8 %	0.8
55	4.7E+09	118	17510	2003	11.4 %	1.1
56	5.3E+09	113	12760	1819	14.3 %	0.9
57	2.4E+09	86	5910	424	7.2 %	0.9
58	3.6E+09	71	3575	495	13.8 %	1.0
59	2.5E+09	81	5140	193	3.8 %	1.0

Table C.10: Coefficient of variation between spermatozoa counts using the software program Image J (1.37v) versus manually counts by hand.

Fish number	Coefficient of variation
1	0.4 %
2	3.0 %
3	2.0 %
4	2.6 %
5	3.6 %
6	5.0 %
7	0.7 %
8	2.8 %
9	3.3 %
10	2.1 %
11	2.8 %
12	8.8 %
13	1.7 %
14	5.8 %
15	5.3 %
16	4.0 %
17	10.8 %
18	0.0 %
19	0.9 %
20	0.6 %
21	0.3 %
22	6.5 %
23	0.9 %
24	5.5 %
25	11.1 %
26	1.0 %
27	1.8 %
28	0.2 %
29	3.9 %
30	4.4 %

Table C.11: Width and length of spermatozoa cells estimated in Image J (1.37 v) with pictures taken in a light microscope (n = 36)

Picture	Cell length/Major (um)	Cell width/Minor (um)	Cell volume (um^3)
LM 1	3.73	2.04	8.13
LM 2	3.27	1.81	5.61
LM 3	2.84	1.85	5.09
LM 4	3.37	2.01	7.13
LM 5	3.40	1.77	5.58
LM 6	3.41	2.01	7.21
LM 7	3.10	1.85	5.55
LM 8	3.15	2.06	7.00
LM 9	2.96	1.89	5.53
LM 10	3.59	1.79	6.02
LM 11	3.10	1.97	6.30
LM 12	3.90	1.77	6.40
LM 13	3.70	2.06	8.22
LM 14	3.08	1.78	5.11
LM 15	3.76	2.23	9.79
LM 16	3.95	2.08	8.95
LM 17	3.11	1.83	5.45
LM 18	3.52	2.08	7.97
LM 19	3.33	1.99	6.90
LM 20	3.09	1.86	5.60
LM 21	3.52	1.86	6.37
LM 22	3.24	1.97	6.58
LM 23	2.55	1.84	4.52
LM 24	3.60	1.80	6.11
LM 25	3.62	2.14	8.68
LM 26	3.37	1.74	5.34
LM 27	3.96	1.91	7.56
LM 28	3.91	2.01	8.27
LM 29	3.54	2.01	7.49
LM 30	3.73	1.73	5.84
LM 31	3.99	1.68	5.89
LM 32	3.26	2.15	7.89
LM 33	3.88	2.08	8.79
LM 34	3.74	1.83	6.56
LM 35	3.80	2.01	8.04
LM 36	3.39	1.79	5.69
Mean	3.46	1.92	6.75
Standard deviation	0.35	0.14	1.30

Table C.12: Width and length of spermatozoa cells estimated in Image J (1.37 v) with pictures taken in a field emission scanning electron microscope (n = 11)

Picture	Cell length/Major (um)	Cell width/Minor (um)	Cell volume (um^3)
SEM 1	2.23	1.23	1.77
SEM 2	2.07	1.24	1.67
SEM 3	2.33	1.21	1.79
SEM 4	2.52	1.12	1.65
SEM 5	2.61	1.12	1.71
SEM 6	2.13	1.18	1.55
SEM 7	2.74	1.02	1.49
SEM 8	2.77	1.13	1.85
SEM 9	2.65	1.2	1.10
SEM 10	2.54	1.29	2.21
SEM 11	2.61	1.14	1.78
Mean	**2.47**	**1.17**	**1.77**
Standard deviation	0.24	0.07	0.20

Table C.13: Testis volume estimated from pieces of testis (3 x 3 cm) from all the male cod collected at the spawning grounds in 2007 (n = 72).

Sample number	Volume/gram (mL/g)	Sample number	Volume/gram (mL/g)
1	1.053	37	1.000
2	1.033	38	1.029
3	0.818	39	0.997
4	0.959	40	0.983
5	0.955	41	1.008
6	1.024	42	0.978
7	1.019	43	0.849
8	1.004	44	1.073
9	0.994	45	1.042
10	1.003	46	1.028
11	0.905	47	0.955
12	0.941	48	0.996
13	0.996	49	1.034
14	1.020	50	0.982
15	0.958	51	1.014
16	0.992	52	0.956
17	1.000	53	0.998
18	0.930	54	1.048
19	0.988	55	0.992
20	1.000	56	1.062
21	0.964	57	1.070
22	0.926	58	1.071
23	0.947	59	0.952
24	1.064	60	1.068
25	0.952	61	1.027
26	0.980	62	1.038
27	1.030	63	0.993
28	0.971	64	1.275
29	0.998	65	0.949
30	0.942	66	0.983
31	0.949	67	1.042
32	0.997	68	1.008
33	0.969	69	1.046
34	0.940	70	1.037
35	0.982	71	0.973
36	0.951	72	0.989

COASTAL COD HISTOLOGY DATA

Table C.14: Histology data of coastal cod (*Gadus morhua*) provided by Dr. Øystein Sæle (Nifes). Different stages of sperm cells in the testes are represented as mean percentages under "sperm counts". The parts that were considered to be connective tissue, and hence removed when estimating the total amount of sperm cells in the testes of Northeast Arctic cod (*Gadus morhua*), are mentioned under the "non sperm counts" as a mean percentage of the total content in testes. Cell stadiums are illustrated a figure of the spermatogenesis (Fig. C.1).

Sperm counts	Percentage content	Non sperm counts	Percentage content
A: Spermatozoa	50.8 %	Blood vessle	0.1 %
B: Elongated spermatids	11.7 %	Lobula	2.7 %
C: Round spermatids	17.9 %	Open space	0.8 %
D: Spermatocytes	15.3 %		
E: Spermatogonia	≈ 0.0 %		
Total	*95.7 %*	*Total*	*3.6 %*

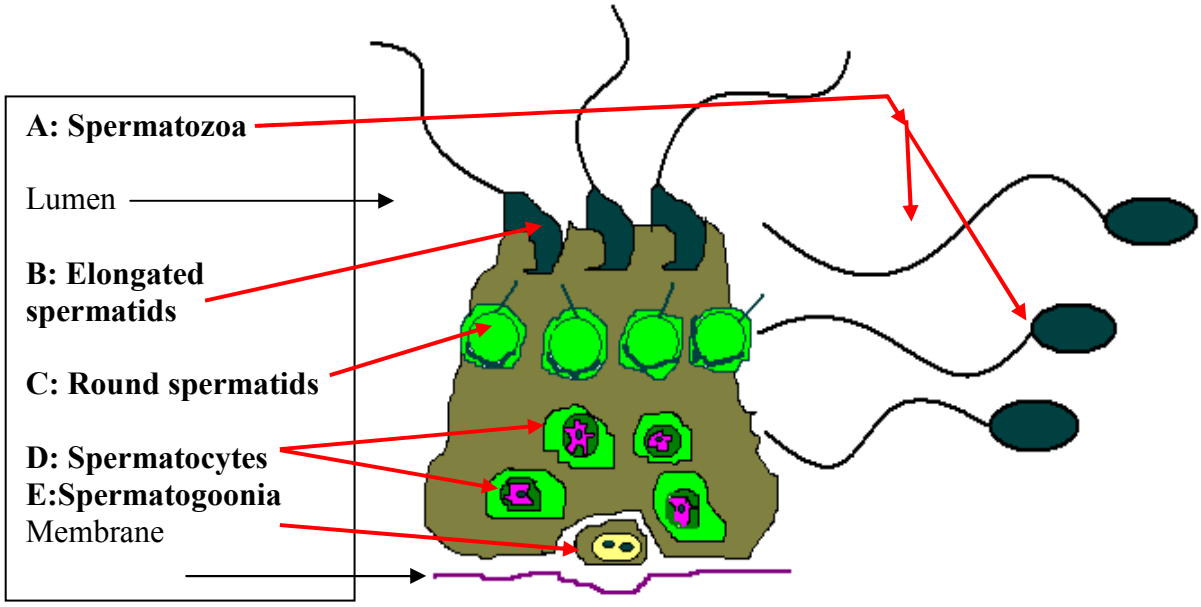

Figure C.1: Spermatogenesis.

Table C.15: Data sperm density, fish weight, fish length, gonad weight, GSI, condition, drumming muscle dry and wet weight in male NEA at spawning grounds in 2008, fish number 1 to 20.

Fish number	Sperm density (cells/mL)	Fish weight (g)	Fish length (cm)	Gonad weight (g)	GSI	K factor	Drum. muscle wet weight (g)	Drum. muscle dry weight (g)
1	8.4E+09	3914	71	372	9.5 %	1.09	1.80	0.30
2	5.9E+09	5404	84	454	8.4 %	0.91	2.20	0.41
3	7.1E+09	6739	88	578	8.6 %	0.99	2.90	0.46
4	1.0E+10	4699	76	458	9.7 %	1.07	4.70	0.97
5	1.1E+10	3554	72	302	8.5 %	0.95	3.60	0.74
6	9.0E+09	2630	69	260	9.9 %	0.80	1.20	0.24
7	9.1E+09	2477	68	250	10.1 %	0.79	0.90	0.16
8	7.5E+09	2378	65	179	7.5 %	0.87	1.10	0.21
9	8.7E+09	2204	64	249	11.3 %	0.84	1.15	0.23
10	1.0E+10	4030	75	551	13.7 %	0.96	2.90	0.59
11	7.3E+09	3200	77	279	8.7 %	0.70	0.95	0.14
12	6.2E+09	8255	95	711	8.6 %	0.96	4.25	0.91
13	9.5E+09	5995	88	589	9.8 %	0.88	3.50	0.63
14	1.4E+10	6420	90	688	10.7 %	0.88	5.30	1.11
15	1.0E+10	5550	84	420	7.6 %	0.94	4.10	0.79
16	1.1E+10	5460	85	482	8.8 %	0.89	6.80	1.48
17	5.8E+09	5035	84	510	10.1 %	0.85	2.75	0.49
18	7.6E+09	4050	80	395	9.8 %	0.79	2.00	0.36
19	9.2E+09	5360	82	643	12.0 %	0.97	2.60	0.46
20	6.5E+09	3485	70	475	13.6 %	1.02	2.20	0.37

Table C.16: Data of sperm density, fish weight, fish length, gonad weight, GSI, condition, drumming muscle dry and wet weight in male NEA at spawning grounds in 2008, fish number 21 to 42.

Fish number	Sperm density (cells/mL)	Fish weight (g)	Fish length (cm)	Gonad weight (g)	GSI	K factor	Drum. muscle wet weight (g)	Drum. muscle dry weight (g)
21	7.4E+09	4370	78	459	10.5 %	0.92	3.00	0.62
22	9.8E+09	4680	79	490	10.5 %	0.95	3.40	0.67
23	8.6E+09	7695	94	974	12.7 %	0.93	6.60	1.44
24	4.0E+09	6805	92	521	7.7 %	0.87	3.40	0.64
25	1.1E+10	8185	95	852	10.4 %	0.95	1.90	0.29
26	8.1E+09	12450	114	1233	9.9 %	0.84	6.10	1.88
27	7.6E+09	9030	102	957	10.6 %	0.85	7.80	1.60
28	1.4E+10	13400	108	1795	13.4 %	1.06	9.30	2.04
29	1.0E+10	5240	84	592	11.3 %	0.88	3.20	0.61
30	7.4E+09	8630	96	1882	21.8 %	0.98	7.60	1.62
31	6.8E+09	5215	83	443	8.5 %	0.91	13.00	4.16
32	5.7E+09	3950	79	232	5.9 %	0.80	2.40	0.40
33	1.2E+10	4680	83	400	8.5 %	0.82	1.60	0.27
34	7.6E+09	6430	87	652	10.1 %	0.98	4.30	0.81
35	8.9E+09	6560	92	907	13.8 %	0.84	2.20	0.36
36	3.8E+09	5170	81	642	12.4 %	0.97	2.65	0.48
37	6.2E+09	2510	68	121	4.8 %	0.80	1.80	0.32
38	1.3E+10	3585	69	360	10.0 %	1.09	2.00	0.36
39	7.4E+09	5240	86	646	12.3 %	0.82	2.00	0.36
40	9.0E+09	8220	88	1530	18.6 %	1.21	5.00	0.96
41	6.6E+09	9340	97	527	5.6 %	1.02	9.70	2.10
42	8.3E+09	6040	85	699	11.6 %	0.98	3.00	0.53

Table C.17: Motility measurements, given in percent, after initial time of activation (T = zero), ten minutes (T = ten) and 60 minutes (T = 60) of activation.

Sample number	Motility (%) at T = zero	Motility (%) at T = ten	Motility (%) at T = 60
1	95	0	0
2	0	0	0
3	80	0	0
4	80	0	0
5	60	0	0
6	80	0	0
7	100	0	0
8	95	0	5
9	50	0	5
10	50	40	0
11	80	10	10
12	60	10	5
13	90	5	0
15	50	0	0
16	70	20	0
17	60	5	0
18	80	0	0
19	65	0	0
20	90	0	0
21	60	0	1
22	95	60	0
23	90	0	1
24	100	20	0
25	40	0	0
26	70	0	0
27	100	0	0
28	100	10	0
29	90	40	0
30	95	70	0
31	80	40	0
33	70	75	0
35	95	0	0
37	100	5	0
38	95	30	0
39	80	45	0
41	50	65	0
42	80	5	0
43	65	15	0
45	100	0	0
46	95	10	0
47	85	5	0
48	95	40	0

SEX RATIOS AT PER STATION ON SPAWNING GROUNDS IN 2007

Table C.18: Sex ratios of NEA cod in selected stations at the spawning grounds 2007.

Station	Females %	Males %
70616	10.0	90.0
70619	31.4	68.6
70621	40.3	59.7
70625	34.5	65.5
70638	6.4	93.6
70641	9.1	90.9
Total	12.4	87.6

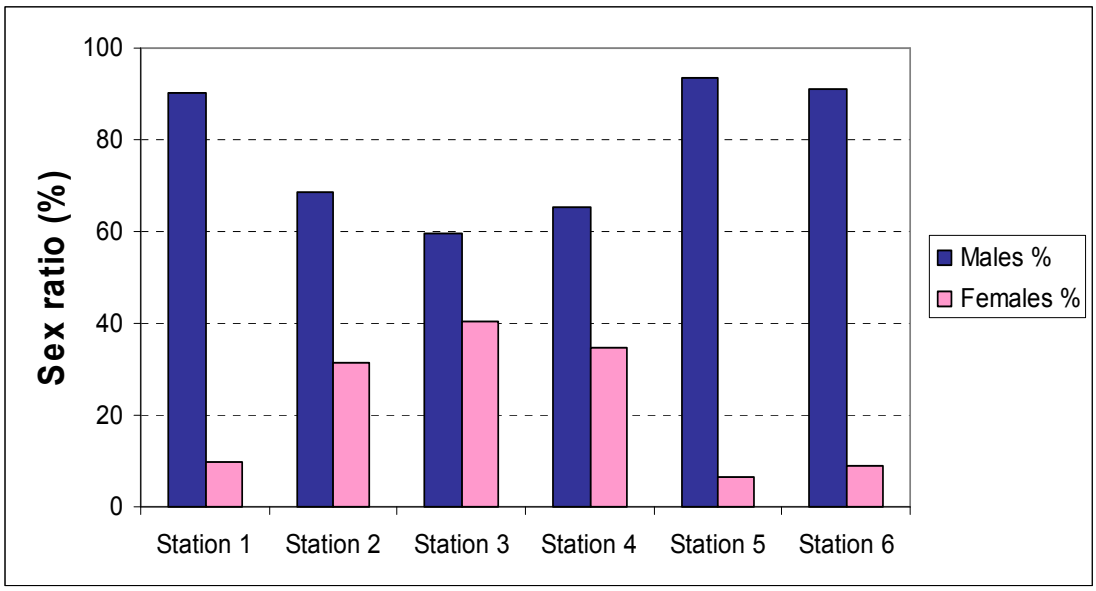

Figure C.2: Sex ratios of NEA cod in selected stations at the spawning grounds 2007.

SEX RATIOS AT PER STATION ON SPAWNING GROUNDS IN 2008

Table C.19: Sex ratios of NEA cod in selected stations at the spawning grounds 2008.

Station	Females %	Males %
71002	40.5	59.5
71003	0.0	100.0
71004	18.1	81.9
71005	38.3	61.7
71008	36.1	63.9
71011	47.9	52.1
71013	25.0	75.0
71018	25.9	74.1
71020	43.2	56.8
71021	37.8	62.2
Total	33.3	66.7

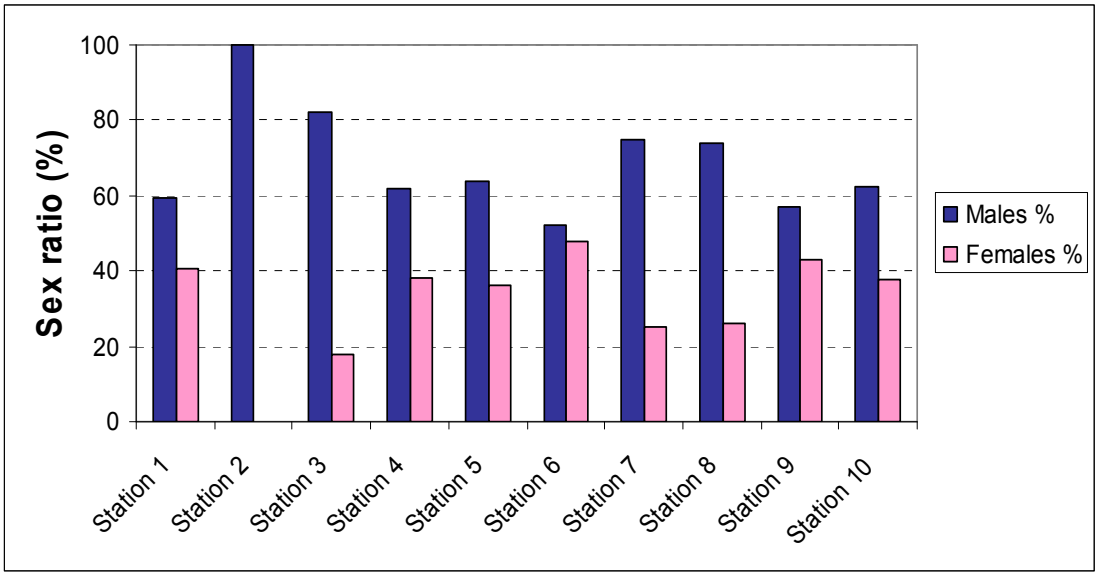

Figure C.3: Sex ratios of NEA cod in selected stations at the spawning grounds 2008.

ANNUAL VIABLE SPERM PRODUCTION

Table C.20: Annual viable sperm production of Northeast Arctic cod, estimated with the parameterised equation and Trippel's equation (1946-2005)

Year	Parameterised eq. (VSP)	Trippel's eq. (VSP)	Year	Parameterised eq. (VSP)	Trippel's eq. (VSP)
1946	4.2E+20	4.2E+19	1976	2.1E+20	1.9E+19
1947	1.3E+21	1.3E+20	1977	5.9E+20	4.9E+19
1948	5.8E+20	5.9E+19	1978	3.8E+20	3.4E+19
1949	1.0E+21	1.0E+20	1979	2.1E+20	1.9E+19
1950	4.6E+20	4.5E+19	1980	2.3E+20	2.1E+19
1951	7.2E+20	6.8E+19	1981	7.9E+20	6.8E+19
1952	4.0E+20	3.9E+19	1982	1.9E+21	1.6E+20
1953	3.4E+20	3.3E+19	1983	7.9E+17	4.6E+16
1954	9.0E+20	8.6E+19	1984	4.2E+19	3.8E+18
1955	5.2E+20	4.8E+19	1985	5.0E+20	4.0E+19
1956	2.7E+20	2.4E+19	1986	3.7E+20	2.9E+19
1957	8.6E+19	7.6E+18	1987	1.3E+20	1.0E+19
1958	3.0E+20	2.7E+19	1988	4.7E+20	3.7E+19
1959	8.1E+20	7.4E+19	1989	6.5E+20	5.3E+19
1960	7.2E+20	6.6E+19	1990	8.8E+20	7.3E+19
1961	1.7E+20	1.5E+19	1991	4.1E+21	3.6E+20
1962	3.7E+20	3.3E+19	1992	2.8E+21	2.4E+20
1963	6.9E+19	6.0E+18	1993	2.1E+21	1.8E+20
1964	3.6E+20	3.2E+19	1994	1.1E+21	8.6E+19
1965	8.0E+19	7.2E+18	1995	1.5E+21	1.2E+20
1966	2.7E+20	2.4E+19	1996	1.7E+21	1.3E+20
1967	1.8E+20	1.6E+19	1997	2.1E+21	1.8E+20
1968	3.1E+20	2.6E+19	1998	1.1E+21	9.7E+19
1969	2.4E+20	2.2E+19	1999	8.7E+20	7.6E+19
1970	4.0E+20	3.5E+19	2000	4.8E+20	4.0E+19
1971	6.5E+20	5.7E+19	2001	1.2E+21	9.3E+19
1972	6.5E+20	5.8E+19	2002	1.5E+21	1.2E+20
1973	5.4E+20	5.0E+19	2003	2.0E+21	1.6E+20
1974	2.2E+20	2.1E+19	2004	1.7E+21	1.4E+20
1975	1.8E+20	1.7E+19	2005	1.5E+21	1.2E+20