Characterization of the Atlantic cod (*Gadus morhua* L) eggs for estimation of spawning time and proportion of spawning females

By

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Thesis submitted in partial fulfilment of the requirements of the
MSc degree in

AQUACULTURE BIOLOGY

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Bergen, Norway
June 2011

Acknowledgements

Many people contributed towards the good and completion of this study, sincerely, I am highly indebted for the word of THANKS to them. First of all, I wish to express my sincere gratitude to my academic supervisors, Professor Audrey Geffen and Associate Professor Andreas Steigen of the Department of Biology, University of Bergen, Norway. Thank you very much; firstly, for accepting me as your student to supervise, secondly, for offering part of your time for our weekly meetings, the time that you could have used for many other responsibilities. Thirdly, for the instructions on how to go about, immediate responses of any query I handed to you and offer of reading materials whenever requested. Fourthly, for the patience and encouragements, among many others. I sincerely thank Prof. Jeppe Kolding of University of Bergen for offering freely his program, the PASGEAR II clx and as well for being available whenever requested for help on the use of the program. This program has been useful and important for the major part of this work.

I thank Anne-Christine Knag (PhD student at University of Bergen) for allowing me to access some of her data which has been useful for this study. Also, many thanks should go to the biology laboratory attendants for their willingness to help and cooperation during my laboratory work (though its analysis is not presented in this study).

I wish to express my sincere thanks to the Norwegian Quota Scheme/ Norwegian State Education Loan Fund (Lånekassen) for taking care of my general welfare during my studies. Everything went well and smoothly as arranged and really I felt safe all the time.

I also very much thank the student administration and the Department of Biology for being highly generous and kind. Frankly I admit, and in fact, you have been of great help. I myself, my family, my home institution (Tanzania Fisheries Research Institute) and generally all Tanzanians will not forget the kindness of yours. To honour you, I promise to put into practice which ever I have acquired during my stay here in Norway.

Special thanks are expressed to my employer TAFIRI, in particular the former Director General Dr. Yohana L. Budeba (a current deputy minister for ministry of

Livestock and Fisheries) for granting me the permission to pursue this study, and to the current Director General Dr. Benjamin Ngatunga of TAFIRI (the former Director of Research) for his best wishes in my study that “wherever I am, my success means the success of TAFIRI and our country”. Also, my special thanks to the TAFIRI-Mwanza Director, Egid F.B Katunzi for his encouragements, advises and his concern and help to my family during my absence.

I also thank my fellow class mates who have been with me for all this period of my two years of study; I value their cooperation and assistance, it has been good all the time sharing the experiences and strategies. Lastly, I thank everybody who in one way or another has contributed to the good and progress of this study, especially those who were involved in data collection and processing. It is difficult to remember and thank everyone here in, however, wherever you are, thank you very much.

In wholly, I thank God the Almighty who gave me strength and good people around through out my study period.

THANK YOU ALL

Sincerely,

Godfrey Wiliiam Ngupula

Bergen-Norway, 1st, June, 2011

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Abstract

A set of eggs from first time spawning Atlantic cod (*Gadus morhua* L) was investigated for the understanding of variations in egg sizes, e.g. with time in the spawning season, daily inter and intra variations, daily distributions and spawning time (early, mid, and late spawning season). Furthermore, the egg sizes were characterized for the estimation of spawning time and the proportion of spawning females in tanks. The egg sizes indicated a decreasing trend between batches spawned early, mid and late in the spawning season. Daily inter and intra variations indicated a decreasing trend towards late the spawning season. The daily egg size distributions indicated high variability during the early spawning season and this variability tended to decrease in batches spawned late in the spawning season. Early, mid, and late phases of the spawning season were categorised through time elapsed since the beginning of spawning and by total daily production of eggs. This study also reviewed whether the size of a fish egg is an indicator of quality and also whether cod production of eggs of high variability in size, amount and quality over the whole spawning season is a reproductive strategy. It has been observed that among many other determinants of egg quality, size is an important indicator as the size of an egg determines the amount of yolk and the size of larvae at hatching. Cod produces eggs of high variability in the season as a strategy of maximizing production efficiency and assuring survival of its offspring.

Key words: Egg quality, spawning season, variability, tanks, egg size, batches.

1. Introduction

1. 1 General overview

1.1.1 Global human population and the demand of protein from aquatic products

Currently, the global human population is approximately 6.9 billion people and is estimated to increase beyond 10.5 billion in 2050 (Naylor et al. 2000: Population reference Bureau 2008). Concurrent with this increase, is a predicted increase in the demand for protein from aquatic products, especially from finfish and shellfish (Naylor et al. 2000; FAO 2006). The increased demand coupled with the increased uncertainty of capture fisheries' future sustainability, means that aquaculture is increasingly being considered as the major alternative supply for the increasing global demands for aquatic products (Naylor et al. 2000; Bryceson 2002; FAO 2006). Globally, aquaculture is the fastest growing food producing sector; its production has doubled during the last 15 years (Naylor et al. 2000; FAO 2006).

1.1.2 Concerns underlying the aquaculture industry

However, despite the aquaculture industry's rapid expansion, there are concerns which underlie its production methods and sustainability, e.g. alternative sources of feeds and fats as substitutes for fish meal and fish oils, environmental pollution, ecological impacts (Naylor et al. 2000; Xuemei and Hawkins 2002; FAO 2006), fish diseases and treatments (Krkosek et al. 2006a), fish welfare issues (Huntingford et al. 2006; Johansen et al. 2006), and the production of quality fingerlings to farmers (van der Meeren and Ivannikov 2006; Paulsen et al. 2009). In many countries production of quality fingerings needed by fish farmers is hatchery-based; starting production cycles with highest quality eggs expected to lead to high hatching success, increased larval survival, reduced costs of production, and increased efficacy (Kjørsvik et al. 2003; Hamoutene et al. 2009; Paulsen et al. 2009).

1.1.3 Descriptions of a good quality fish egg and factors influencing its quality

One of the major problems as far as fish eggs and quality of larvae are concerned has been describing what actually comprises a good quality egg and what factors influence its quality (Brooks et al. 1997; Marteinsdottir and Steinarsson 1998; Treasurer and Ford 2010). To most scientists a good quality egg has been described and classified by (a) fertilization and hatching rates, and egg cleavage pattern (Kjorsvik et al. 2003: Hamoutene et al. 2009;Treasurer and Ford 2010), (b) egg diameter or size (Ouellet et al. 2001;Treasurer and Ford 2010), (c) egg survival, morphological features of the larvae after hatching, and the appearance of the egg e.g. shape, transparency, distribution of oil globules, and zona pellucida (Brooks et al. 1997; Marteinsdottir and Steinarsson 1998), (d) egg ability to float or sink and egg wet and dry weights (Brooks et al. 1997; Vallin and Nissling 2000; Treasurer and Ford 2010).

1.1.3.1 Biological factors influencing the quality of fish eggs

Concerning the biological factors influencing the quality of fish eggs many contributions exist, the most important include (a) spawning season (Rideout et al. 2005; van der Meeren and Ivannikov 2006), (b) age of the female fish (Kjesbu et al. 1996; Berkeley et al. 2004; Carr and Kaufman 2009), (c) batch effect[1] (Kjesbu 1989; Chambers and Waiwood 1996), and (d) maternal (female) condition[2] (Ouellet et al. 2001; Hamoutene et al. 2009).

Despite a general consensus on the factors influencing the quality of fish eggs, there are still many uncertainties. Extensive research has led to detailed knowledge of various aspects of egg quality in fish; this includes the studies by Chambers and Waiwood (1996), Ouellet et al. (2001), Kennedy et al. (2007), and Hamoutene et al. (2009). Chambers and Waiwood (1996) investigated maternal condition and seasonal effects on eggs sizes of captive Atlantic cod, *Gadus morhua* L. They found that female condition and batch effect influenced egg diameters by 35% and 26%, respectively. While female size did not relate to egg sizes, the batch effect was largely due to female condition which varied during the spawning. One female Atlantic cod in the spawning season can produce up to a total of 19 batches varying in quality parameters (egg size, quality of larvae) with highest quality produced at the peak of the season and more variability in quality in the later batches (Kjesbu 1989). Ouellet et al. (2001) studied the maternal characteristics[3], egg quality (mean egg diameter, dry mass, and energy content), and viability in the Atlantic cod, and their results indicated a strong relationship between maternal characteristics and egg quality and viability. However, no batch effect was observed. Kennedy et al. (2007) studied the maternal

[1] Batch effect: Maternal influences on fish eggs (for batch spawners) due to differences in time on which eggs were spawned (GW Ngupula).
[2] Maternal (female condition): Refers to generally, healthy and reproductive energy status of the female fish
[3] Maternal characteristics (effects): Refers to factors like size, condition, and age of the female fish (Kennedy et al. 2007).

influences[4] on egg and larval characteristics of plaice (*Leuronectes platessa* L), and determined size of eggs related to maternal size and yolk sac volume, and decreased during the season. In Hamoutene et al.'s (2009) study on cod egg quality and its predictors, e.g. the effects of spawning time, maternal and paternal influences on egg diameters, cleavage pattern, fertilization and hatching rates, maternal influences dominated.

1.1.3.2 Environmental factors influencing the quality of fish eggs

Various environmental factors directly or indirectly influence egg quality in fish (Campana et al. 1995; Chabot and Dutil 1999; Hansen et al. 2001). The most important include (a) the diet of the brood fish (Ali and Wootton 1999; Rideout et al. 2005), (b) physico-chemical conditions of the water in which the fish are exposed e.g. temperature, light regimes, pH, salinity, oxygen, and generally the quality of the husbandly practices (Campana et al. 1995; Chabot and Dutil 1999; Hansen et al. 2001). Water temperature influences the metabolism of fish thus affecting food intake and it may also affect food availability because temperature is normally linked to biological productivity in waters (Chabot and Dutil 1999). Water temperature and levels of dissolved oxygen are among the most important factors which determine the metabolic rate in fish and ultimately its growth, behaviour, and activity levels (Campana et al. 1995; Chabot and Dutil 1999),(c) physico-chemical conditions (especially temperature) of the water in which the fish egg is incubated. The water temperature where fish eggs were incubated is known to influence the rate of the cleavage processes (Kjesbu 1989; Kimmel et al. 1995; Hall et al. 2004), and for cods kept in captivity, mean water temperature has been found to influence spawning frequency as well (Kjesbu 1989).

1.2 The objectives and hypotheses of the study

So far, studies on the quality of fish eggs and factors affecting it show that maternal influences dominate. This has the implication that fish eggs reflect much of the maternal characteristics and its life welfare as well. Therefore, developing methods to extract information from fish eggs for the purpose of understanding the maternal

[4] Maternal influences: Effects of female fish on its eggs (e.g. size, quality, etc) and larvae (e.g. .hatching successes, survival, etc) as well (GW Ngupula).

characteristics and its life conditions can be appreciated and of importance during field and experimental studies. The purpose of this study is to extract information from fish eggs to understand the maternal spawning activity. The study analyses and explores a set of Atlantic cod (*Gadus morhua*) egg data from the experiments conducted at the Institute of Marine Research in Bergen in 2004-2006 (Knag 2007).

1.2.1 The objectives of the study

The overall study objective was to:

Characterize cod eggs to estimate spawning time and the proportion of spawning females in the tanks.

The three specific objectives were:

(i) To understand patterns of egg size variation over time in the spawning season and daily patterns of intra and inter variation in egg sizes.

(ii) Characterization of the daily spawned cod eggs for sorting of egg groups on a daily basis in the production season and establishment of methods for the estimation of spawning time and proportion of spawning females in the experimental tanks.

(iii) Make a brief review on egg size and reproductive investment, focussing on the following points: (a) the use of egg size as an indicator of egg quality (b) the role of egg size in an individual's reproductive strategy (c) the relationship between duration of spawning (spawning time) and spawning intervals for first and second time spawners (d) the relationship between duration of spawning and spawning intervals for fish spawning singly and /or in groups, and (e) differences in egg sizes between forced (stripping) and naturally spawned eggs.

1.2.2 The hypotheses of the study

The study main hypotheses were:

(i) Cod egg sizes decrease with time in the spawning season and show batch effects on egg sizes, however, the intra and inter daily variation patterns over the production season contrast the maternal and batch effects patterns.

(ii) Groups of spawning fishes produce a mixture of eggs which generally reflect the maternal characteristics, therefore, on characterising the given egg mixtures, the resulting groups reflect the proportion of the spawning females.

2. Material and methods

2.1 Brief descriptions of the 2004 and 2006 experiments

In 2004, at the Institute of Marine Research (IMR) in Bergen an experiment was conducted to study the effects of oil exposure on cod reproduction. Cod fish (either from egg to late larval stages or from early larval stages to juvenile) were kept and exposed to different concentration levels of "produced water" (contaminated waste from oil drilling platforms) and then reared to maturity. In 2006, at IMR a second set of experiments was conducted as continuation of the 2004 experiments, when the previously exposed cod became sexually mature and began to spawn naturally. In the 2006 experiments these cod (of more or less the same size) were fed fish pellets in tanks until spawning for the first time and eggs were collected daily, photographed, and egg production was analysed (for detailed descriptions of procedures refer the study by Knag 2007). Based on information from the 2004 experiment, the set-up of nine tanks in the 2006 experiment were as given in Table 1 below. Each tank contained six females and six males. In both tanks, temperature range of 4.6 to 6.8 ^{o}C was monitored throughout the duration of the experiments.

Table 1: Set up of the 2006 experiments based on 2004 experimental set up

No.	Groups	Tank name
1	Control	1(control 1)/5(control 2)
2	Low oil	2/6
3	Medium oil	3/7
4	High oil	8
5	Endocrine disrupters (+ve control)	4/9

NB: The group 5: the endocrine disrupters or positive (+ve) control group comprised of fishes which were exposed to oestrogen.

Soon after onset of the spawning season (when fish in the tanks started spawning), the eggs from the five groups were collected daily and then photographed, and their sizes (measured as diameters) and developmental stages (staged according to Fridgeirsson 1978) recorded. The obtained data were registered (e.g. picture numbers, date spawned, diameter of the egg, egg developmental stage, and tank number) into excel spreadsheets. Those data set are what formed the basis of this study.

2.2 Extracting and reorganising the data

Therefore, being given the aggregated data files on cod eggs and backed up with this information, the first major work of this study was to reorganise and assemble the given data files and make separate files that contained date, egg size, and developmental stage, by tank and as well on a daily basis for the whole spawning season. Data sets from each tank contained daily spawned eggs labelled by date and month of the year.

2.3 Analysis of data

2.3.1 Variations in daily egg sizes

The second major work of this study constituted analysis of the data to meet the intended objectives. In examining how the daily egg mean diameters and as well daily egg sizes (or daily frequency distributions) varied with time over the spawning season, the PASGEAR II clx program (version 2.4) was used. The compiled size frequency histograms generated for the daily egg size shifts were used to further elaborate the shifts observed in the daily mean diameters plots. The egg data files from the individual experimental tanks were easily used for this purpose as they were stored in the format required by the program. It was indicated from the produced patterns of daily egg mean diameters as well as daily egg size variations over the spawning season; a strategy of producing eggs of various sizes in the spawning season could be deduced. The PASGEAR II clx program was also used for visualising intradaily variations whilst interdaily variations were calculated in excel program using egg mean diameters. Also, the program (PASGEAR II clx) was used for the general visualisation of the egg size distributions in the nine tanks.

2.3.2 Identifying daily egg groups

For obtaining daily egg groupings (given in mean egg diameters as modal sizes) the daily mixture of egg sizes was characterised and modes isolated to define different egg batches. For this purpose egg of tanks 1 and 5 (referred to as control tanks) were used. For characterising daily egg sizes mixture and as well isolation of the different egg groups, daily egg data were first analysed using Microsoft excel (procedures outlined in appendix I) to generate the bins[5], frequency distributions, and as well the histogram (e.g. Figure 1). The histogram was important for the preliminary visualization of the egg groups before applying a mixed distribution analysis using mixdist[6].

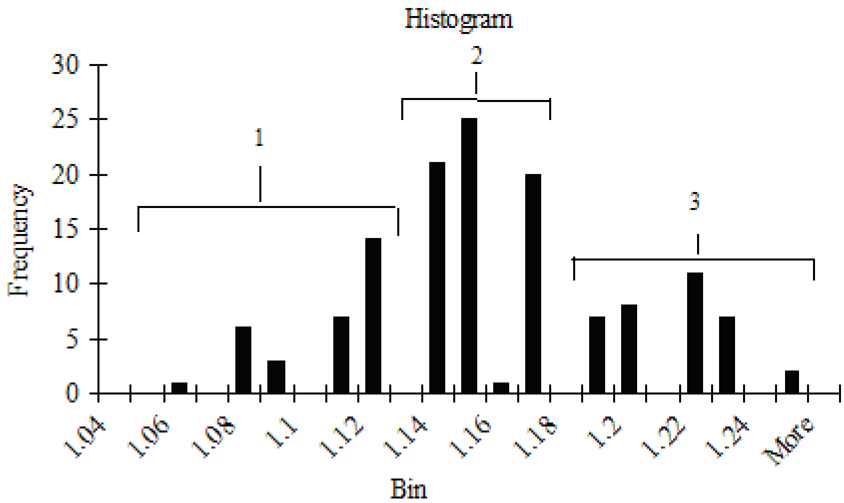

Figure 1: An illustration of the excel analysis outcome during daily analysis of Atlantic cod (*Gadus morhua*) eggs. Three modes of egg diameters are suggested, but further mixed distribution analysis is required to test for the real modes.

The egg class intervals and their frequencies were then taken into mixdist analysis using "R" (procedures outlined in appendix II). The final outcome from this program also was the outline of the groups (or modes) generated in mean diameters with their corresponding proportions and the figure (e.g. Figure 2) which outlined the general egg frequencies and as well the different egg groups (or modes).

[5] Bins: As used in this study refer to the egg size classes or size intervals for size frequency distributions, obtained using the excel program applied to egg diameters

[6] Mixdist analysis: Is among the packages in "R program" working specifically on mixtured (or grouped) and condition data on where it reveals the different groups and as well indicate their proportions.

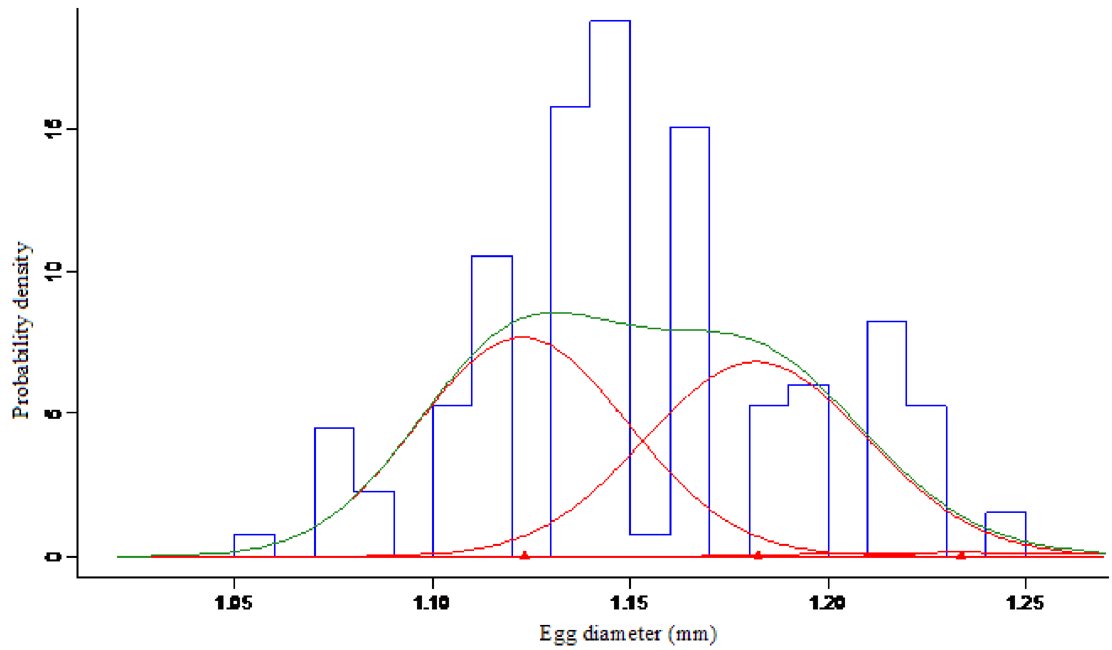

Figure 2: An illustration of the mixdist analysis outcome during daily analysis of Atlantic cod (*Gadus morhua*) eggs. Three groups of eggs (two major and one minor) are indicated (marked by small red triangles) as has been estimated in Figure 1.

2.3.3 Estimation of spawning time and proportion of spawning females in tanks

Two procedures were followed to estimate the proportion of spawning females in the respective tanks. The first procedure was to relate egg size distributions with the developmental stages (referred to as egg category) as well as the outcome of the mixdist characterization (procedure 2.3.2 above). In the given data files, the egg developmental stages ranged from stages 1 to 17, however, important to this study were a range of stages 1 to 10 (Table 2).

Table 2: Developmental stages of Atlantic cod (*Gadus morhua*) eggs as has been used in this study

Egg developmental stage	Description of the stage
1	Unactivated 1
2	Unactivated 2
3	1 cell
4	2 cells
5	4 cells
6	8 cells
7	16 cells
8	32 cells
9	64 cells
10	128 cells/morula stage

The second procedure was based on observation of the patterns of the mean egg diameters over the spawning season as has been isolated by mixdist analysis, and relating its estimation with overall egg size characterization of the respective tanks.

2.3.3.1. First procedure: The relation of the egg size distributions with egg developmental stages plus the egg size characterizations by mixdist

To estimate the proportion of spawning female fishes on each day in the selected tank, five days were randomly selected from the eggs of the control (1) group. For this purpose, in addition to examining the size structure of the eggs produced on each day as well their corresponding developmental stages, knowledge of the indoor temperature of which the eggs were incubated was important for calculating the time taken from fertilization to first cell cycle and the like. A fertilized cod egg incubated at 7.0 ± 0.2^{o}C takes about 5:15 hours (5 hours and 15 minutes) post fertilization to one cell cycle and about a mean cell cycle time of 2:15 hours (2 hours and 15 minutes) for each of the first six cell cycles which occur synchronously (Hall et al. 2004). This fact implies that it takes about 7:30 hours since fertilization for an egg at 1 cell stage to develop to 2 cells stage, and about 9:45 hours from 2 cells stage to 4 cells stage. Likewise, about 12:00 hours from 4 cells stage to 8 cells stage, about 14:15 hours from 8 cells to 16 cells stage, and about 16:30 hours from 16 to 32 cells stage. And, it took about 18:45 hours from 32 to 64 cells stage and about 21:00 hours from 64 cells stage to 128 cells stage (Hall et al. 2004) (Table 3).

Table 3: Different stages of Atlantic cod (*Gadus morhua*) egg development during cleavage period and the corresponding time taken since fertilization as has been described by Hall et al. 2004. NB: 5:15 = 5 hours and 15 minutes, 7:30 = 7 hours and 30 minutes….

Description of the stage	Time taken since fertilization to the stage (hours: minutes)
1 cell	5:15
2 cells	7:30
4 cells	9:45
8 cells	12:00
16 cells	14:15
32 cells	16:30
64 cells	18:45
128 cells/morula stage	21:00

NB: 5:15 = 5 hours and 15 minutes, 7:30= 7 hours and 30 minutes….

NOTE: Because age of the egg can be calculated and knowing the time of sampling it is possible to estimate the approximate time of spawning of each fish.

2.3.3.2 Second procedure: Patterns of egg mean diameters and the egg size characterization by mixdist

To observe patterns of mean egg diameters over the spawning season, daily eggs of the control (1 and 2) group of spawning fishes were characterized by mixdist analysis to isolate different egg groups (or modal sizes). The SPSS program was used for generating a plot to indicate patterns of generated egg groups. The mean egg diameters of the generated egg groups were tested for their correlation with time using linear regression analysis as has been given by PASGEAR II clx program. It was indicated by closely examining the patterns whilst linking with Atlantic cod spawning activity over the season that the proportion of the spawning fishes in the tanks could be estimated.

The information from the first and second procedures was then combined for the final estimation on the number of fish spawning in tanks. Furthermore, eggs of the control (1) and (2) groups were characterized on tank basis to generally indicate the number of egg groups of each tank. The generated egg groups (or modal sizes) were expected to indicate the number of spawning fishes in the respective tanks.

3. Results

3.1 Spawning season and the size of cod fish eggs

3.1.1 Spawning season

Fishes in the different experimental tanks started spawning on different dates, however the differences were small. In this study, spawning season is the term used to refer to the time elapsed in each experimental tank from the spawning of the first batch of eggs to the spawning of the last batch. Hence, the fish in each tank had their own spawning season. NOTE: If narrowed further, each fish of the same spawning tank had its own spawning season too. Fishes of the control (1) and (2) had a spawning season of 70 and 69 days, respectively. This study's division of the time periods into early, mid and late spawning season was based on dividing the spawning season of each group of spawning fishes into three equal parts, each of which was 23 days for the fishes of the control (1) and (2) groups (Figure 3).

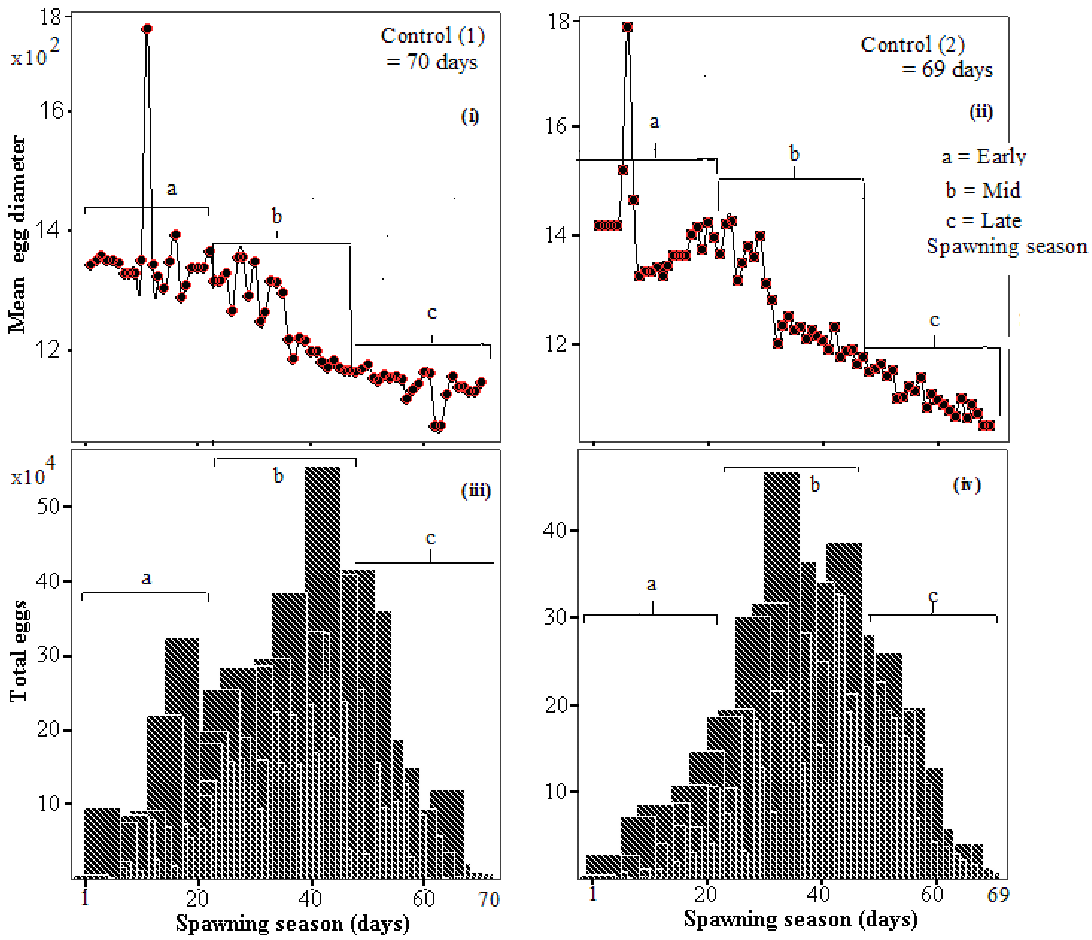

Figure 3: Early, mid and late spawning season of Atlantic cod eggs (*Gadus morhua*) as has been estimated basing on dividing the spawning season of each group of spawning fishes (control (1) & (2)) into three equal parts.

3.1.2 Changes in the size of cod eggs over the spawning season

3.1.2.1 Mean egg diameters

Changes in the size of the cod eggs, as observed for the control groups indicated a decreasing trend through the spawning season. The linear regression analysis to test the significance of the indicated correlation returned positive results for both groups (Figure 4). Batches of eggs spawned early in the season were significantly larger than those spawned in the mid and late season (One-way ANOVA followed by Tukey HSD contrast, $p < 0.01$). Also, the egg curves indicated a continuous rapid decline between eggs of the initial and final batches (Figure 3 (i) & (ii) above).

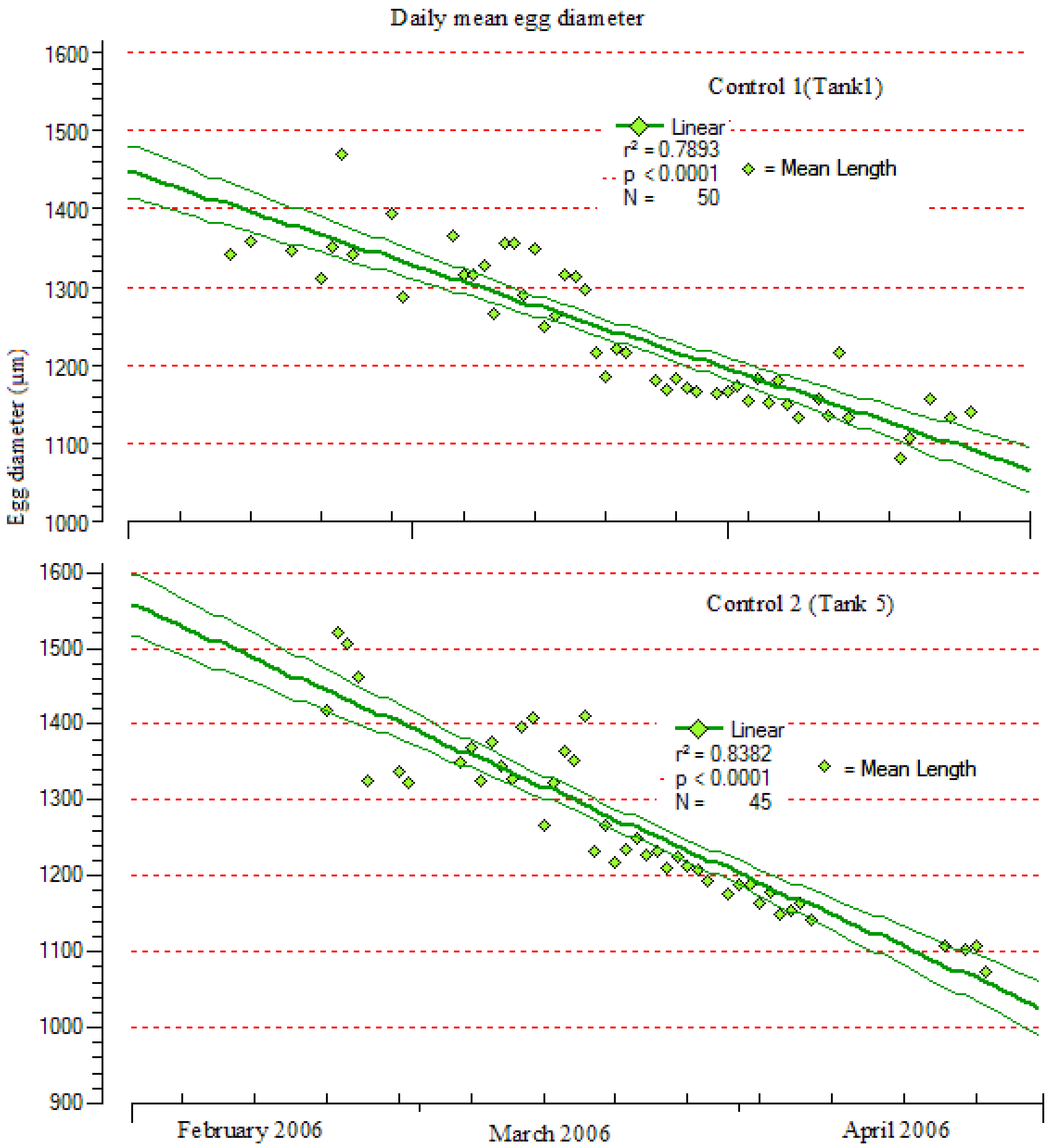

Figure 4: The correlation of daily mean egg diameter of Atlantic cod (*Gadus morhua*) with time (e.g. start of spawning until late in the season) of the control (1) and (2) groups of spawning fishes. Each spawning group of fishes had six spawning females. Correlations were calculated using the PASGEAR II clx statistical package.

3.1.2.2 Daily egg sizes distributions

Whilst the daily mean egg diameters decreased over time in the spawning season (as observed in Figure 4 above), the daily egg size distributions varied among spawning days with a decrease in variation over time in the season. Also, the pattern of shift on daily basis (the zigzag) indicated no trend, for example, it was impossible to tell whether the mean size of eggs of any succeeding day will be greater or smaller than those of the previous day (Figures 5 and 6).

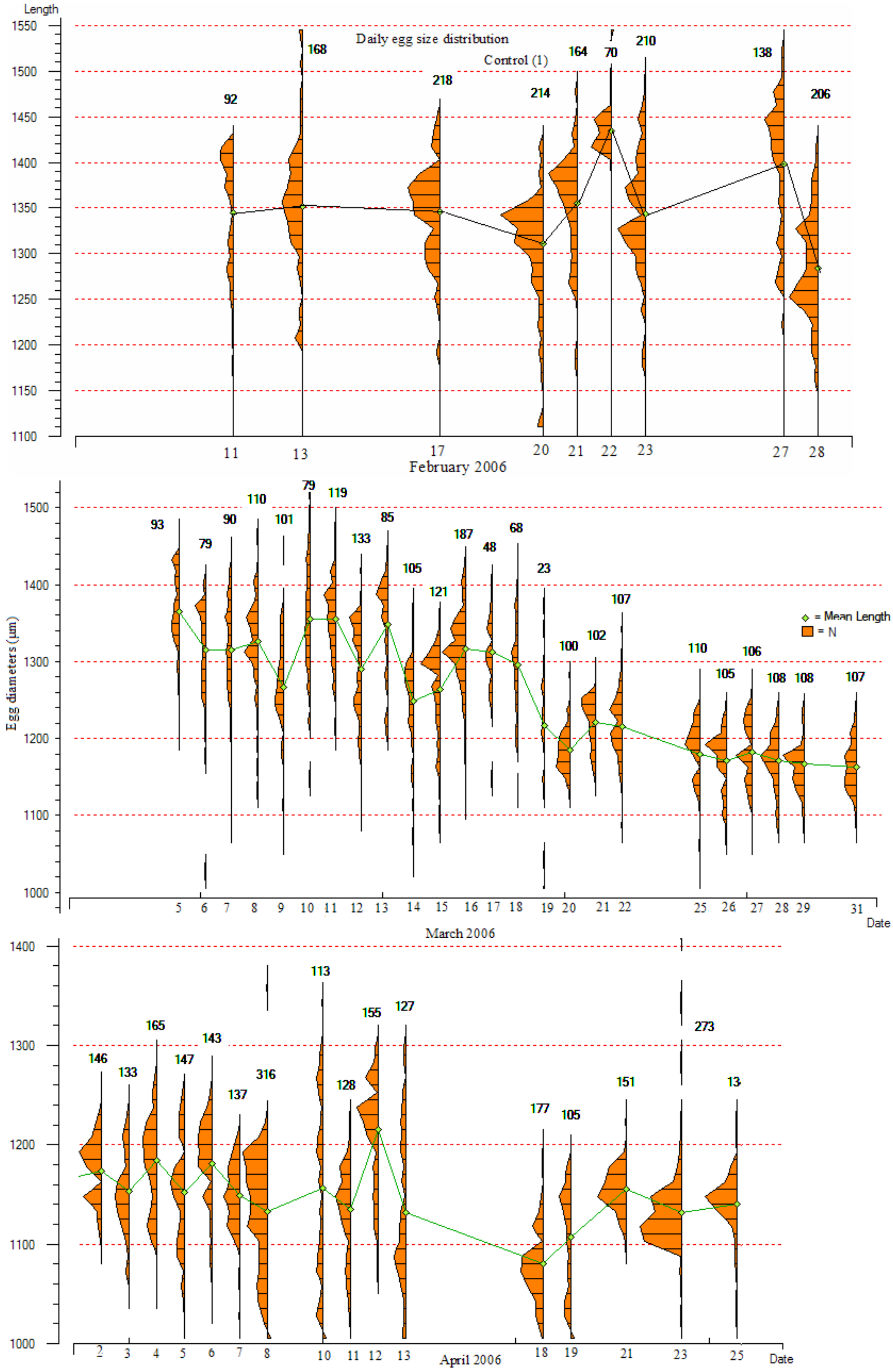

Figure 5: Shifts in daily egg sizes (size distributions) of Atlantic cod (*Gadus morhua*) at the start of spawning until late in the season of the control (1) group of spawning fishes visualized using the PASGEAR II clx statistical package.

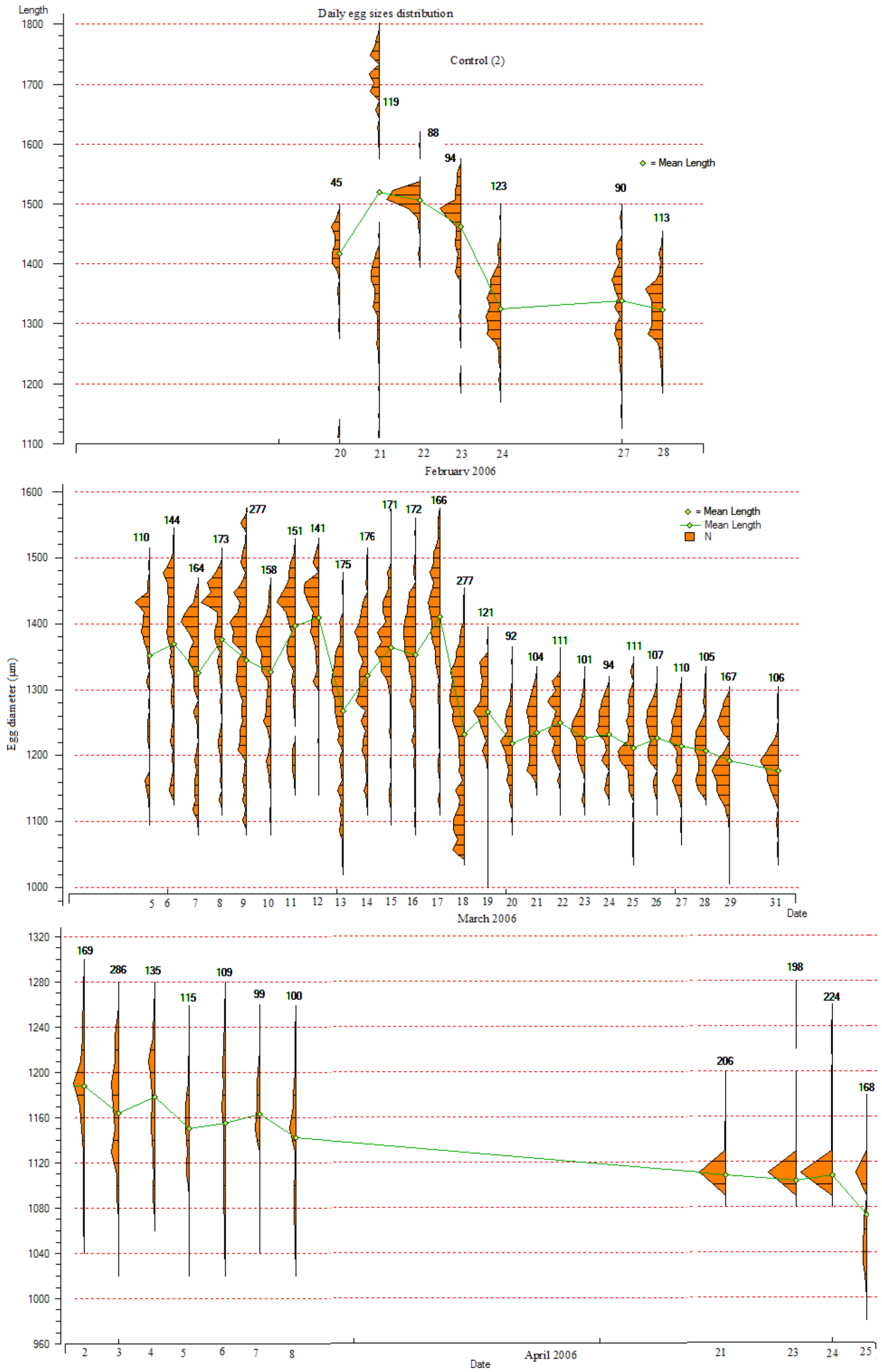

Figure 6: Shifts in daily egg sizes (size distributions) of Atlantic cod (*Gadus morhua*) at the start of spawning until late in the season of the control (2) group of spawning fishes visualized using the PASGEAR II clx statistical package.

Aiming at comparing the daily egg sizes distribution of the control groups (Figures 5 and 6 above) with one of the treatments, the daily egg sizes distribution of the medium oil group indicated some differences. For example, its egg sizes variation did not decrease with time in the spawning season (Figure 7).

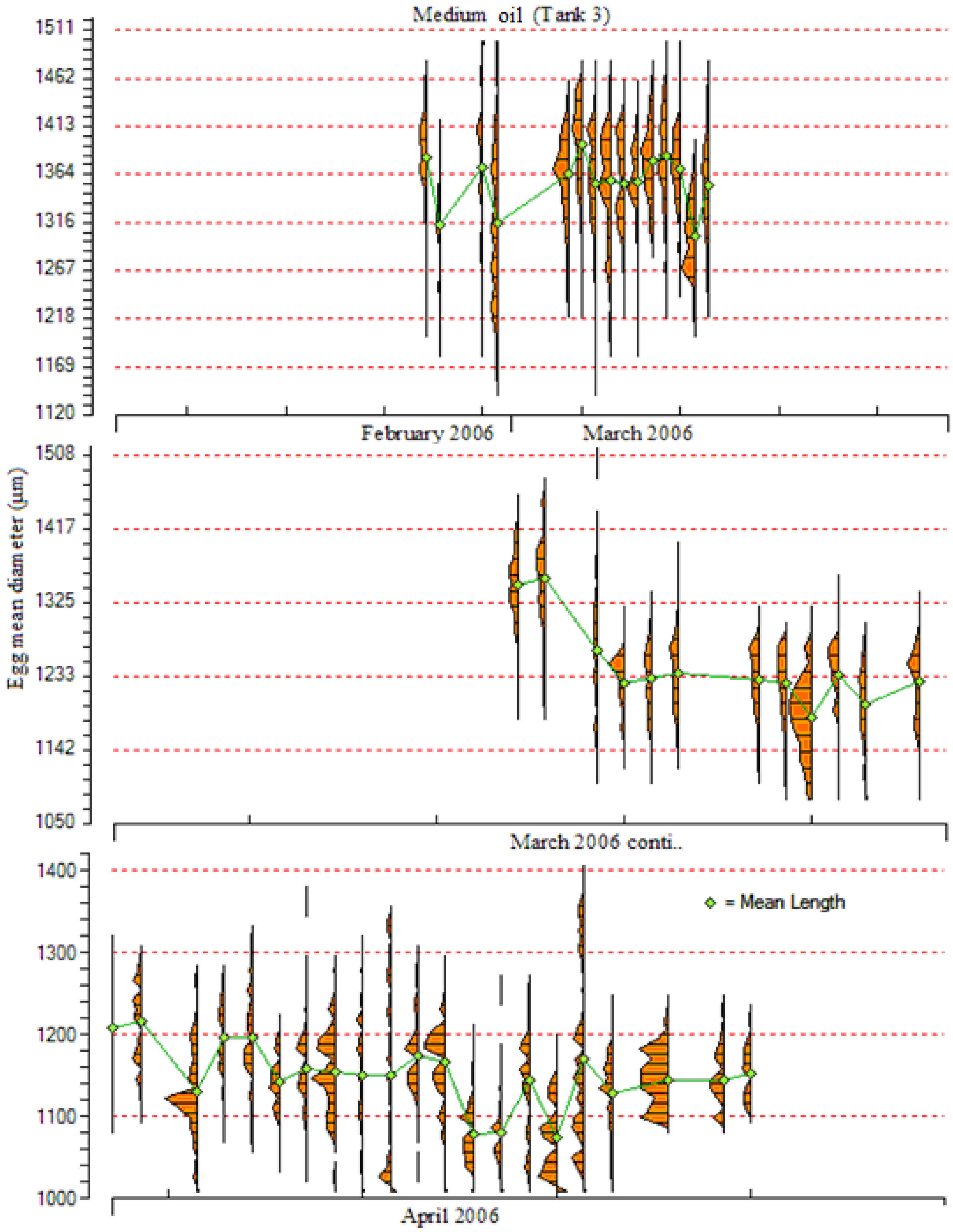

Figure 7: Shifts in daily egg sizes (size distributions) of Atlantic cod (*Gadus morhua*) at the start of spawning until late in the season of the medium oil group of spawning fishes as visualized using the PASGEAR II clx statistical package.

3.1.3 Intra and inter variations in eggs sizes of different batches

Generally, for the eggs in the control groups (tanks 1 and 5) there were greater intradaily (around the mean) and interdaily (differences between previous day and the succeeding day) variations in egg sizes for batches of eggs spawned early in the season compared to batches spawned in the mid and late season (Figure 8).

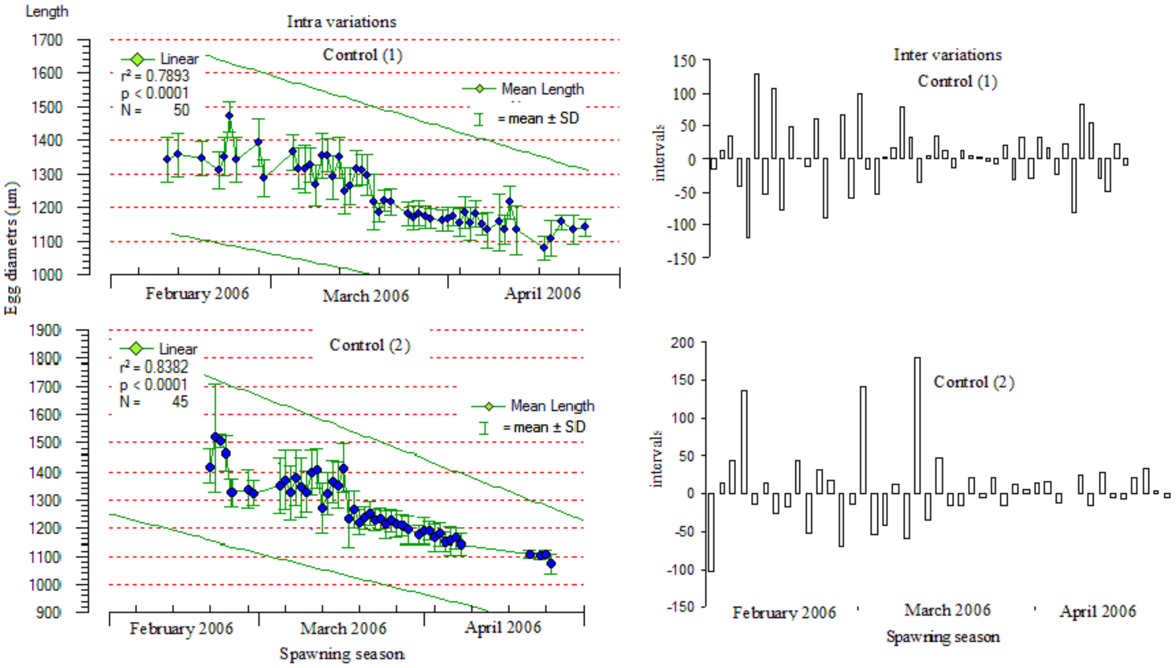

Figure 8: Intra and interdaily variations in mean egg sizes of Atlantic cod (*Gadus morhua*) at the start of spawning until late in the season of the control (1) and (2) groups of spawning fishes.

For comparison purposes, the eggs of the medium oil group (e.g. tank 3) were also examined for the intra and intervariations in batches. The eggs of this group contrasted the eggs of the control group (refer Figure 8) as indicated neither intravariations trend nor intervariations trend (Figure 9).

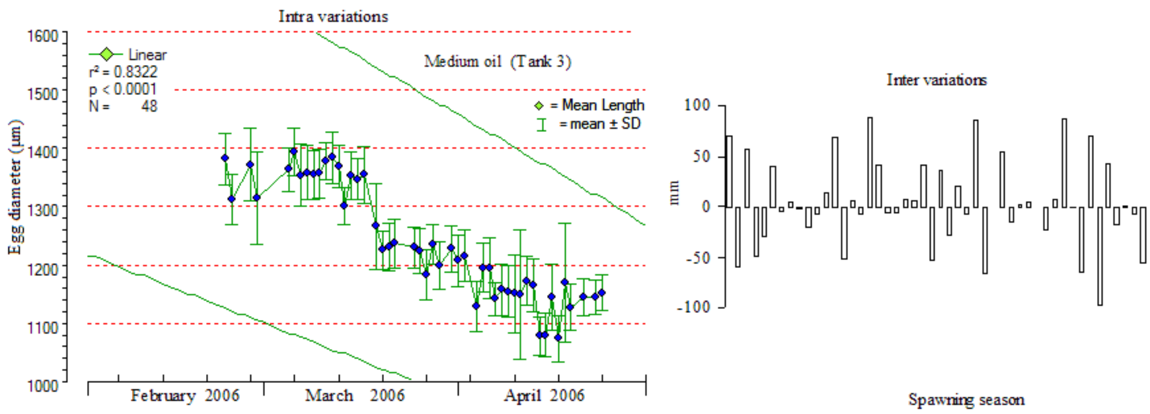

Figure 9: Intra and inter daily variations in mean egg sizes of Atlantic cod (*Gadus morhua*) at the start of spawning until late in the season of the medium oil group of spawning fishes.

3.1.4 General overview of the egg size distributions of the nine experimental tanks

Egg size distributions of the nine tanks indicated high variability in the distributions, for example, tanks 1(control), 3(medium oil), 4(endocrine disr.), 5(control), and 6(low oil) had wide and slightly skewed to the left egg size distributions whilst tanks 2(low oil), 7(medium oil), 8(high oil), and 9(endocrine disr) had narrow and more normal egg size distributions. The eggs from tank 5 had the largest mean egg diameter (e.g 1261.4 ± 128.7 µm) whilst tank 2 (e.g. 1169.8 ± 68.8 µm) had the lowest. The overall mean diameter of cod eggs was 1216.2 ± 113.6 µm (mean ± SD) (Figure 10).

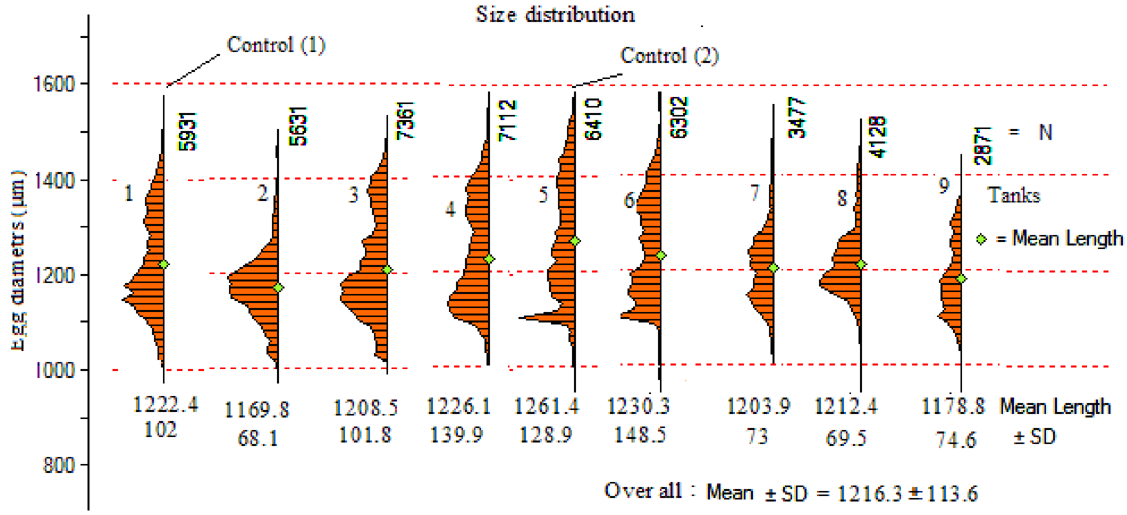

Figure 10: Total egg size distributions in the nine tanks (experimental groups) and their respective mean ± SD as visualized and calculated using the PASGEAR II clx statistical package.

3.2 The estimation of proportion of spawning females in tanks

3.2.1. The relation of the egg size distributions with egg developmental stages plus the egg size characterizations by mixdist (1st procedure)

To estimate the proportion of spawning female fishes in tanks, five days were randomly selected: 13th, 17th, and 27th of February, 22nd of March, and 5th of April of the control (1) group of spawning fishes.

Eggs spawned on the 13th of February: egg sizes at developmental stages 4 (number: 56, diameter range: 1.212 to 1.505mm), 8 (number: 8, range: 1.230 to 1.505mm), and 9 (number: 15, range: 1.308 to 1.438mm) were most abundant (highest in number). The wide range in egg sizes, as well the greater number of eggs at developmental stage 4, implied that this group contains the eggs of more than one female. Eggs at stages 8 and 9 were from only two different females because of the small time interval (e.g. 2:15 hours) and as well small number. Therefore, on this day the estimation led to more than three spawning fishes (out of six females) in the tank. Furthermore, for this day, the egg characterization by mixdist analysis (Figure 11) indicated that there were in total four groups of eggs. Combining these two outcomes suggests that a total of four fishes were spawning in this date.

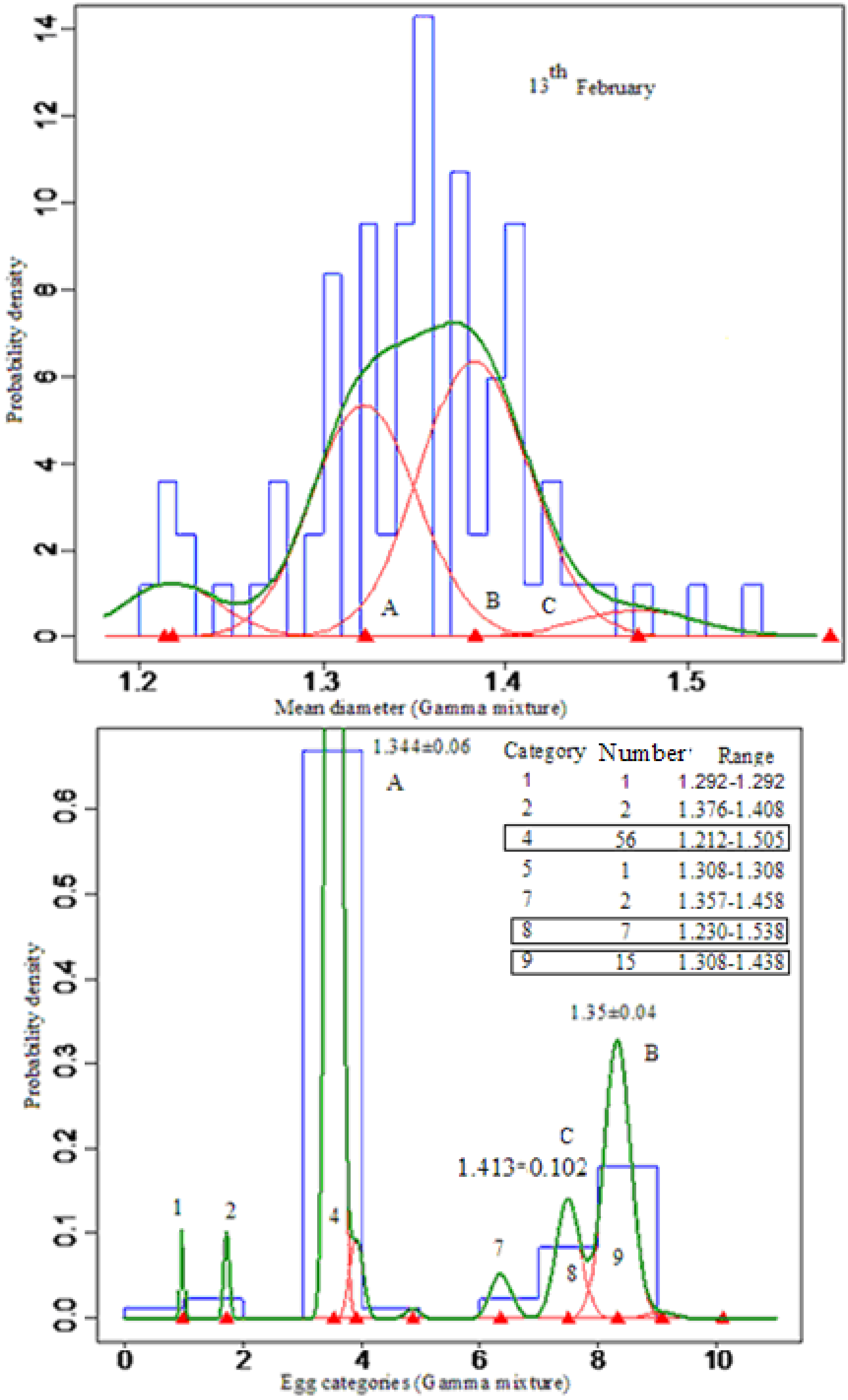

Figure 11: Characterization of the eggs of 13th February of the control (1) group of spawning Atlantic cods (*Gadus morhua*) using the mixdist analysis package in R. Egg category = egg developmental stage.

As another example, the eggs spawned on the 17th of February, major proportions comprised eggs at developmental stages 8 (frequency: 18, range: 1.259 to 1.426 mm), 9 (frequency: 79, range: 1.193 to 1.426mm), and 10 (frequency: 12, range: 1.275 to 1.456mm). As eggs at developmental stage 9 had a wide size range and were most abundant, they were probably spawned by more than one fish in the tank. The eggs at developmental stage 8 had a moderate range in diameters, but because of their small number they were probably from a single female. The eggs at developmental stage 10 (more than 21 hours since fertilization) were probably spawned the previous day. Therefore, on this day (February 17th) the estimation led to more than two spawning fishes in the tank. The characterization of the egg sizes of this day by mixdist analysis (Figure 12) indicated four groups of eggs. By excluding eggs with developmental stage of 10, the estimation led to three spawning fishes in the tank.

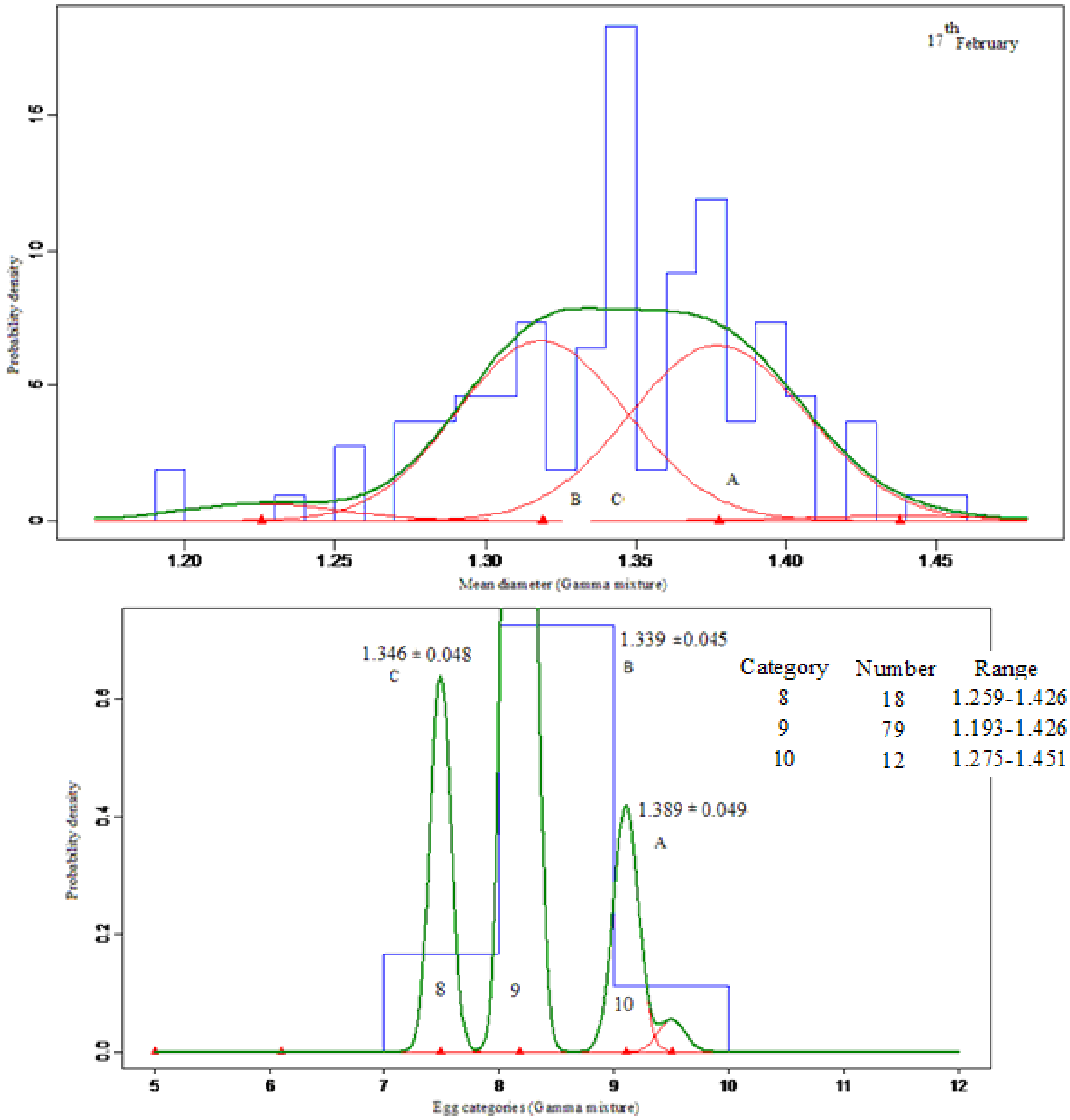

Figure 12: Characterization of the eggs of 17th February of the control (1) group of spawning Atlantic cods (*Gadus morhua*) using the mixdist analysis package in. Egg category = egg developmental stage.

Eggs which were spawned on the 27th of February comprised developmental stages 7 (number of eggs: 60, size range: 1.261 to 1.523 mm) and 10 (number of eggs: 9, size range: 1.275 to 1.405mm). The eggs at developmental stage 7 had a wide range in sizes and were most abundant, so this implied that they were spawned by more than one female in the tank. Therefore, on this day it seems there was more than one female spawning in this tank. Egg sizes characterization (of this day) by mixdist analysis indicated four groups of eggs (Figure 13). Excluding eggs at developmental stage 10, it was estimated that there were a total of three spawning fishes in the tank.

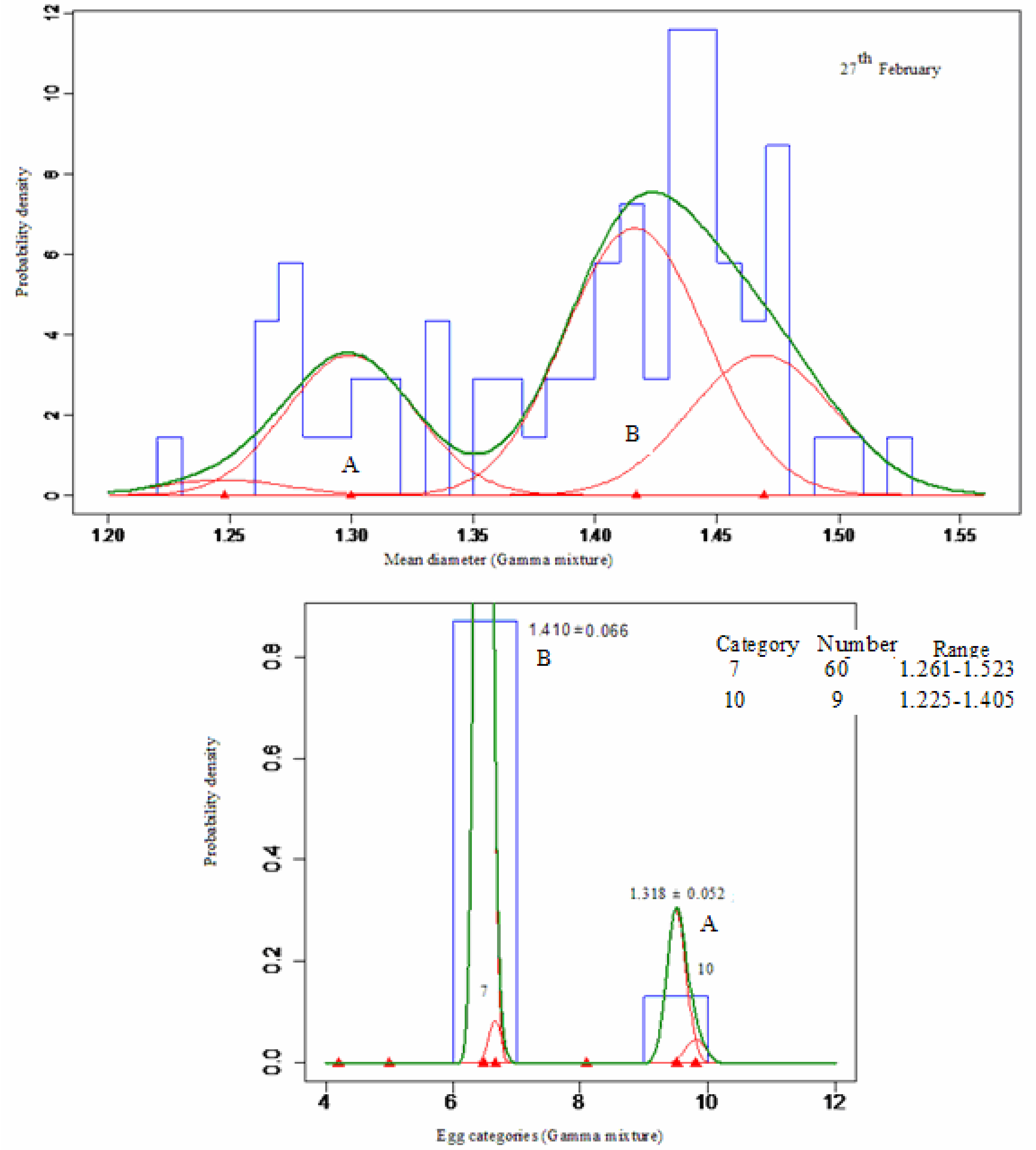

Figure 13: Characterization of the eggs of 27th February of the control (1) group of spawning Atlantic cods (*Gadus morhua*) using the mixdist analysis package in R. Egg category = egg developmental stage.

Eggs which were spawned on the 22nd of March comprised developmental stages 5 (number: 10, range: 1.210 to 1.235mm), 6 (number: 4, range: 1.221 to 1.246 mm), 8 (number: 104, range: 1.129 to 1.242 mm), and 10 (number: 32, range: 1.203 to 1.333mm). Although the eggs at developmental stage 8 were most abundant, the small range in diameters implied that were from one fish. The same also applied to eggs at developmental stages 5 and 6, thus making an estimation of three spawning fishes in the tank. Figure 14 indicated that on 22nd of March there were four groups of eggs. Excluding eggs at developmental stage 10 (the leftovers of the previous spawning day), and as well correlating the two outcomes, led to the estimation of three spawning fishes in the tank.

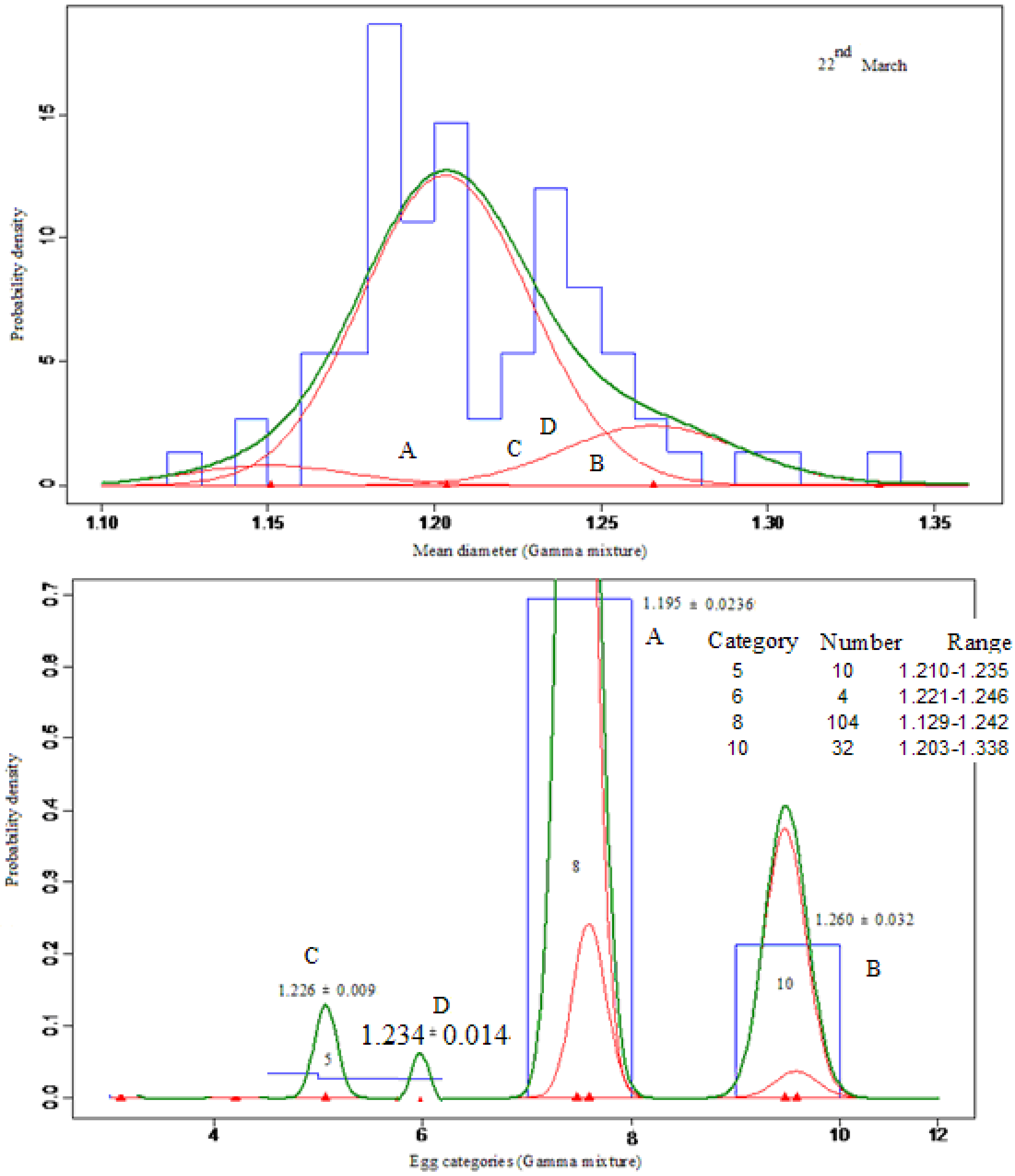

Figure 14: Characterization of the eggs of 22nd March of the control (1) group of spawning Atlantic cods (*Gadus morhua*) using the mixdist analysis package in R. Egg category = egg developmental stage.

The date of 5th of April had eggs which comprised developmental stages 4 (number: 85, range: 1.106 to 1.428mm), 6 (number: 17, range: 1.106 to 1.153 mm), and 10 (number: 58, range: 1.058 to 1.247mm). The eggs at developmental stage 4 were most abundant and had a wide size range, thus were spawned by two or more fish. The batches of eggs at developmental stages 6 and 10 were each spawned by one fish as they were small in number and had a narrow size range as well. Thus, after excluding the eggs in stage 10, the estimation led to three spawning fishes or more. An outcome of egg characterization of this day (Figure 15) indicated that there were three groups of eggs. By correlating the two outcomes, on this day there were three fishes spawning.

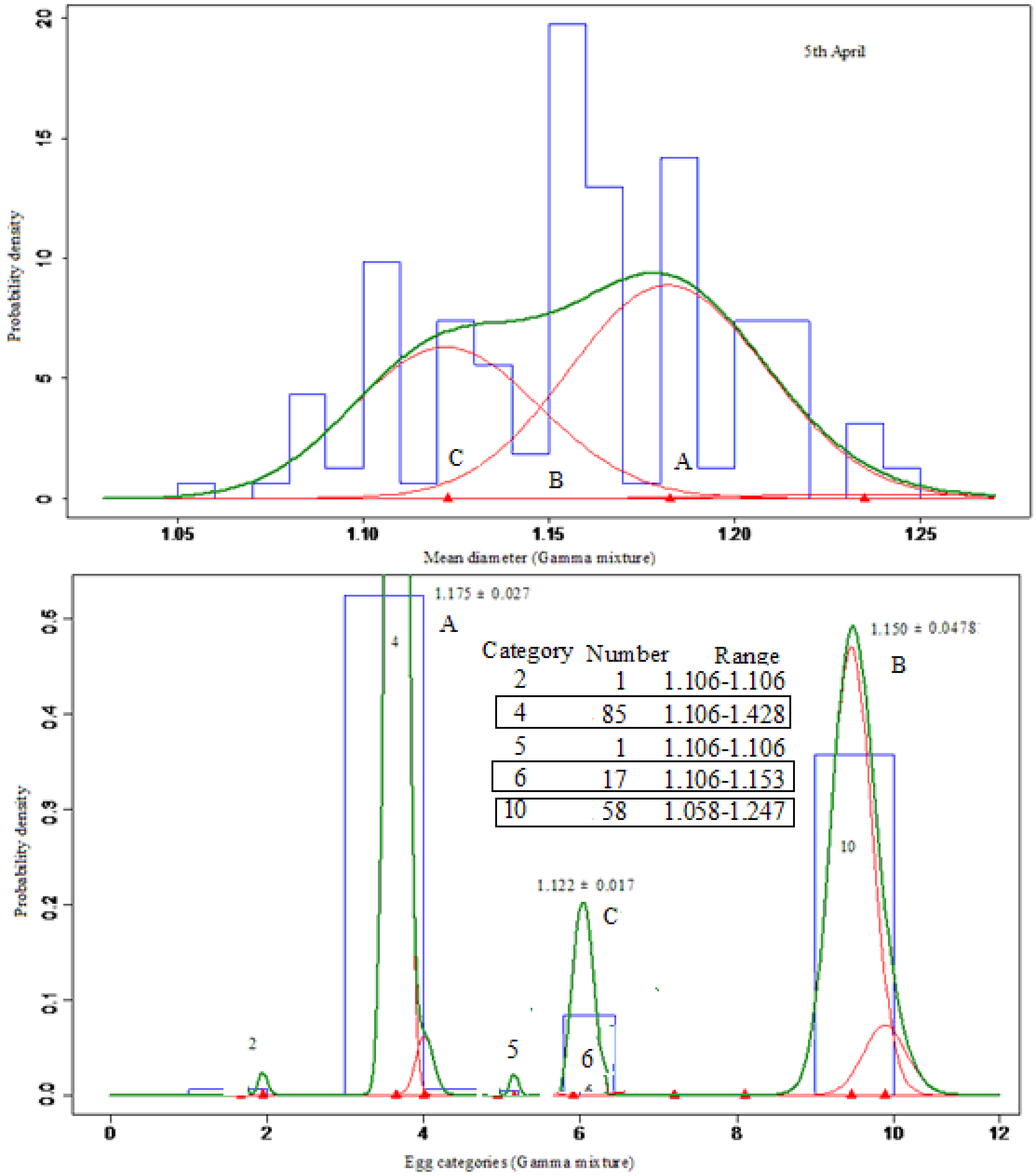

Figure 15: Characterization of the eggs of 5th April of the control (1) group of spawning Atlantic cods (*Gadus morhua*) using the mixdist analysis package in R.

NOTE: By looking at the time intervals between developmental stages of daily eggs, it can as well be stated that, cod spawned both during day and night.

3.2.2. Patterns of mean egg diameters and the egg sizes characterization by mixdist (2nd procedure)

The mixdist analysis of the daily egg sizes to identify different groups of eggs of the control (1 and 2) produced six groups of mean diameters as shown in Figure 16. Accordingly, the six groups of mean egg diameter represent the number of female fishes spawning in the tanks. The PASGEAR II clx tested the relationship between the mean egg diameters (six egg groups of the control 1) with time, four egg groups (groups 1, 2, 3, and 4) indicated declining trends with time with significant linear relationships, contrasting groups 5 and 6 which indicated no significant relationships with time (Figure 17).

Because the egg groups 1, 2, 3, and 4 were continuously represented on daily basis over the spawning season, this implies that four fishes (out of six female fishes of the control (1) group) spawned regularly contrasting fishes 5 (egg group 5) and 6 (egg group 6) which indicated occasional spawning. However, alternatively, the patterns of mean egg diameters as indicated in the Figure 16 probably only indicate that largest eggs are a characteristic of early the spawning season whilst large and small eggs, the midst and late the season, respectively.

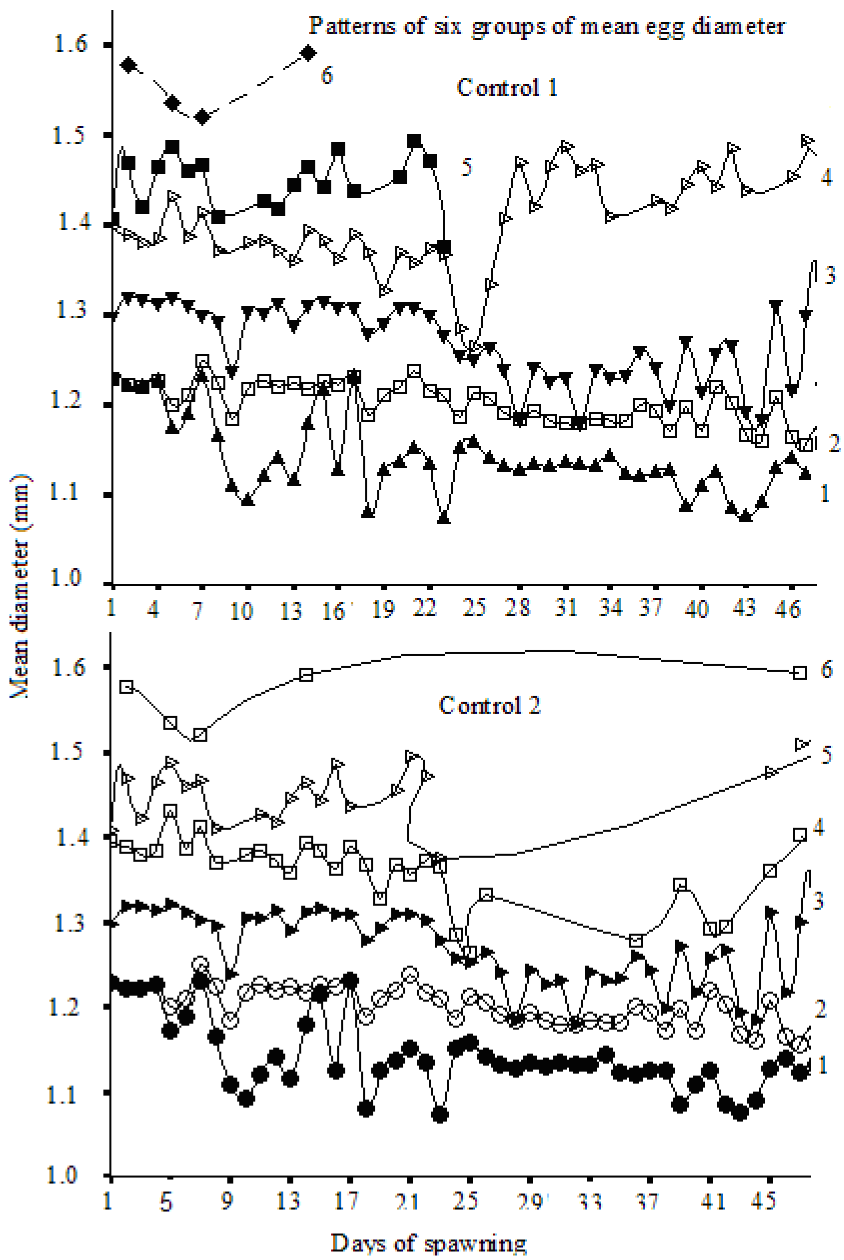

Figure 16: Patterns of the six groups of daily mean egg diameter (identified using the mixdist package in R) of Atlantic cod (*Gadus morhua*) observed over time during the spawning season. The dashed line between points indicates zero values of spawned eggs in that day. These patterns were drawn using SPSS statistical package.

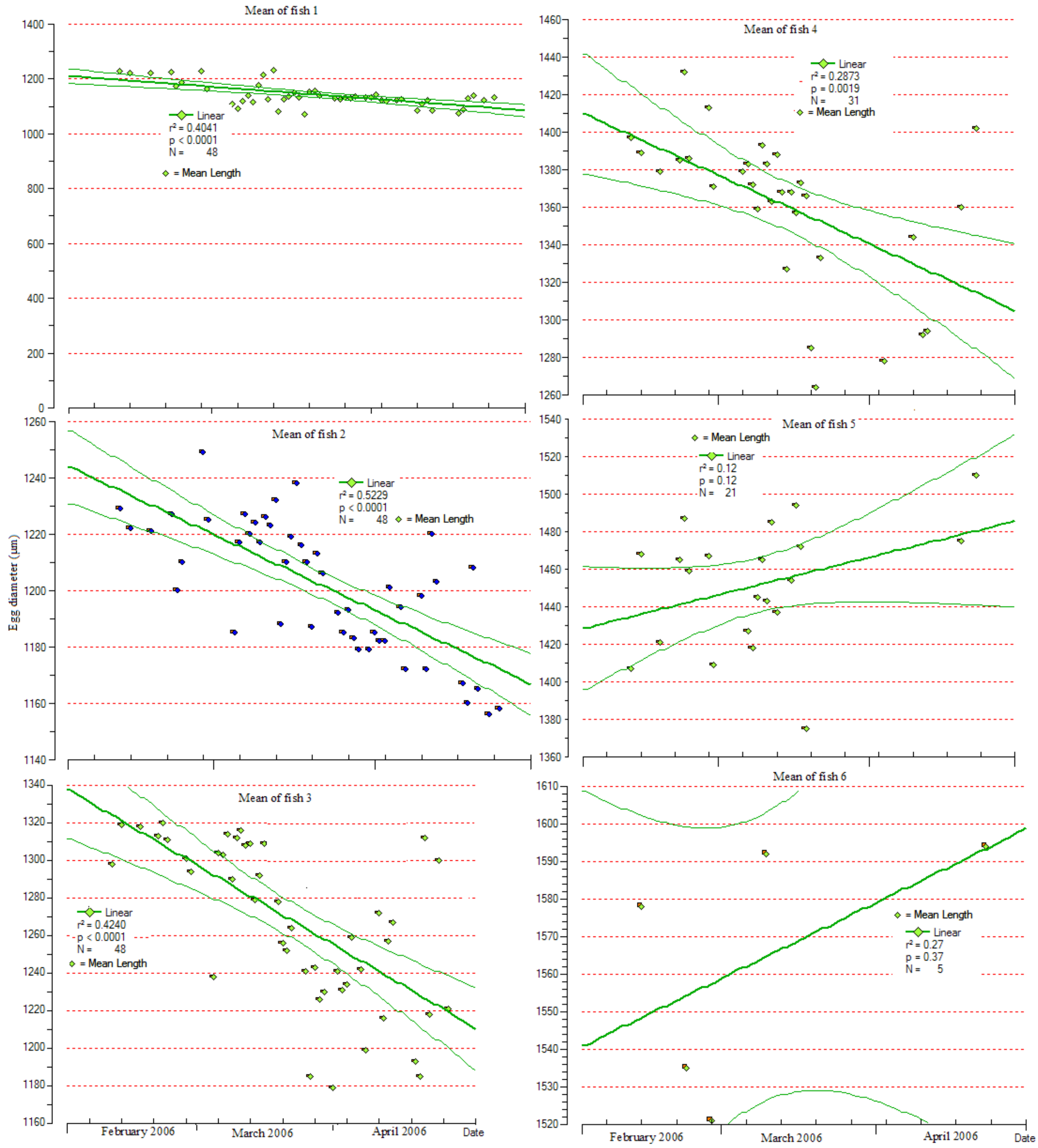

Figure 17: Regression analysis of the six groups of average daily egg sizes versus time of the control 1 group of spawning fishes analyzed using the PASGEAR II clx statistical package.

The combined information from the first and second procedures indicated that there were a maximum of four females spawning on each spawning day. Furthermore, a general characterization of the eggs in the control (1) and (2) groups has also detected six groups of mean egg diameters (Figure 18).The six groups of mean egg diameters probably indicate that all the six fishes in the control (1) and control (2) participated in spawning during the season. The largest proportions of egg groups 1, 2, 3, and 4 in the control (1 and 2) probably indicate that four fishes spawned regularly. If this holds true that on average four fishes spawned each day, then cod spawning interval (between spawning one batch until the next) might be $\geq 24 \leq 48$ hours.

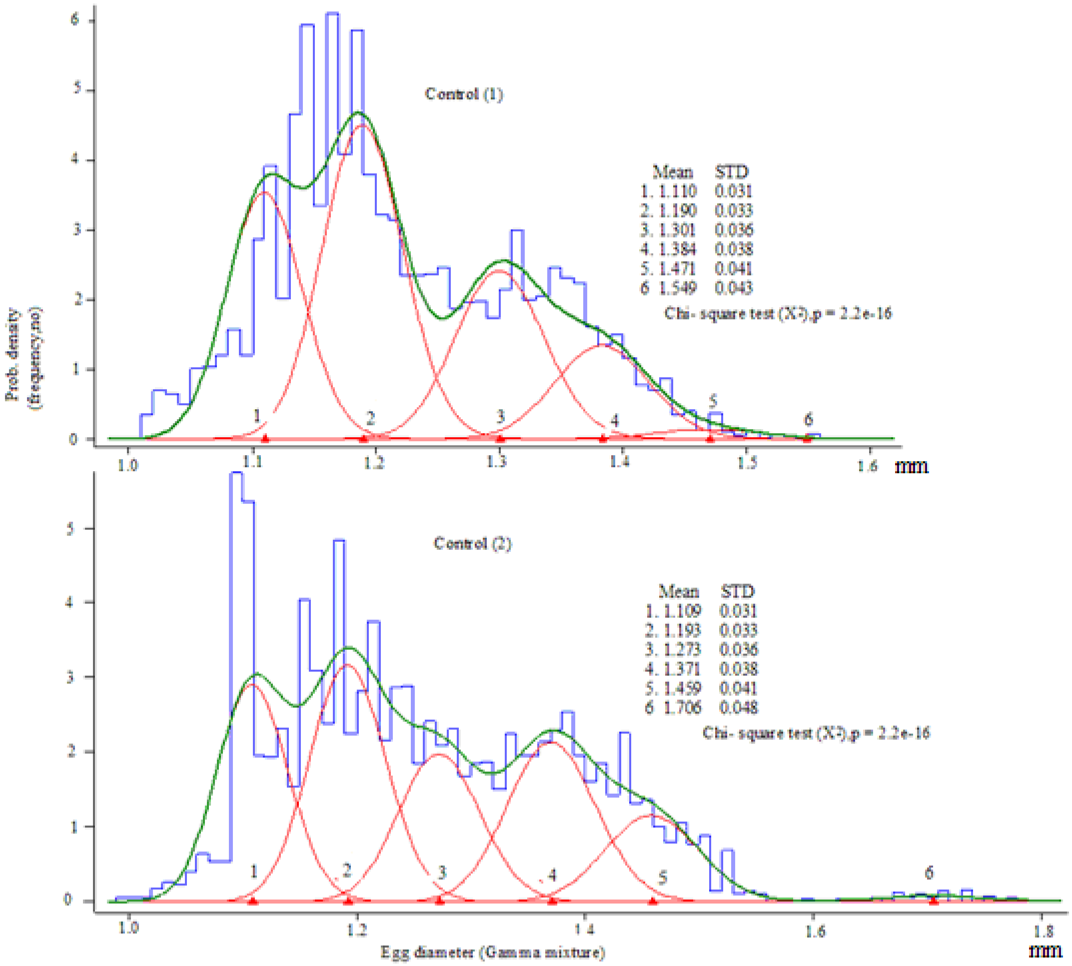

Figure 18: Groups of Atlantic cod (*Gadus morhua*) eggs as indicated by the mixdist in R, for the control (1) and (2) groups observed for 50 days and 45 days, respectively, over the spawning season.

4. Discussion

4.1 The categorization of the spawning season into early, mid and late season.

Although this study categorises spawning season into early, mid and late spawning season based on the time (total) elapsed by the fishes whilst spawning (see Figure 3 on page 11), the same categorization can be achieved based on daily total number of eggs over the production season (as illustrated in Figure 19(x)). And, because the categorisation based on total time elapsed compares that of the total number of eggs, then this fact implies that either of the two alternatives is correct.

This study's peak production season falls into the mid season where egg production was greatest (see Figure 19(x)) and egg sizes were average (see Figure 3).

NOTE: The eggs of a single female are not expected to show the same increasing trend of total eggs as observed for the group of spawning fish. The large increase observed in Figure 19(x) is an outcome of the increased number of fishes which spawned, as normally fish spawning in groups start spawning on different dates. The trend of total egg daily production (Figure 19(x)) contrasted the number of egg production per ml which increased (Figure 19(y)).

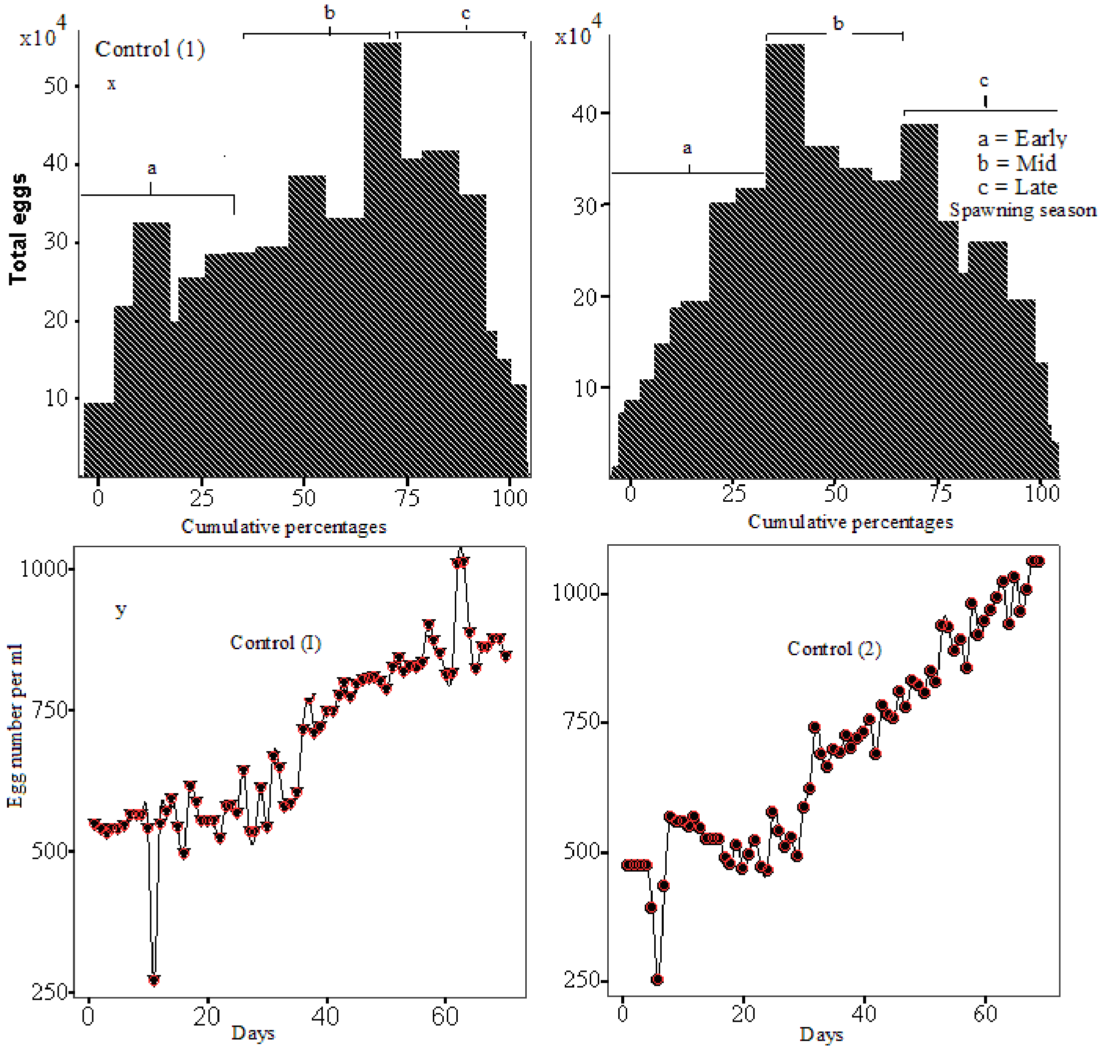

Figure 19: Early, mid and late spawning season of Atlantic cod eggs (*Gadus morhua*) as estimated from daily total eggs production and the number of egg per ml over the spawning season.

4.2 The size of cod eggs over the spawning season and the appearance of egg curves

4.2.1 The size of cod eggs over the spawning season

Cod eggs spawned earlier in the season were significantly larger than those spawned in the mid and late the season (One-way ANOVA followed by Tukey HSD contrast, $p< 0.01$). This finding concurs with the findings of Kjesbu (1989) and Ouellet et al. (2001) on Atlantic cod (*Gadus morhua*) eggs. Also, this pattern was described by Rideout et al. (2005) on haddock *(Melanogrammus aeglefinus*) and Kennedy et al. (2007) on plaice (Pl*euronectes platessa* L).

Generally, there is a consensus that the gradual decrease in size of eggs of many batch spawners in the spawning season is an outcome of depletion of energy resources of the maternal fish. However, Ouellet et al. (2001) tested the hypothesis that "the decreasing egg size is not due to depletion of the female's energy reserves", and they found stable total lipid/yolk protein ratios in the season. They did not find any negative relationships between total egg dry mass in a batch with batch number, and during spawning each female's total egg dry mass in a batch was not related to either post-spawning somatic protein or lipid reserves. They therefore concluded that depletion of a female's energy reserves (in cod) is not the cause for the decrease in egg size over the spawning season Generally, as regards reproduction in cod, despite of the best nutritional resources and a high quality rearing environment, the main strategy is to invest in increased fecundity as the first priority rather than to invest in making larger eggs (Kjesbu 1989; Karlsen et al. 1995; Jorgensen and Fiksen 2006). In nature, the variation in the size of fish eggs besides female status (e.g. condition, size, age, etc) and season effects also is influenced by population origin, temperature, latitude, and salinity (Chambers and Waiwood 1996; Marteinsdottir and Steinarsson 1998; Vallin and Nissling 2000).

4.2.2 The shape of egg size curves

The egg curves indicated a continuous rapid decline in size between eggs of the initial and final batches (see Figure 20 for a further illustration). This finding contrasts with Kjesbu's (1989) observation. However, concurs with the results of Trippel's (1998) study on the egg size variation in the first-time spawners contrasting the second spawners which exhibited a parabolic curve (e.g. a slight increase followed by a decrease).The high variation observed in this study's curves (as indicated in Figure 20) is because each curve is a combination of many spawning females whereas Kjesbu (1989) and Trippel (1998) used the eggs of one female fish.

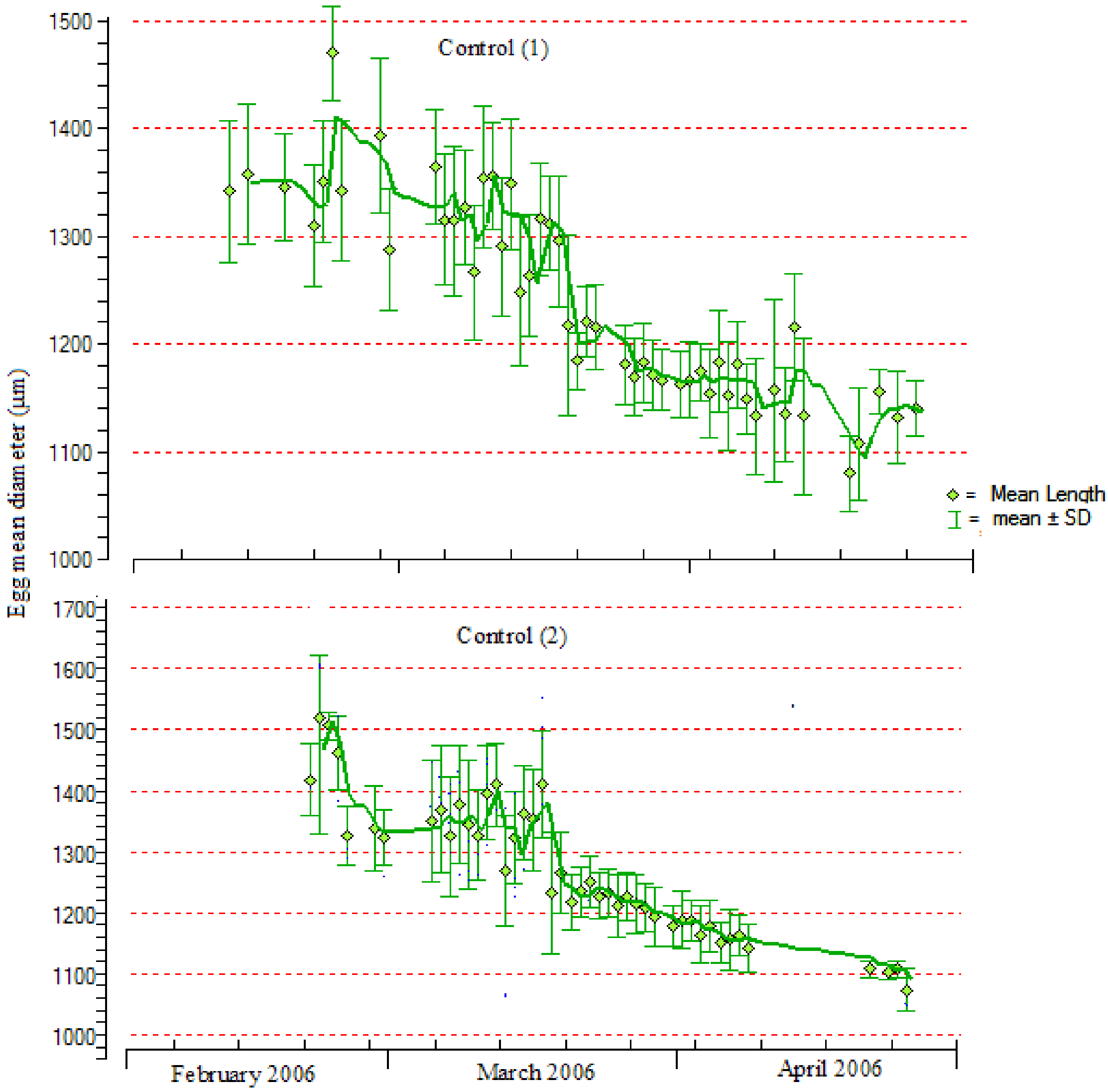

Figure 20: Curves of Atlantic cod (*Gadus morhua*) average egg diameters of the control (1) and control (2) groups of spawning fishes over the spawning season.

4.3 Inter and intravariations of cod egg sizes in the spawning season

Generally, the egg sizes exhibited higher intra and inter variations in the batches spawned early in the spawning season compared to those spawned in the mid and late spawning season. The observed intervariations concur with Kjesbu's (1989) finding in which the variability was higher in the early than mid and late spawning season. So far, the trend of the *intravariations* is a new observation. However, Kjesbu (1989) described the egg size distribution of each batch and showed that the size variations lead towards a dome shaped curve with time. This study also observed the same trend in daily egg size distributions where the decreasing trend of the daily intra variations produced a dome shaped curve (e.g. Figures 5 and 6 on pages 14 and 15, respectively).

4.4 Differences in egg sizes between various treatments

Eggs of the various treatments indicated low differences in average sizes, the same also was observed by Knag (2007) on which this study is based upon. Knag (2007) as regards this observation, concluded that oil pollution had no major effects on cod egg sizes variability. However, the main reason for the lack of oil effect could be due to the fact that oil was introduced to cods during their very early life stages (eggs to larvae). A fact which may as well imply that the life condition a cod fish is exposed to during its very early life stages doesn't have major effects on its reproductive condition later on. This study involved cods which were firstly grown under differing life conditions from eggs/larvae stages to juveniles during the IMR-2004 experiments and then re- reared under the constant environment during the IMR-2006 experiments from juveniles to spawning. The life condition (e.g. temperature, nutrition status, etc) the female fish is exposed whilst developing gametes (as a preparation for spawning) is a major determinant of the extend of its reproductive investments (Wieland et al. 2000; Rideout et al. 2005; Jorgensen and Fiksen 2006).

4.5 Spawning time intervals in cod

4.5.1 Cod behaviour during its spawning activity

Based on the estimated number of fish spawning per day and the eggs developmental stages, it was observed that during the experiments cods exhibited day and night spawning. The fact that cod spawn during day and night is also supported by Kjesbu (1989) and Trippel (1998).

4.5.2 The length of spawning time in cod

Knag's (2007) study (with experimental temperature range of 4.6 to 6.8 ^{o}C) observed a spawning time of 69 to 70 days for the control (1) and (2) groups of spawning fishes which were first time spawning fishes. The finding contrasts with Kjesbu (1989) (experimental temperature range of 5 to 7^{o}C) who found 47 days and 50 to 60 days for the first and second time spawning cods, respectively. Trippel (1998) (experimental temperature range of 3 to 4^{o}C) estimated only 23 to 24 days spawning time for first time spawners, and an average of 41 days for second time spawners. Trippel (1998) and Kjesbu (1989) based their conclusions from the observation on single females where as the Knag's (2007) study based on the group of spawning fishes which spawned asynchronously, and this could be the reason for the differences. However, Ouellet et al. (2001) and Hamoutene et al. (2009) put forward the speculation that the length of spawning time in cod is variable depending on many factors, especially the nutritional and environmental factors, e.g. water temperature.

4.5.3 The spawning interval (time between batches)

Based on the conclusion that 3 to 4 fishes out of 6 spawned every day, this study estimated a spawning interval (time between batches) of one to two days for each fish. The finding contrast with the finding of Trippel (1998) who found an interval of about 6 days for first time spawners and about 5 days for second time spawners. This study finding also differs with the finding of Kjesbu (1989) who found a spawning interval of around 2 to 5 days. As has been on the length of the spawning time, so too on the spawning interval where many factors contribute to the observed discrepancies. For example, increased food ration resulted in decreased spawning intervals in female three -spined sticklebacks (Ali and Wootton 1999).

4.6 The methodologies used for the estimation of proportion of spawning fishes

4.6.1 Observations on the ranges of egg sizes and the corresponding developmental stages

Although generally a single female produces eggs of differing sizes with varying proportions, in a single day of spawning the variation is expected to be insignificant (Kjesbu 1989).Therefore, for each spawning day the assumption that eggs of the same developmental stage presented in large proportion and of wide range were from more than one female in the tank and vice versa, is most probably true. Fish eggs even of the same batch can advance differently in the cleavage process after fertilization, however, under normal condition, those eggs normally comprise a minor proportion (Kimmel at al.1995). This study neglected those minor proportions, and only eggs of large proportion were considered as a fish or fishes.

4.6.2 The characterization of egg sizes using mixdist package

Whilst characterizing the egg sizes, the groups generated by the program more or less correlated to those generated by the excel histogram plot. Thus, the mixdist program performance was as expected. Because eggs sampled each day in a tank were spawned by more than one fish, the assumption that groups of egg sizes (especially those with large proportion) generated by mixdist analysis run on daily basis represented individual fish is highly probable.

4.7 Brief review on the formulated questions in 1.2.1 (iii) page 4

4.7.1 Is the size of the fish egg an indicator of quality?

Many scientists, i.e. Kjesbu (1989), Ouellet et al. (2001), and Kennedy et al. (2007) correlate fish egg size with female size where large females are known to produce large sized eggs. And, the increased size of fish egg has been found to result into increased larval size and survival potential (Srivastarv and Brown 1991; Rideout et al. 2005; Paulsen et al. 2009). However, the size of fish eggs has as well been correlated with age of the female fish (Kjesbu et al. 1996; Marteinsdottir and Steinarsson 1998; Berkeley et al. 2004). Kjesbu et al. (1996) found that cod egg size mainly corresponds to age of the female, and cod's first batches (in the spawning season) most often are comprised of few eggs and/ or of poor quality (Ouellet et al. 2001). There are however still some doubts whether the size of the eggs is related to egg survival and hatching successes (Ouellet et al. 2001: Hamoutene et al. 2009).

Generally, although the size of the fish egg is positively correlated with high larval survival rate as large egg leads to large yolk-sac volume (Kennedy et al. 2007; Higashiani et al. 2007) and large hatchlings (Srivastarv and Brown 1991; Rideout et al. 2005; Paulsen et al. 2009), lack of correlation between the size of an egg with fertilization rate and hatching success is a drawback towards the conclusion that the size of the fish egg is an indicator of quality.

It is generally agreed that high quality eggs lead to high egg survival rates, fertilization rate and hatching success (Kjesbu 1989; Ouellet et al. 2001; Hamoutene et al. 2009), but there is no clear demarcation on which eggs are of high quality from physical indicators. Still, irrespective of the many discrepancies, so far the size of the egg remains to be an important criterion as an indicator of egg quality in fish. Cod fish like other batch spawners (e.g. haddock *Melanogrammus aeglefinus*), maximises its egg production during the mid of the spawning season by producing eggs of high number and moderate sizes, the period of which favourable condition for the offspring is expected (Kjesbu 1989; Rideout et al. 2005; Jorgensen and Fiksen 2006).

4.7.2 Is fish investing on the size of its eggs a strategy?

As regards reproduction, two major options have been observed in fish, either (a) invest on fecundity where number is important (e.g. cods, turbots, herrings, halibuts, etc) for best utilization of richly pelagic environment or (b) invest on egg size (e.g. salmonids, wolf fish, elasmobranches) for assured survival as large sized eggs lead to large larvae which compete positively for food and space, and to reduce predation (Marteinsdottir and Steinarsson 1998; Higashiani et al. 2007; Kjørsvik et al. 2007).

Generally, between different fish species, egg size and fecundity are inversely related (Kjørsvik et al. 2007). Contrasting small sized egg, large egg has more yolk available for the larva which is important for its survival before the commencement of first feeding (Srivastarv and Brown 1991; Kennedy et al. 2007; Higashiani et al. 2007). First feeding period present a critical period for the larva as normally has to competitively find food before starving to point of no return and ultimately death (Berkeley et al. 2004; Kjørsvik et al. 2007). Normally, the larvae of most marine pelagic fishes (e.g. cods) which are generally small, face high mortality as they are forced to start their first feeding very early after hatching (e.g. 4 to 5 days) contrasting the larvae, for example, of salmonids and wolf fishes which commence first feeding at around 44 and 104 days, respectively (Kjǿrsvik et al. 2007).

Evidence exist which relate fish internal coordination and external environmental conditions (e.g. photoperiod, food availability, temperature, water quality, etc), especially for seasonal spawners which help determine a proper time for spawning or otherwise would postpone spawning (Wieland et al. 2000; Rideout et al. 2005; Taranger et al. 2009).

Now, because fish is aware of what is or will happen on its surrounding environment, cod's behaviour of producing few large eggs during early the spawning season, then high amount of medium sized eggs during mid the season (peak), and few small eggs during late the season might be a strategy for assuring high survival of its offspring.

In nature, cods normally start spawning during late winter (few food resources for the larvae), spring (abundant food resources for the larvae) to summer (decreased food resources due to stratification) (Wieland et al. 2000; Taranger et al. 2009). Johnston and Leggett (2002) reported an existence of relationship between the environment which a fish inhabit and offspring (number and size) it produces, e.g. a fish inhabiting environment with relatively poor conditions for survival and growth of its offspring will produce few and large offspring.

Furthermore, cod preference for investing on fecundity rather than on size of its eggs, and the fact that its size of eggs correlate mostly with the size and age of the females, implies that in hatchery productions, requirements for large eggs for high survival of the larvae is mainly a matter of the brood stock sizes and age whilst rearing conditions are important for determining its fecundity (Karlsen et al. 1995; Kjesbu et al. 1996; Ouellet et al. 2001).

4.7.3 Is there a relationship between spawning time (duration of spawning) and spawning time intervals for first and second time spawners?

Kjesbu (1989) whilst establishing the spawning time interval of Atlantic cod (*Gadus morhua*) using second time spawners, fish exposed to the same temperature showed no notable differences in spawning time intervals. Comparisons of spawning time intervals of fishes reared under different temperature regimes showed differences. Furthermore, stressed fish indicated irregularity in spawning time intervals and behaviour as compared to normal fishes. Also, fecundity and size of eggs depended on the size of the female fish (Kjesbu 1989). Higashiani et al. (2007) indicated that in marbled sole *Pseudopleuronectes yokohamae* fecundity is related to body size whilst large females spawned large eggs.

Trippel (1998) whilst monitoring the egg and larval production of first and second time spawners captive Atlantic cod (*Gadus morhua),* among other findings, also indicated that first time spawners bred for shorter period (duration of spawning) and produced fewer egg batches than second time spawners. Also, first time spawners are likely to experience problems in timing the proper time for reproduction which is important for assuring survival (Trippel 1998; Carr and Kaufman 2009). And, as regards the spawning time intervals, first time spawners indicated longer spawning time intervals (e.g. 6 days) than the second time spawners (e.g. 5 days) (Trippel 1998). Generally, the Canadian cods (with spawning time intervals of around 5 to 6 days) contrast the Norwegian cods by having short spawning time intervals between batches (e.g. 2 to 3 days). Ali and Wootton (1999) found that in three-spined sticklebacks increase in food ration resulted in decreased spawning intervals.

4.7.4 Is there relationship between spawning time and spawning time intervals for fish spawning singly and /or in groups?

Unlike other fishes, for example Zebra fish which indicate remarkable behaviours whilst spawning and thus influencing spawning activity, generally cod does not express any remarkable social behaviour during spawning (Kimmel et al.1995; Trippel 1998). It remains relatively motionless between batches and do not feed (due to loss of appetite) until approximate 90% of its eggs has been shed in the season (Trippel 1998). In nature cods normally forms relatively large groups whilst spawning (Wieland et al. 2000; Paulsen et al. 2009) therefore, avoiding feeding behaviour possibly is an evolutionary strategy/or advantage to avoid predation of its own eggs. Kjesbu (1989) observed some fish moving around whilst other resting at the corners of the experimental tanks. Due to lack of any special social behaviour that cod express during the spawning activity period, most probably singly or group spawning affect not the length of the spawning time and spawning time intervals between batches.

4.7.5 As regards fish egg sizes, are there differences between forced and natural spawned eggs?

The term egg size as has been used in this study refers to the average egg diameters measured from the individual eggs (unpreserved) which were collected daily. Because cod embryo development rate is sensitive to temperature, the collected eggs were kept on ice prior to analysis (Knag 2007). The use of the term egg sizes (referring to average egg diameters) has as well been used by many other studies like the studies by Kjesbu (1989), Trippel (1998) and Chambers and Waiwood (1996), etc. As regards egg sizes, differences between preserved (e.g. on formalin, ice, etc) and unpreserved eggs have been demonstrated. For example, Chambers and Waiwood (1996) pointed out that cod eggs preserved with formalin can cause a shrinkage of 2% the diameter of fresh eggs and a difference of 3% can be accounted between a fertilized egg (being greater) undergoing cleavage and unfertilized egg. They however stated that normally these variations are assumed negligible. Most of the eggs used in this study were fertilised and were of different developmental stages as regards cleavage processes. The egg sizes as used in this study did not take any consideration of the variations of the egg diameter due to developmental processes. Egg diameter was measured at an orientation that its diameter represented the actual size of the egg (Knag 2007). In cod, like other fishes, for example, the zebra fish, salmonids, etc, a fully developed egg is externally fertilized before the cleavage starts (Kimmel et al. 1995; Hall et al. 2004).Therefore, comparing the egg sizes obtained by stripping a fish (forced) and those consciously spawned by the fish itself, most likely will present no differences other than differences due to developmental processes because nothing special happens to the fish egg when is fully grown except fertilization and the following embryonic developmental process (Kimmel et al. 1995; Hall et al. 2004).

5. Conclusions

Fish eggs reflect much of the maternal characteristics on which when carefully examined, is possible to obtain information which can be useful in various field studies, for example, in estimating the proportion of spawning maternal fishes in experimental tanks or in natural environments. Cod investment on egg size and its behaviour of maximizing egg production (number and quality) during its mid spawning season is a strategy towards assuring high reproductive efficiency and survival of its offspring. Early, mid and late spawning season of individual fishes in various experimental studies can be observed on total daily egg production and as well on time elapsed whilst spawning. As regards daily egg production in cods, normally high variation of egg sizes is the characteristic of early production season and the variation tends to decrease towards late the season. Egg sizes characterization by mixdist program and coding of its outcome with egg developmental stages gives the possibilities for estimation of the proportion of maternal fishes. Cods which spawn for the first time contrast the second time spawners in their egg production curves, length of spawning time and spawning time intervals between batches.

6. References

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7. Appendices

Appendix I: Brief descriptions of excel procedures followed for the observations of daily cod egg distributions and as well obtaining the bins and their corresponding frequencies important for mixdist analysis.

Despite having excel sheets with dates, daily egg sizes, and tank number, to observe the frequency distributions of egg sizes in daily basis, it was necessary to obtain the class bins for the plotting of histograms. To fill the excel cells with bins the following steps were observed.

(1) Find out the minimum and maximum sizes from the daily egg sizes distribution (e.g. observed manually after sorting the data or calculated using the formula as has been given by the excel program).
(2) Find out the value just below the minimum egg size (e.g. predicted after observing the minimum value)
(3) Fill the predicted minimum value in the first cell in an excel sheet, and then determine an interval of space where the bins were filled automatically after following step (4).
(4) And, by using the command edit, then fill in series, choose a stepping interval (e.g. 0.01), and a stop value (normally the value close the maximum value from the class interval), the series were filled within a highlighted space in an excel data sheet. These values were termed as bins.

After having the bins, the next step was to plot the egg sizes to observe the frequency distribution. To plot the egg sizes - frequency distributions, the function data analysis was chosen. Under data analysis, the sub item histogram was selected, then input range (referring to the egg sizes distribution), bin range (referring to the bins), an output range (referring to the area where the program should post the results) and as well chart output. The final out come of this procedure was the egg sizes – frequency distribution.

Note: The bins (that generally represented the egg sizes) and their frequencies given on daily bases constituted the raw data used for “mixdist analysis” in R program. For mixdist analysis, it was important that the last value of the bin indicate the infinity prediction, as indicated in the example below.

Dia	Freq
1.1	0
1.11	7
1.12	6
1.13	0
1.14	15
1.15	18
1.16	0
1.17	14
1.18	0
1.19	25
1.2	6
1.21	1
1.22	9

1.23	2
1.24	0
1.25	5
inf	0

Appendix 11: Brief descriptions of the mixdist procedures followed whilst using the package in R program.

When using R program, it was necessary that the file to be worked upon is imported first in the program from the clipboard, and made available for analysis. To import the file from the excel sheet into the clipboard, the function"file1<-read.table ("clipboard", header=T)" was used. When copying the contents from the excel sheet, it was important that only the part with data was copied, and names given to the columns should be the same referred too while calling the functions. To make the data copied in the clipboard available for analysis in the program, the function "attach (file1)" was used. To perform any kind of analysis in R program using the mixdist, it was necessary that the package is installed and then loaded in the program, and this was simply done from the R main menu. However, even after installing and loading the mixdist package, it was important to call the package in every analysis done using the function "library (mixdist)". The mixdist analysis (as given by R program) demands that the data to be analyzed should be mixture data, and therefore must be tested first. For testing the data, function "file1 <- as.mixdata (file1) was used, followed by function "png ("tmpPic1.png")" which was important for any query in the attached file. For plotting the frequency distributions in R in the same manner as would appear in the excel plot, the function "plot (file1)" was applied followed by the fuction "dev.off ( ) ".

To isolate the mixtured data into separate groups accordingly,the function "fitfile1a<-mix(file1,mixparam(c(1.1,1.2,1.3,1.4,1.5,1.6),0.03),"gamma",mixconstr(consigma="CCV")) was used. The numbers, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6 were the approximate guesses of the expected means in the data, and 0.03 was the guess for the standard devation. Thereafter, followed the function "png ("tmpPic2.png") "which was important for any query in the fitted file. To make another plot with frequency distributions as well the indications of the isolated groups, the function "plot (fitfile1a)" was applied, followed by the functions "dev.off( )", and sink("eggsummary.txt", append=FALSE, split=FALSE) important for making the summaries of the above plot.To recall the above made summary,the function "summary (fitfile1a)" was applied. To observe the final outcome as has been worked above, the functions "sink()", "plot(file1)" ,"plot(fitfile1a)", and "summary(fitfile1a)" were applied.