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Estimating cod egg developmental stage based on DNA concentration

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Pelagic egg surveys are commonly used to map Atlantic cod spawning areas in the northeastern Atlantic. However, a sampling location may not necessarily indicate a spawning location, because more developed eggs may have drifted long distances in coastal currents. Newly spawned eggs have only a few embryonic cells, whereas eggs in later developmental stages have progressively larger numbers of cells and hence greater amounts of DNA. The progression through developmental stages largely depends on temperature, which influences cell division and growth. Preservation of the eggs for later analysis presents logistical dilemmas. Preservation in ethanol is suitable for DNA extraction but obscures developmental stage, as the eggs shrink and turn opaque. On the other hand, preservation in formaldehyde retains visual characteristics, but this method limits the ability to extract intact DNA. Here we report a method to estimate developmental stages in Atlantic cod eggs by assaying the amount of DNA from eggs preserved in ethanol. We used a correlation between the amount of DNA in an egg and its embryonic developmental stage in samples from western Norway and used this correlation to estimate developmental stages of eggs sampled in northern Norway.

Keywords: Atlantic cod, DNA extraction, embryonic developmental stages.

Introduction

Sampling newly spawned pelagic eggs is widely used to map spawning areas to predict future recruitment and to study the dynamics of larval development among stocks (Ciannelli *et al.*, 2010; Börjesson *et al.*, 2013; Espeland *et al.*, 2015). Atlantic cod (*Gadus morhua*) spawn large numbers of pelagic eggs that have a relatively long pelagic period that depends in part on water temperature. At 4°C, egg development progresses from zygotes in newly spawned eggs to hatched pelagic larvae in 20–24 days (~500 h), whereas in colder waters (near 0°C), development times may extend beyond 40 days (Westernhagen, 1970). During this long pelagic period, developing eggs often experience a large amount of variation in oceanographic processes and may be advected far from the spawning area (Myksvoll *et al.*, 2014; Espeland *et al.*, 2015). The analysis of developmental stage can provide important information about the dynamics of a population; newly fertilized eggs are likely to have been spawned nearby, whereas more developed eggs may have originated from a distant spawning area and possibly from a different population. The sampling of eggs may be more efficient than sampling adult fish to collect DNA for genetic analysis. For instance, in marine protected areas it may be more acceptable to sample eggs from highly fecund species, as eggs have a high expected mortality, while fishing mature adults may be undesirable and unnecessary.

Embryonic growth is marked by five developmental stages (I–V) that can be determined visually by size and appearance of an embryo in translucent eggs (Hiemstra, 1962; Thompson and Riley, 1981). The first stage can be subdivided into stages I (2) and I (4), depending on whether two or four cells are visible. The identification of more than 8 cells is difficult, so eggs with more than eight cells are classified as stage I. The transitions between developmental stages are approximate and are difficult to determine exactly. For

instance, a stage II embryo covers about half the egg, whereas a stage III embryo covers from 1/2 to 3/4 of the egg. The developmental stages roughly correspond to egg age, although the relationship depends on temperature and is not linear.

Egg size is often used to determine the species, with fresh material or with samples fixed in formaldehyde. Early stage cod eggs are sometimes misidentified as haddock eggs, as the eggs of these species have overlapping size ranges. In such cases, accurate species identifications can be made only by using species-specific genetic markers. TaqMan probes can be used to assay DNA variability in eggs preserved in formaldehyde (Goodsir *et al.* 2008), but with lower success rate (84–96%) than in samples preserved in ethanol.

The method of egg preservation is a tradeoff between preserving visual characteristics for stage classification and facilitating DNA extraction for genetic analysis. When the goal of a study is to assay DNA, the eggs must be preserved in ethanol, not formaldehyde, as the latter denatures DNA but preserves visual features of development (Srinivasan *et al.*, 2002; Karaiskou *et al.*, 2007). Ethanol-preserved eggs shrink, lose pigmentation and become opaque (Steedman, 1976; Goodsir *et al.*, 2008).

One solution would be to identify developmental stages of the eggs visually upon sampling, then preserve them in ethanol for genetic analysis. Eggs would have to be stored in individual containers to be able to link developmental stage with an individual egg. However, field protocols for sampling eggs are usually restricted by on-board logistics. The preservation of individuals in separate containers, expert identification and determination of stage may not be feasible during the sampling of large numbers of eggs at numerous stations (Fox *et al.*, 2008). Hence, eggs are generally preserved in batches by location, but batch preservation with ethanol precludes matching the stage of development with an individual egg.

The choice and standardization of the DNA extraction method is also important. Early stage eggs contain only a few cells, and may be more prone to PCR failure because of the lack of a sufficient amount of template DNA. If eggs at various developmental stages in a batch sample have different origins, age (stage)-biased success of DNA extraction may produce misleading conclusions. Thus, DNA extraction methods producing maximum yields are essential.

In this study, we show how measures of DNA concentration after standardized extraction protocols can be used to estimate the developmental stage of an egg. We used a sample of Atlantic cod eggs of known developmental stages from western Norway to establish a relationship between egg stage and DNA content. We then examined another sample from northern Norway to estimate developmental stage based on DNA concentration and compared the estimates with observed stages of eggs to show the cross-region validity of this method.

Material and methods

Eggs used in this study were sampled in 2015 and 2016 from two areas in Norway as a part of "National programme for mapping of biodiversity—coast". The goal of this programme was to map spawning areas of coastal cod along the Norwegian coast through sampling of cod eggs during the spawning season. Three surveys were conducted, one in Sogn and Fjordane county in western Norway in 2015 and two in Finnmark county in northern Norway in 2015 and 2016. Stations sampled were placed in a grid mainly covering the inner parts of the fjords (Figure 1) to sample

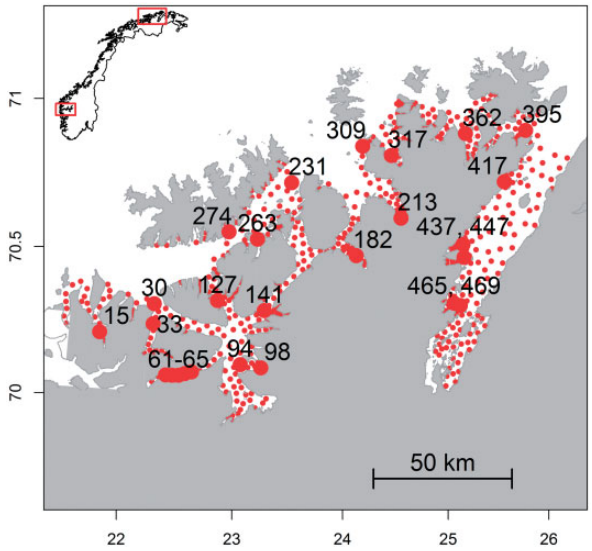

Figure 1. Large map show the area studied in Finnmark, northern Norway. Small dots represent the stations sampled. Larger dots with numbers correspond to station numbers and locations where eggs were subsampled for genetic analysis. The inset map show the entire country of Norway with a northern red box indicating the Finnmark sampling area and a box in western Norway indicating the area of Sogn and Fjordane County.

both spawning areas with a high density of cod eggs and areas with background densities of eggs. Background densities consisted of older eggs advected from nearby spawning areas.

Stations were sampled by vertical net hauls with a WP2 plankton net, which is a conic net with an upper diameter of 0.6 m and mesh-size 500 µm. Hauls were made at an optimal speed of 0.5 m/s (Barnes, 1949). All fish eggs were extracted from the sample and were separated into cod eggs or eggs of other species. Early stage cod eggs were identified by size (1.2–1.5 mm), whereas older stages were identified by larval pigmentation (Hiemstra, 1962). Cod eggs were placed into one of five developmental stages (Thompson and Riley, 1981). In addition to these five stages, the first stage was separated in two sub stages, stage I (2) with only two visible cells inside the egg and stage I with more than 2 visible cells. The eggs of both cod and others species were photographed and preserved in 96% ethanol in glass bottles by sampling station.

To calibrate the relationship between DNA concentration and egg stage, eggs from selected stations in Sogn and Fjordane County were sorted and preserved separately by stage. Samples for analyses were selected by the availability of an adequate number of eggs in each stage, preferably 24 eggs from each stage. In Sogn and Fjordane a total of 3876 cod eggs were sampled from 337 stations. From these, a total of 108 eggs from the six stages, as well as 11 cod larvae, were selected to establish the relationship between stage and DNA concentration (Table 1). For further analysis, "larvae" were classified as one of the stages, although this is not technically an issue as larvae are easy to separate from eggs in fresh as well as preserved material.

In Finnmark, a total of 45 711 cod eggs from 497 stations were sampled in 2015 and 2016 and preserved in ethanol. Eggs were sampled with a WP2 net using the same procedure as in Sogn and Fjordane. Eggs were visually classified to stage and preserved in ethanol in batches by station.

Table 1. The number of eggs sampled in Sogn og Fjordane county by different stages.

Development stages	No. eggs	Mean DNA (ng μl^{-1})	SD (ng μl^{-1})
I (2)	14	31	4.19
I	24	40	5.86
II	24	42	7.62
III	24	65	12.83
IV	12	112	30.39
V	10	141	17.69
Larvae	11	175	49.07

The mean DNA concentration in ng μl^{-1} is given for each stage.

Only information of the total number of eggs in different stages from stations was available for analysis. From the 497 stations, 26 were selected for further genetic examination. No larvae were used in the genetic material from Finnmark. Up to 24 eggs from each station were subsampled for a total of 504 eggs (Table 2). Some station samples contained <24 eggs; in these cases, all available eggs were used. As the purpose of the larger project was to map spawning areas for coastal cod, these 26 stations were selected as geographically representative to be further analysed (Taylor *et al.*, 2002; Stenvik *et al.*, 2006). The results of the spawning area survey are not reported here, except to indicate that all samples for DNA analysis consisted of cod eggs, with various portions of coastal and North East arctic cod.

DNA was extracted using kits with single filter tubes that were specially designed for small amounts of DNA (omegabiotek.com). In addition to manufacturer's protocol, additional measures were taken to increase the DNA yield by equilibrating filters, repeating elutions and adjusting extraction volumes. After extraction, the DNA concentration (ng μl^{-1}) for each single egg was measured with a NanoVue spectrophotometer (GE healthcare).

The average and standard deviation of DNA concentration for each stage were used to create a Gaussian distribution of DNA concentration at the given stage, which can be interpreted as the probability that an egg with a given DNA concentration belongs to a particular stage (Figure 2). The intercept between probability distributions was used as cut-off points for delineating developmental stages. An ANOVA and a Tukey's Honest Significant Difference test (Chambers *et al.*, 1992; Yandell, 1997) was used to test for differences in mean DNA concentrations between all stages. These tests revealed pairwise statistical differences between stages except between the two sub stages of stage I and between stages I and II (Table 3). Hence, eggs in stages I (2), I and II were pooled into an "early life stage" group for further analysis.

To test the assignments to developmental stage, the original eggs and larvae from Sogn and Fjordane were reassigned to the stage of highest probability and compared with their original visual assignments. Further, eggs from Finnmark (2015 and 2016) subsampled for DNA analysis were assigned a stage based on DNA concentration without prior visual assignment. Since "Larvae" was included as a stage in the trial data for Sogn and Fjordane, two eggs from Finnmark were assigned the "larvae" stage due to high DNA concentrations. These samples were not used in further analysis.

The proportion of eggs predicted to be in the various stages from the genetic analysis was compared with the visual classifications of developmental stages in the entire sample from each station. Comparisons were made using the "prop.tes" function in the "stats" library in R (Newcombe, 1998a,b; R Core Team, 2016). This procedure uses a binomial distribution to test the null hypothesis that the proportions in several groups are the same. One test was performed for each stage from each station to compare predicted vs observed proportions. Stages tested were Early stage, stages III–V, for a total of 104 tests.

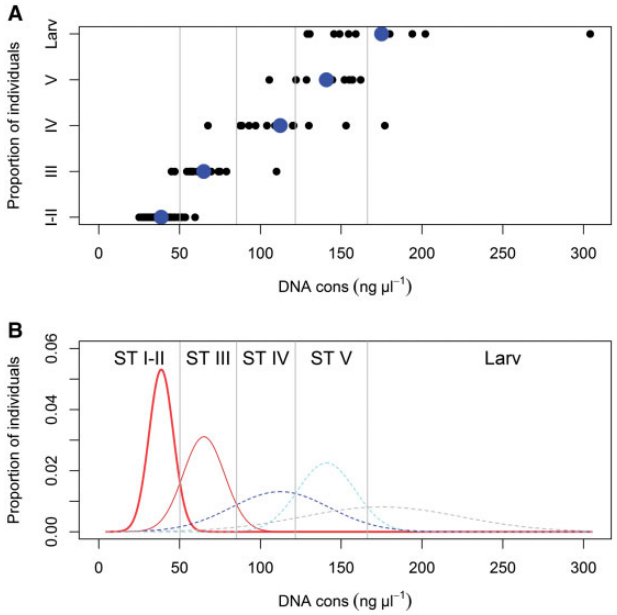

Figure 2. DNA concentration in relation to egg stage. Black diamonds in the upper panel (a) plots observed DNA concentrations as measured by the NanoVue spectrophotometer method from eggs. Symbols are grouped according to egg stage given on the y-axis. Blue points give the average for each stage, while vertical grey lines indicate transitions between stages. In the lower panel (b), a Gaussian distribution is created for each stage from the mean and *SD* of the observed DNA concentrations. This can be interpreted as the probability that an egg with a given DNA concentration belongs to the different stages. Vertical grey transition lines are constructed where the distributions intersect.

Results

Of the 119 eggs and larvae collected from Sogn and Fjordane that were stored in stage-specific batches, DNA was successfully extracted in concentrations ranging from 25 to 304 ng μl^{-1} in 100% of the eggs. Of the 504 eggs selected for DNA extraction in Finnmark, one egg did not yield a traceable amount of DNA.

Eggs with <50.25 ng μl^{-1} were considered "Early stages", based on the distributions of DNA concentration among stages. Eggs with DNA concentrations >50.25 ng μl^{-1} and up to 85.35 ng μl^{-1} were placed in stage III. Stage IV eggs had up to 121.65 ng μl^{-1} DNA, and stage V eggs had up to 166.35 ng μl^{-1}. Eggs yielding larger concentrations of DNA were most likely larvae (Figure 2).

The average DNA concentration for a given stage was consistently lower than for older stages. The results of Tukey's Honest Significant Difference test (Table 3) revealed pairwise significant differences in the mean DNA concentrations of the different stages, except the first three (stage I (2), stages I and II). Younger eggs at stage 1(2) tended to have less DNA than either stages I and II eggs, but this difference was not significant.

Table 2. Numbers of total eggs sampled and subsamples for genetic analysis at 26 stations in Finnmark (shown in Figure 1).

Station number	Total number of eggs	For DNA analysis	Station number	Total number of eggs	For DNA analysis
182	70	24	417	52	24
213	198	24	465	17	12
231	267	24	469	14	12
263	408	24	30	22	22
274	144	24	61	5	5
437	53	24	62	13	7
447	64	24	63	6	6
15	41	24	64	3	3
33	129	24	65	3	2
309	52	24	94	134	26
317	190	24	98	33	24
362	195	24	127	46	24
395	27	24	141	49	24

Table 3. Results from pairwise differences in DNA concentration between stages.

		Developmental stages						
		I (2)	**I**	**II**	**III**	**IV**	**V**	**Larvae**
Developmental stages	I (2)		0.81695	0.66886	**0.00002**	**0.00**	**0.00**	**0.00**
	I	9.04		0.99999	**0.00048**	**0.00**	**0.00**	**0.00**
	II	10.71	1.66		**0.00145**	**0.00**	**0.00**	**0.00**
	III	34.00	24.95	23.29		**0.00**	**0.00**	**0.00**
	IV	81.25	72.20	70.54	47.25		**0.01576**	**0.00**
	V	109.85	100.00	99.14	75.85	28.60		**0.00229**
	Larvae	144.00	134.96	133.29	110.00	62.75	34.15	

Numbers below the diagonal is the absolute difference of DNA concentration in ng μl^{-1} between average of the stage given by the horizontal columns against the average given by the vertical rows. The number above the diagonal line gives the *p*-value of the pairwise significant test between the average DNA concentrations of the two stages. Significant numbers are given in bold.

When the eggs from Sogn and Fjordane were reassigned to a stage based on their DNA concentration, 84% were given the same stage as in the original visual classification. A total of 19 eggs were predicted to be in another stage than in the visual classification. Of these 19 eggs, one was visually classified as stage IV but predicted with DNA concentration to be a larva, whereas all others were predicted to be either one stage earlier or later.

In Finnmark, the observed proportion of eggs in the various stages resembled the predicted proportion based on the DNA concentrations. In 104 tests between visual identifications of egg stage and DNA estimates of egg stage, 66% were not significantly different. These tests included most of the stations for which nearly all eggs were subsampled for genetic analysis (stations: 30, 61, 62, 63,64 65, 98, and 465). At 6 stations (stations: 15, 231, 263, 317, 469, and 141), the DNA predicted distributions of stages of eggs were slightly larger (greater development) than visual classification. No geographic pattern was found in relation to discrepancies between visual and DNA classifications of developmental stages.

Discussion

Overall, the DNA concentrations in eggs gave relatively accurate estimates of the developmental stage of the egg, indicating that DNA concentrations can be used to estimate the unknown developmental stages of eggs preserved in ethanol (Figure 2). However, two caveats are in order. First, the amount of DNA extracted from an egg can be influenced by various DNA extraction methods and by adherence to a particular extraction protocol. Second, variability of DNA concentration among eggs at each stage adds error to estimating the developmental stage of a single egg. Embryonic growth is a continuous process, whereas developmental-stage classifications impose artificial boundaries on this process. When eggs move from one stage to another, visual assignments by experts may differ.

The accuracy of using DNA-based classifications of developmental stage was acceptable for both areas examined in our study. DNA-stage classifications of eggs from Sogn and Fjordane differed from visual classifications in 16% of the eggs examined, most likely because the visual determination of stages may have been difficult for these samples. In Finnmark, our estimates of developmental stage showed no or minor deviations between observed and predicted distribution of stages at most stations. Samples from three stations were moderately different, while three stations (stations: 263, 15, and 469) showed large deviations. All distributions are provided in the Supplementary information and two stations (15 and 469) are shown in Figure 3. The genetic subsample from station 469 that was used to predict stages was based on 12 out of 14 eggs, so stage proportions should be similar, while the proportion test revealed significant differences in the early stages and stage 4. The range of DNA concentrations of individual eggs from this station were close to transitions between stages. Since only 12 eggs were used, every egg accounted for a proportion of 0.08. One egg was predicted to be at stage IV based on a low DNA concentration, but was still

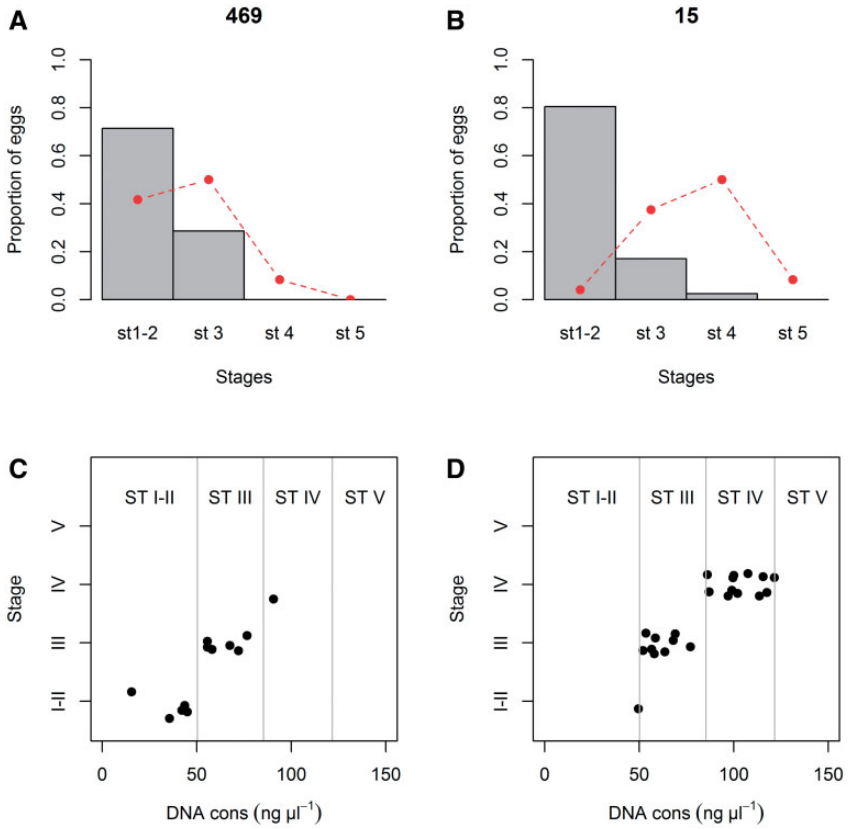

Figure 3. Comparison of the predicted and observed distribution of stages of eggs from a two station (469 and 15). The two upper panels (a and b) show solid bars that represent the proportion of observed eggs of a given stage in the station. Circular dots connected with lines show the predicted proportion of eggs from a given stage based on DNA concentration for a subsample from the given station. The two lower panels (c and d) show the DNA concentration measured for each egg in the two stations as dots along the x-axis. The symbols are grouped on the y-axis according to the stage predicted from the DNA concentration.

above 85.35 ng µl^{-1}. This egg could have been visually classified as stage III during the field sampling. Further, three eggs in the lower part of the DNA concentration interval of stage III could have been visually classified as stage II. Thus these four eggs together could account for the significant discrepancies in proportions of visually determined stages and stages assigned by DNA concentrations.

The proportions of eggs from station 15 (Figure 3) also differed between visual stage classification and the predicted stage based on DNA concentration. This could partly be attributed to eggs that are close to transitions between stages, as in station 469. In this sample, the genetic subsample included 24 of 41 eggs, so this subsample may not have been a true representation of the entire sample. In this case, too few early stage eggs may have been subsampled for genetic analysis.

Nevertheless, DNA concentration provides accurate estimates of developmental stage that correspond well with visual classifications of fresh eggs. The results of our study validate the assumption that a larger number of cells in more developed embryos yield a greater amount of DNA, so that DNA concentration can be used to estimate developmental stages of eggs in a batch sample. Unlike tissue samples from a large individual, the analysis of an individual egg includes tissue of the entire individual. Since early developmental stages of a cod egg is characterized by rapid cell division, DNA concentration provides considerable power to distinguish among stages.

Supplementary data

Supplementary material is available at the *ICESJMS* online version of the article.

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