TaqMan DNA technology confirms likely overestimation of cod (*Gadus morhua* L.) egg abundance in the Irish Sea: implications for the assessment of the cod stock and mapping of spawning areas using egg-based methods

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Abstract

Recent substantial declines in northeastern Atlantic cod stocks necessitate improved biological knowledge and the development of techniques to complement standard stock assessment methods (which largely depend on accurate commercial catch data). In 2003, an ichthyoplankton survey was undertaken in the Irish Sea and subsamples of 'cod-like' eggs were analysed using a TaqMan multiplex, PCR (polymerase chain reaction) assay (with specific probes for cod, haddock and whiting). The TaqMan method was readily applied to the large number of samples ($n = 2770$) generated during the survey and when combined with a manual DNA extraction protocol had a low failure rate of 6%. Of the early stage 'cod-like' eggs (1.2–1.75 mm diameter) positively identified: 34% were cod, 8% haddock and 58% whiting. As previous stock estimates based on egg surveys for Irish Sea cod assumed that the majority of 'cod-like' eggs were from cod, the TaqMan results confirm that there was probably substantial contamination by eggs of whiting and haddock that would have inflated estimates of the stock biomass.

Introduction

Many cod (*Gadus morhua*) stocks in the northeast Atlantic are severely depleted because of overfishing combined with recent adverse environmental conditions (Beaugrand *et al*. 2003). For ecological research, changes in spawning grounds over time are an important consideration. Ichthyoplankton surveys can be a powerful tool to monitor such changes. Conservation efforts also rely on assessments of the size of the stock and virtual population analysis (VPA) is the most widely used assessment method in the northeast Atlantic. Unfortunately, for stocks at low abundance significant bias can be introduced into this method from misreporting of landings, undeclared discarding and

by the model assumption of constant natural mortality (Cotter *et al*. 2004). Ichthyoplankton surveys can be used as an additional stock assessment tool that is free from these underlying assumptions. In the annual egg production methods (AEPM), one uses ichthyoplankton surveys to estimate egg production over the spawning season. This information is combined with estimates of weight specific fecundity of the female fish, levels of atresia (degradation and absorption of oocytes prior to spawning), and the sex ratio of the population to produce an estimate of the spawning stock biomass.

In 1995, AEPM was applied to cod (*Gadus morhua* L.), plaice (*Pleuronectes platessa* L.) and sole (*Solea solea* L.) in the Irish Sea. For cod, the spawning stock biomass estimates generated were considerably in excess of those derived from VPA (Armstrong *et al*. 2001). One potential reason for this discrepancy may have been the difficulty in unambiguously identifying the early stage eggs of cod from those of other species with similar appearance and overlapping size

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ranges, in particular haddock (*Melanogrammus aeglefinus*) and whiting (*Merlangius merlangus*). As only small quantities of mature haddock were being caught in the area at the time, it was assumed that the majority of eggs between 1.2 and 1.75 mm diameter and lacking oil globules were those of cod (Fox *et al.* 1997; Armstrong *et al.* 2001). Since then, the amount of mature haddock in the Irish Sea has increased and the assumption that the majority of 'cod-like' eggs are truly cod is no longer tenable (Dickey-Collas *et al.* 2003).

In 2000, the AEPM was repeated and iso-electric focusing (IEF) was used to identify subsamples of eggs to species (Mork *et al.* 1983). In practice, this method had a number of disadvantages which presented practical problems at sea and possibly led to a relatively high proportion (36%) of failed reactions (Heffernan *et al.* 2004). Despite the high failure rates, the data suggested that the eggs contained a significant fraction of species other than cod.

In an attempt to overcome the problems with IEF, we have developed a highly specific and sensitive DNA-based egg identification method (Taylor *et al.* 2002). In this study we report the results from the first field application of this method and evaluate the relative advantages/disadvantages of the TaqMan approach compared to IEF. We also address the hypothesis that misidentification of cod eggs could have contributed to the apparently elevated spawning stock biomass estimates generated by AEPM.

Materials and methods

Field sampling

A single survey was undertaken using research vessel (RV) Corystes between 18 February and 4 March 2003 following the design employed in 1995 and 2000 (Armstrong *et al.* 2001). At each station, plankton were collected using a Gulf VII high-speed sampler deployed in a double-oblique manner to within 3 m of the seabed at a towing speed of 3–4.5 knots (Nash *et al.* 1998). On shallow stations, multiple oblique tows were undertaken to ensure that a sufficient volume of water was filtered. Upon recovery, the plankton sample was transferred into a jug and kept cool on ice. Aliquots of the sample were examined in Pyrex pie dishes and 'cod-sized' eggs removed using wide-bored pipettes. These eggs were transferred into drops of seawater on a Petri dish and measured using an interactive image-analysis system (PISCES, Perceptive Instruments). Eggs falling within the size range 0.9–1.75 mm diameter and not possessing oil globules or other characteristic features (such as the segmented yolk of sprat eggs, *Sprattus sprattus*) were classified as 'cod-like'. These eggs were assigned a developmental stage according to Thompson & Riley (1981) and transferred into individually labelled tubes containing 1.5 mL of ethanol. Shipboard sorting was continued until up to 100 eggs had been

removed from the sample. The remainder of the plankton sample was then fixed in 4% formalin [4% formaldehyde in distilled water buffered with 2.5% sodium acetate trihydrate (w/v)]. Fish eggs and larvae were subsequently sorted, identified and enumerated on the basis of size and appearance (Russell 1976). All eggs larger than 1.2 mm in diameter were identified, measured and assigned to development stage. On stations where there were very high abundance of smaller eggs, a Folsom splitter was used for subsampling (UNESCO 1968).

Application of TaqMan probes to presorted eggs

The technical details of the TaqMan method for distinguishing eggs of cod, haddock and whiting are described in Taylor *et al.* (2002). In summary, the technique is a polymerase chain reaction (PCR) monitored in real time by the release of up to three fluorogenic dyes with unique emission spectra in a multiplex reaction. Each dye is linked to a species-specific probe and a quencher so that the release of the dye from the quencher results in an increase in detection signal proportional to the rate of amplification for that specific probe.

Total DNA was extracted from individual eggs in 96-well plates using Proteinase K digestion and either the Bilatest Magnetic Bead Extraction Kit (Bilatec AG) running on a Roboseq 4204 S robot (MWG), or salt precipitation using a manual protocol modified from Aljanabi & Martinez (1997). PCR was undertaken using 1 μL (*c.* 50 ng) of extracted DNA per well amplified with 200 nM of each species-specific probe (COD-P, FAM labelled, HAD-P, TET labelled, and WHI-P, VIC labelled), 300 nM of the GAD-F and GAD-R primers, 8.3 μL of TaqMan Universal PCR Master Mix (ROX passive reference) (Applied Biosystems) and 4.9 μL tissue culture H_2O (Sigma). Plates were run under real time conditions on three dye layers with eight no template controls (NTCs) and three positives (DNA extracted from eggs from hatchery cod, haddock and whiting) per 96-well plate. The assay was run using the default cycling conditions. Post PCR, the results were analysed using the SEQUENCE DETECTION SOFTWARE version 1.71 (Applied Biosystems). The ΔR_n values for each cycle and dye layer were then exported to MS Excel, and processed further as described in Taylor *et al.* (2002).

Samples which had been manually extracted and failed to react with the TaqMan probes were amplified using universal teleost cytochrome B primers GLU (L)-TGACTT-GAAGAACCAC/TCGTTG-3' (Palumbi 1996) and CB2-(H)-AAAC TGCAGCCCCTCAGAATGATATTTGTCCTCA-3' (Kocher *et al.* 1989). PCR was performed in an MJ Research PTC-200 thermal-cycler under the following conditions: 94 °C for 120 s; followed by 30 cycles of 94 °C for 30 s, 47 °C for 30 s, 72 °C for 45 s; followed by 72 °C for 10 min. Ten μL reaction mixes consisted of 1 μL (*c.* 20 ng) template DNA, 0.5 μm of each primer, 5 μL of 2× PCR Master Mix (ABGene),

and 2 μL H$_2$O. PCR products were resolved on 1.2% agarose gels run in 0.5× TBE buffer and stained with ethidium bromide. Samples that produced amplification products were classed as 'null' reactions, i.e. not cod, haddock or whiting but another species for which specific probes have not yet been developed. Samples that produced no amplification products were classified as 'failed extractions'.

Statistical treatment of data

The accuracy of the subsampling undertaken at sea was assessed by comparison of egg diameter and developmental stage frequency distributions in the subsamples and in the subsequent laboratory analyses of the remaining formalin-fixed eggs. Second, the reliability of the TaqMan egg identification method was assessed by consideration of percentages of samples positively identified and producing 'null' or 'failed' reactions. The data were then filtered to include only eggs at the early developmental stage (stage I). At the water temperatures recorded during the ichthyoplankton survey (4–8 °C), cod eggs take between 3.5 and 5 days to develop through the end of stage I so the location of stage I eggs will be close to site of spawning (Thompson & Riley 1981). At each sampling location, the number of 'cod-like' eggs in the laboratory-sorted portion of the plankton sample was apportioned between cod, haddock, whiting and other species on the basis of the ratio determined from the TaqMan analyses of the presorted eggs from that location.

Results

On stations with low to medium abundance of 'cod-like' eggs (< 500 eggs), we were usually able to presort for subsequent TaqMan analysis more than 20% of the eggs from the total available. On stations where eggs were very abundant, the percentage presorted varied between 1% and 10% of the eggs available. The frequency distribution of developmental stages in the presorted subsamples and in the remainder of the total plankton sample was in good
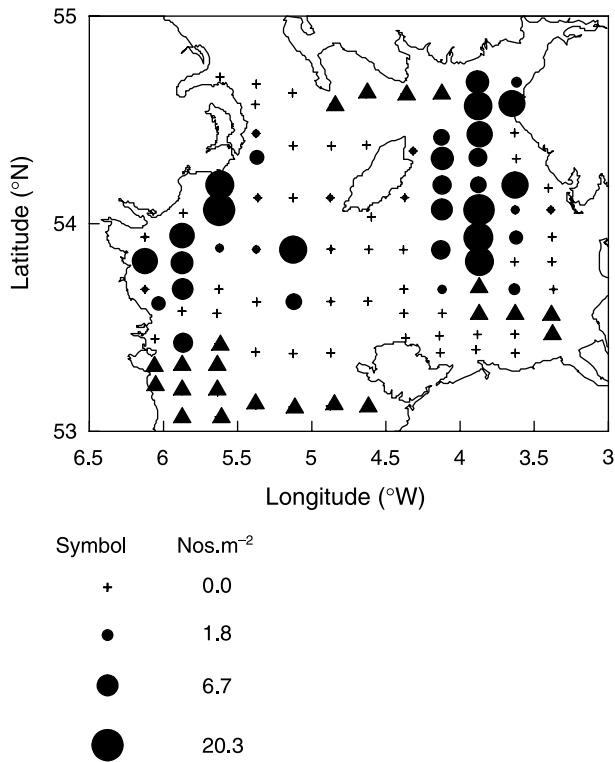
agreement although there may have been a slight bias towards presorting too many stage V eggs (percentages presorted compared with percentage of stages in the remainder of plankton sample: stage I, 43.4% cf. 44.1%; stage II 23.4% cf. 28.4%; stage III 23.8% cf. 20.2%; stage IV 7.1% cf. 6.6%; stage V 2.4% cf. 0.7%). These results support the conclusion that presorting at sea was capable of producing a representative subsample from the total eggs present.

The majority of eggs lacking oil globules collected in the survey were smaller than 1.0 mm diameter and were most likely produced by dab (*Limanda limanda*) and flounder (*Platichthys flesus*). The size range of eggs positively identified as cod by the TaqMan assay was 1.17–1.63 mm (95 percentiles of size distribution) and was 1.17–1.62 mm for haddock. These ranges were in good agreement with those quoted in Russell (1976). Using a lower limit of 1.2 mm diameter as a cut-off for staging eggs in the formalin-fixed portion of the sample would have encompassed 97% of cod eggs present and 98% of haddock. The maximum size of positively identified whiting eggs was slightly greater than literature estimates, 95% of the observations lay within the range 0.97–1.41 mm (Russell 1976 quotes a min–max range of 0.97–1.32 mm).

The results of TaqMan analysis of robotically and manually extracted eggs are given in Table 1. An unacceptably high proportion of eggs extracted using the robotic procedure failed to react with TaqMan probes (27%). In the majority of cases this appeared to be caused by the robot having failed to deliver reagents to the correct wells on the plate, which caused high levels of cross-contamination and irrecoverable loss of the samples. Because of this, the robotic DNA extraction was replaced with a manual procedure. When the remaining samples were extracted using the manual protocol, the failure rate dropped to 6%. Failure rates were not related to egg development stage indicating that the TaqMan PCR probes are sufficiently sensitive to identify even the earliest stage eggs that contain low levels of DNA. The occurrence of failures was also unrelated to

Table 1 Numbers of eggs presorted and genetically analysed and success rates of TaqMan species identification. Includes all eggs collected (0.9–1.75 mm diameter)

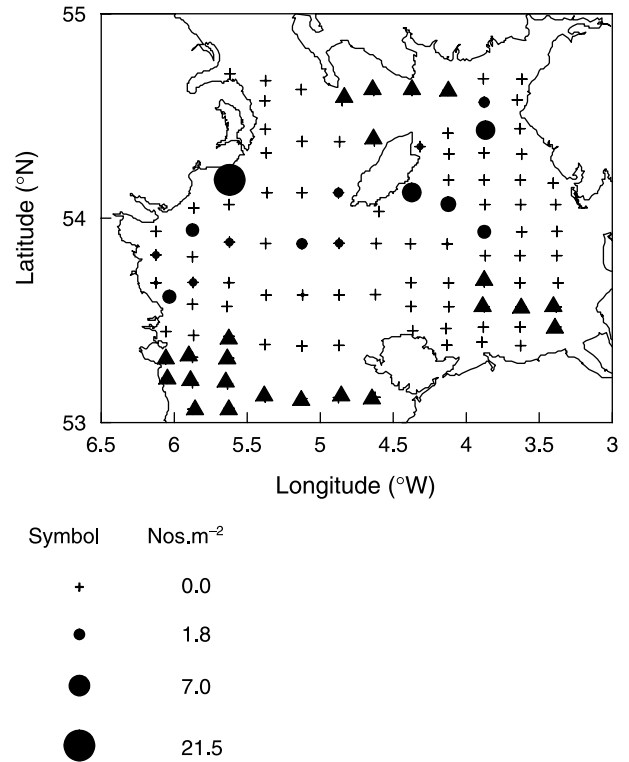| Stage	Number eggs — robotic DNA extraction	Number eggs — manual DNA extraction	Failed TaqMan — robotic DNA extraction (%)	Positively identified by TaqMan — robotic DNA extraction (%)	Failed TaqMan — manual DNA extraction (%)	Null reaction TaqMan — manual DNA extraction (%)	Positively identified TaqMan — manual DNA extraction (%)
I	608	563	24.6	75.4	7.3	5.5	87.2
II	238	428	27.3	72.7	4.9	4.9	90.2
III	210	463	30.0	70.0	5.6	5.0	89.4
IV	98	107	35.7	64.3	6.5	6.5	86.9
V	21	34	38.1	61.9	2.9	0.0	97.1
Total	1174	1595	27.2	72.8	6.0	5.2	88.8

Fig. 1 Chart showing the distribution of stage I cod eggs based on number of 'cod-like' eggs in the plankton samples raised by the proportion of positively identified cod eggs by TaqMan. The data are expressed as nos m^{-2} sea surface, bubble diameter represents abundance following a logarithmic transformation. Solid triangle symbols indicate stations where there were insufficient genetic results to apply proportional raising.

Fig. 2 Chart showing the distribution of stage I haddock eggs based on number of 'cod-like' eggs in the plankton samples raised by the proportion of positively identified haddock eggs by TaqMan. Key as Fig. 1.

sample number within each station (C.J. Fox, unpublished). This supports the view that failures were not related to handling time on-board the research vessel and we conclude that sample collection and presorting at sea does not cause significant mitochondrial DNA degradation (at least within the 80 min taken to sort 100 eggs).

Early stage 'cod-like' eggs (> 1.2 mm) were most abundant in areas close to the Irish coast, around the Isle of Man and in the eastern Irish Sea. Based on positive identification by TaqMan (excludes failed extractions), 58% of these were whiting, 34% were cod and 8% were haddock. The distribution of positively identified cod eggs was generally similar to the distribution of 'cod-like' eggs but cod were absent off the North Wales coast (Fig. 1). Stage I haddock eggs were confined to a few stations off the Irish coast and to the southeast of the Isle of Man (Fig. 2). The TaqMan analyses showed that the majority of 'cod-like' eggs sampled were in fact whiting. The eggs of this species were widely distributed across the shallower stations. The loss of several complete plates of samples (during robotic processing)

prevented estimation of egg abundance by species at a number of stations. However, these largely coincided with stations with low overall numbers of 'cod-like' eggs and it is not anticipated that their absence would markedly alter the resulting species-specific distribution patterns.

Discussion

Three key outcomes have arisen from this study. First, around one-third of the 'cod-like' eggs analysed were positively identified as cod. Second, early stage haddock eggs were identified proving that a spawning stock now exists in the Irish Sea. Third, the TaqMan technique can be readily applied to the large numbers of samples collected during ichthyoplankton surveys with good reliability in egg identification (failure rate of 6% using manual DNA extraction procedure).

These results demonstrate that the size fraction of eggs previously assumed to be mostly cod will be contaminated with a substantial proportion of whiting as well as haddock

eggs. Without the application of molecular identification, the assumption that all 'cod-like' eggs are cod will lead to inaccurate spawning maps and inflate spawning stock biomass estimates made by egg production methods. The present results, along with those of Heffernan *et al.* (2004), show that at least part of the discrepancy between the stock biomass estimate produced from the 1995 AEPM and from fishery-dependent methods (e.g. VPA) was probably because of egg misidentification (Armstrong *et al.* 2001; Armstrong & Dickey-Collas 2002).

Ideally, any species identification method should be capable of positively identifying close to 100% of the samples analysed. This removes any element of doubt and simplifies the apportioning of species ratios determined on subsamples. In the Irish Sea, a few species in addition to cod, haddock and whiting produce eggs with overlapping size ranges and similar appearance. Based on IEF results (Heffernan *et al.* 2004) and the occurrence of larvae (Fox *et al.* 1997; Bunn & Fox 2004), these include: lemon sole (*Microstomus kitt*), witch (*Glyptocephalus cynoglossus*), pollack (*Pollachius pollachius*), saithe (*Pollachious virens*), bib (*Trisopterus luscus*) and Norway pout (*Trisopterus esmarkii*). Because of high development costs, we have so far only produced specific TaqMan probes for the three main commercial species. Eggs of other species can thus produce a null reaction with the TaqMan probes but by re-amplifying with universal teleost primers we were able to distinguish these samples from those where DNA extraction had failed or DNA had degraded. The percentage occurrence of these other species in the Irish Sea was relatively low (*c.* 5% based on eggs of all sizes which had been manually extracted, failed to react with TaqMan probes and were successfully re-amplified with universal primers) but in other sea areas they could form a larger proportion of eggs sampled. The capacity to expand the number of species that can be identified in a multiplex TaqMan reaction is currently limited by the number of wavelength specific dyes available.

Surveys for mapping spawning locations should ideally cover the whole spawning season. Because this was a demonstration project and timing was constrained by ship availability only a single survey was undertaken. It is thus not possible to describe the temporal extent of spawning from these data. Historical data, based on the appearance of late stage eggs and larvae, suggest that the peak of cod spawning occurs from early to the middle of March in the western Irish Sea, and a little later in the eastern area (Brander 1994; Fox *et al.* 1997; Bunn & Fox 2004). The 2003 survey was therefore probably too early to hit the peak but coincided with the onset of cod spawning in both areas.

Comparing the application of DNA-based and IEF identification to ichthyoplankton surveys: the DNA-based method has greater sensitivity and reliability [comparing results in this study with those in Heffernan *et al.* (2004)]; samples for DNA-based analyses can be stored at room temperature whilst material for IEF must be kept frozen (ideally at −80 °C but at −20 °C for short periods) which can prove problematic at sea and when transporting samples between laboratories; the main disadvantage of the DNA-based method is cost. A significant limitation of applying both techniques to ichthyoplankton surveys is the requirement to presort target eggs from the bulk of the plankton at sea in order to preserve them in a manner that does not degrade DNA (or protein for IEF). This is time consuming and only feasible under reasonably calm sea conditions. Using DNA-based molecular methods, it may be possible to overcome this problem as there are reports of successful PCR from formalin-fixed samples (France & Kocher 1996; Shedlock *et al.* 1997; Chase *et al.* 1998; Kirby & Reid 2001). In contrast, formalin renders material totally unsuitable for IEF. Recent success with the use of monoclonal antibodies with formalin-fixed eggs has also been reported (Hiroishi *et al.* 2004).

In conclusion, the TaqMan method proved suitable for application to the large numbers of samples generated during an ichthyoplankton survey. Early stage haddock eggs were positively identified demonstrating that a spawning stock exists in the Irish Sea. The TaqMan analysis also showed that the majority of 'cod-like' eggs sampled were from whiting supporting the idea that egg misidentification was one potential reason for previous elevated estimates of stock biomass using egg production methods in this region. Wider application of molecular-based identification methods in ichthyoplankton surveys will significantly increase the accuracy of spawning maps and stock assessments for species producing eggs that cannot be separated using classical methods.

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