Short communication

The use of species-specific TaqMan probes for identifying early stage gadoid eggs following formaldehyde fixation

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Goodsir, F., Armstrong, M. J., Witthames, P. R., Maxwell, D. L., and Fox, C. J. 2008. The use of species-specific TaqMan probes for identifying early stage gadoid eggs following formaldehyde fixation. – ICES Journal of Marine Science, 65: 1573–1577.

Surveys of fish eggs are increasingly being used to monitor the spawning areas and stock status of commercially important species such as Atlantic cod (*Gadus morhua*), but early stage cod eggs are visually indistinguishable from those of several other common co-occurring species, including haddock (*Melanogrammus aeglefinus*) and whiting (*Merlangius merlangus*). In recent surveys in the Irish and North Seas, a molecular identification technique (TaqMan multiplex real-time polymerase chain-reaction) assay has been used to overcome this problem. The method needs high-quality DNA, so the current protocol requires that individual "cod-like" eggs are "presorted" from plankton hauls on board ship and immediately preserved in ethanol. This increases seagoing staff costs, can be a difficult process at sea, and means that plankton sampling cannot be undertaken from non-specialized vessels such as fishing boats. Successful application of TaqMan probes to DNA from eggs preserved in formalin would overcome these problems, but previous attempts have resulted in poor success. In this study, batches of hatchery-sourced cod, haddock, and whiting eggs were fixed in 4% buffered formalin for up to 3 weeks, then transferred to a formaldehyde-free solution for 1, 2, or 3 months. After these periods they were assessed visually for fixation quality and analysed using species-specific TaqMan probes. Eggs, which had been fixed for up to 3 weeks in formalin, were identified successfully, although the positive rate (84–96%) was slightly lower than samples preserved throughout in ethanol (92–99%). There was no increase in the percentage of eggs misidentified comparing formalin-fixed and ethanol-preserved material. These results suggest that TaqMan probes can be applied successfully to fish eggs fixed in 4% buffered formalin for up to 3 weeks.

Keywords: fluorogenic 5′ nuclease assay, gadoid, multiplex, real-time PCR, TaqMan.

Received 4 April 2008; accepted 9 October 2008.

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Introduction

Several European cod (*Gadus morhua*) stocks are currently at historically low levels (see the latest stock assessment advice at www.ices.dk). Low stock abundance is often accompanied by increased levels of uncertainty in stock assessments when these are based on commercial fishery and research trawl survey data (Reeves and Pastoors, 2007). In these cases, fishery-independent assessment methods can provide additional sources of information for tracking stock status. One example is the estimation of total egg production in a spawning season which, when combined with estimates of weight-specific fecundity, can be used to estimate the biomass of spawning fish within a region. This is known as the total annual egg production method (AEPM; Armstrong *et al*., 2001). A variant, the daily egg production method (DEPM) is routinely applied to species such as the South African anchovy (*Engraulis encrasicolus*; Shelton *et al*., 1993). Both AEPM and DEPM can only be applied where it is possible to identify the early developmental stages of the eggs in plankton samples to species level. Identification has been traditionally undertaken using egg size and the presence of features such as oil globules (Russell, 1976). This has tended to limit the range of species to which AEPM and DEPM have been applied. Given the poor status of European cod stocks, research has recently been undertaken to see if egg production methods can provide useful additional assessment tools for this species (Armstrong *et al*., 2001). However, the early stage eggs of cod (*G. morhua*) are visually indistinguishable from those of various other species that overlap in geographic distribution and spawning season. The most common of these species are haddock (*Melanogrammus aeglefinus*) and whiting (*Merlangius merlangus*), and misidentification of eggs from these species as cod has the potential to bias egg-based stock assessments (Fox *et al*., 2005b). Taylor *et al*. (2002) developed a sensitive real-time polymerase-chain-reaction (PCR) method using species-specific TaqMan probes. This method has subsequently been applied in the Irish Sea (Fox *et al*., 2005b) and North Sea (Fox *et al*., 2005a) to map cod spawning areas.

Formalin is the fixative of choice for ichthyoplankton samples, being cheap and leading to good preservation of morphology

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(Steedman, 1976a). However, formaldehyde interacts with DNA (Karaiskou *et al*., 2007), and previous attempts to apply the TaqMan method to eggs fixed in formalin were not successful (M. Taylor, Bangor University, pers. comm.). Ethanol is a reliable preservative for DNA but causes fish eggs to shrink and become opaque, leading to difficulties in visually identifying or assigning developmental stages. Molecular identification methods are still relatively expensive, so it is uneconomic to identify all the eggs collected in a plankton sample by molecular means (Lindeque *et al*., 2006). Genetic identification of a targeted subsample (in this case a subsample of "cod-like" eggs) is currently the only practical approach. Consequently, a field-sampling protocol has been used in which a subsample of up to 100 early stage "cod-like" eggs (defined as eggs of diameter 1.1–1.75 mm and lacking oil globules or other distinguishing features) is immediately sorted from each plankton haul upon recovery of the net. These eggs are then preserved in individual vials containing ethanol. The rest of the plankton sample is fixed in 4% formalin, and the fish eggs subsequently sorted and identified using traditional methods to as low a taxonomic grouping as possible. This includes the group "cod-like" eggs. The ethanol-preserved eggs are analysed by TaqMan and the relative proportions of cod, haddock, and whiting eggs in the subsamples then used to allocate the remaining early stage "cod-like" eggs in the bulk of the plankton samples to species (Fox *et al*., 2005a, 2008). The necessity to presort a subsample of the "cod-like" eggs at sea adds to the overall expense of the surveys. Presorting requires a relatively large, stable ship along with additional sea-going staff with the necessary plankton-sorting skills. Presorting 100 eggs can take up to 1 h and this can slow the survey, especially where plankton stations are close together. Data quality can also suffer if deteriorating sea conditions cause poor adherence to subsampling protocols or prevent presorting at particular stations.

Successful application of molecular methods to the identification of formalin-fixed eggs could have a number of potential benefits: survey costs could be reduced; vessels other than dedicated research ships could be used to collect the plankton samples; additional variance introduced through shipboard presorting would be eliminated because samples can be placed directly into formalin and processed back in the laboratory; and genetic analyses could be better targeted because the spatial distribution of the "cod-like" eggs would be fully known before the application of genetic analyses.

Material and methods

Batches of good quality (based on egg buoyancy), early (I and II) and late-stage (III–V) cod, haddock, and whiting eggs were collected from a commercial hatchery (Viking Fish Farms, Ardtoe, Scotland). Eggs were fixed in acetate-buffered 4% formalin (pH 7) for periods of 2, 5, and 21 d. They were then rinsed with fresh water and transferred into Steedman's observation fluid (propylene phenoxetol 0.5 ml; propylene glycol 4.5 ml; seawater or distilled water 95 ml; well shaken; Steedman, 1976a) for periods of 30, 60, or 90 d, respectively. Overall, this resulted in 54 treatment groups. After these times, ~30 eggs from each treatment level were visually assessed for fixation quality. These eggs were then placed into random order and individually labelled using a double-blind approach (i.e. the molecular analyst was not aware of the true identity of each egg). In parallel, ~10 ethanol-preserved eggs per group were interlaced into the series to act as positive controls. The eggs were then analysed using the TaqMan method.

Molecular species identification followed Taylor *et al*. (2002), with minor modifications. DNA was extracted from individual eggs using a Qiagen DNeasy tissue kit (Qiagen). TaqMan chemistry was applied to each template sample in Optical 96-well reaction plates (Applied Biosystems), to a final volume of 25 μl. Each reaction contained 9 μl of TaqMan Universal PCR Master Mix (UNG+ROX passive reference), 5 μl of ultra-pure H_2O, 300 nM of GAD-F and GAD-R primers, and 200 nM of each species-specific probe. These plates were processed under real-time conditions with universal cycling conditions on an Applied Biosystems 7900 real-time sequence-detection system in a three-dye layer set (Cod-FAM, Whiting-VIC, and Haddock-TET) with four "no template" controls per plate. The endpoint (40 cycles) of the PCR was analysed using Sequence Detection System Version 2.1 (Applied Biosystems), and the eggs assigned as cod, haddock, whiting, or a null result. Following molecular analysis, the true identities of the eggs were revealed and compared with the species identities according to the TaqMan results, to determine the accuracy of the method for the different treatment groups.

The results consist of a response with two outcomes (successful, i.e. TaqMan result equal to true identity, or unsuccessful identification, either misclassification or failed PCR), and five potential explanatory variables (ethanol vs. formalin fixation, species, development stage, days in formalin, days in observation fluid), which may act separately or in combination. The results showed a very low level of misclassification (see Results), so the major negative responses were attributable to failed PCR reactions. A series of binomial generalized linear models (GLMs) was used to evaluate differences in the rates that eggs were successfully identified. Fixative effects were tested by fitting models with species, stage, and fixative terms, and their interactions, to the whole dataset, followed by testing the effect of fixative on separate datasets for each combination of species and stage. Next, the results for formalin-fixed eggs (Table 1) were analysed. Differences between species were tested by models including species, stage, days in formalin, and days in observation fluid. Finally, the effects of time in formalin and observation fluid were tested for each species separately using models with these two terms and stage. All models were fitted using the statistical language R v2.6.0 (R Development Core Team, 2007). The p-values presented test the change in deviance against a χ^2 distribution.

Results and discussion

All the eggs visually assessed were classified as being sufficiently well fixed and preserved to allow measurement and assignment to developmental stage. Some eggs showed artefacts such as bubbling and discolouration of yolks (Figure 1a and b), but this has also been noted with eggs fixed and stored in 4% formalin since collection (S. Milligan, Cefas, pers. comm.).

Of a total of 1815 eggs analysed by TaqMan, the overall success rates for species identification were 96% for eggs preserved in ethanol and 91% (averaged across species) for eggs fixed in formalin. None of the ethanol-preserved eggs that produced successful PCR reactions was misclassified by TaqMan ($n = 254$), and only three formalin-fixed eggs, which produced successful PCR reactions, were misclassified. The overall accuracy for positive identifications of formalin-fixed eggs by TaqMan was therefore 99.8% ($n = 1561$).

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Table 1. Percentage of gadoid eggs fixed in formalin correctly identified by TaqMan against their true identity.

Days in formalin	Days in observation fluid	Cod				Haddock				Whiting			
		Early*		Late**		Early*		Late**		Early*		Late**	
		%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*
2	30	97	30	94	31	87	30	–	0	92	50	67	9
5	30	80	30	73	30	81	31	–	0	84	58	50	2
21	30	88	32	96	28	100	30	100	30	83	58	50	2
2	60	91	32	96	28	100	30	100	30	90	52	88	8
5	60	93	30	97	30	100	30	100	30	82	45	87	15
21	60	100	30	100	30	100	30	93	30	84	43	82	17
2	90	97	30	100	30	97	30	100	30	80	30	73	30
5	90	97	30	100	30	97	30	100	30	77	31	100	29
21	90	90	30	93	30	100	30	90	30	82	33	93	27

%, percentage correctly identified; *n*, number of eggs per treatment group.
*Development stages 1 and 2 (pre-embryo).
**Development stages 3, 4, and 5 (embryo formed).

Identification success rates by species for the formalin-fixed eggs were 93% for cod ($n = 541$), 96% for haddock ($n = 481$), and 84% for whiting ($n = 539$). In comparison, the overall identification success rates for eggs fixed in ethanol were 98% for cod ($n = 85$), 99% for haddock ($n = 84$), and 92% for whiting ($n = 85$). The GLM tests indicated that the 5% difference in success rates between the two fixatives was statistically significant ($p = 0.006$). Testing the effects of fixative separately by species and developmental stage, the difference in successful identification rates between fixatives was only significant for early stage whiting eggs ($p < 0.001$), but not for late-stage whiting or for any cod or haddock eggs ($p = 0.14$–0.81). Overall, whiting tended to have a lower success rate of identification than either cod or haddock ($p < 0.001$), an observation made previously for eggs fixed in ethanol. This may be due to the difficulties in multiplex interactions and spatial overlap of the three-dye-layer set FAM, VIC, TET. For formalin-fixed eggs, the egg stage did not significantly influence identification success in any of the three species ($p > 0.2$; Table 1).

There were statistically significant variations between some combinations of treatments ($p < 0.001$), but the variations were small and with no obvious trend or pattern about the time spent in formalin or observation fluid before analysis (Figure 2).

In general, the overall success rate of identification for eggs fixed in formalin for periods of up to 21 d, followed by up to

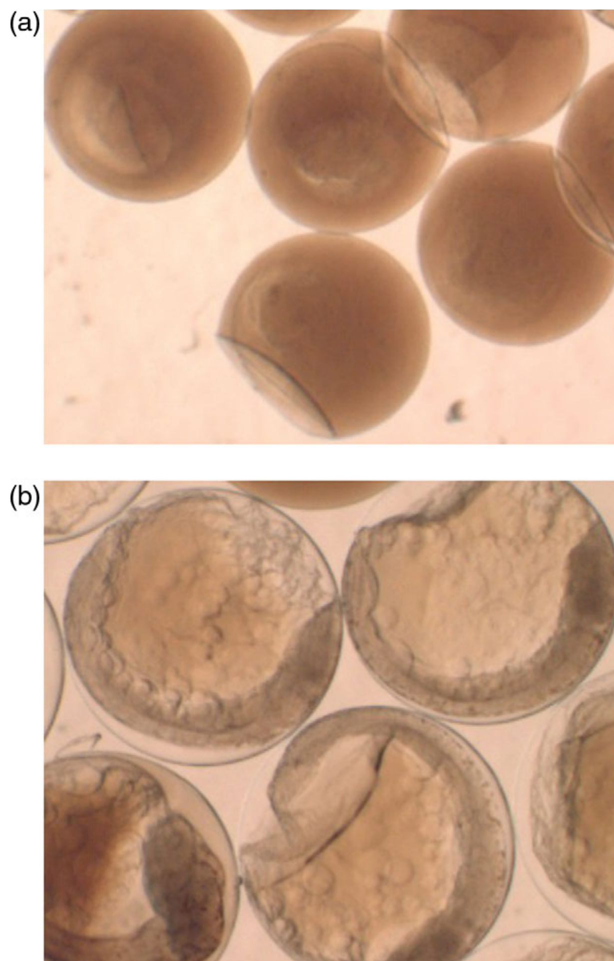

Figure 1. (a) Early stage and (b) late-stage whiting eggs fixed for 21 d followed by storage for 90 d, showing slight darkening of the yolk in (a) and a clear embryo, but with bubbling of the yolk in (b).

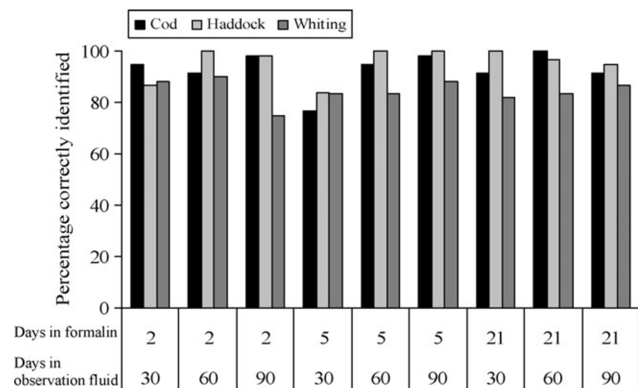

Figure 2. Percentage of eggs correctly identified by TaqMan against their true identity with different combinations of days in formalin and observation fluid (early and late developmental egg stages pooled).

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90 d in observation fluid, reveals both a highly successful (>75%) and highly accurate (99.8%) method. We therefore conclude that the TaqMan method can be used to identify gadoid fish eggs to species even when the eggs have been fixed in formalin for up to 3 weeks. Our success rate was higher than we have attained previously and much better than the 20% success in identifying formalin-fixed horse mackerel (*Trachurus* spp.) eggs reported by Karaiskou *et al*. (2007). The difference between our protocol and that used for previous less successful trials is that the eggs were transferred to a medium free of formaldehyde following fixation. Our results did not demonstrate significant declines in PCR success with fixation periods of up to 3 weeks. It would be worth repeating the experiment with longer fixation periods to determine the point at which PCR success declines significantly. However, it should be practical in most field applications to rinse and transfer the complete plankton sample to a formaldehyde-free solution within 3 weeks, either on board ship or on return to the laboratory. We also took care to ensure adequate buffering of the formalin, because acidification has been reported to be a specific issue (Steedman, 1976b; Koshiba *et al*., 1993).

The effects of formaldehyde on DNA are complex (O'Leary *et al*., 1994), and potentially include interaction with hydrogen bonds, cross-linking of outer proteins, and hydroxymethylation of nucleic acids (Karlsen *et al*., 1994). The exact reactions depend on multiple factors, including specimen type, temperature, fixation pH, and ion concentration (O'Leary *et al*., 1994). It has also been reported that formalin fixation causes infidelity in PCR-amplified DNA fragments, although base-length is not affected (de Giorgi *et al*., 1994). In addition, any formaldehyde residues present will inhibit PCR.

For these reasons, applying molecular methods to formalin-fixed specimens has been considered difficult (Schander and Halanych, 2003). However, many of the reactions between formaldehyde and DNA appear to be reversible (O'Leary *et al*., 1994), and there is an increasing literature on successful PCR from formalin-fixed material. Early success came with *in situ* hybridization using formalin-fixed, paraffin-embedded histological sections (Goelz *et al*., 1985; Rogers *et al*., 1990; Coombs *et al*., 1999). There have been suggestions that paraffin-embedding itself may protect the DNA from some of the degradation effects of formaldehyde. Other workers have reported successful amplification of DNA from archived samples such as museum collections (France and Kocher, 1996; Shedlock *et al*., 1997) or the Continuous Plankton Recorder programme (Kirby and Lindley, 2005). In such cases, even a relatively low rate of success may be sufficient to yield valuable scientific data (Kirby and Reid, 2001). In contrast, in AEPM or DEPM, a relatively high rate of success is needed or the costs of processing eggs that subsequently fail in PCR will rapidly outweigh the cost savings made by eliminating the step of presorting eggs at sea.

The TaqMan method does have a major limitation in the number of dyes (and hence species-specific probes) that can be multiplexed. Although in areas such as the Irish and North Seas, the three gadoid species (cod, haddock, and whiting) provide most "cod-like" eggs found in plankton (Fox *et al*., 2008), in certain areas there are significant numbers of eggs from other species, e.g. saithe (*Pollachius virens*). Failure to produce a positive TaqMan identification can either be due to the presence of eggs of these other species or because the PCR or DNA extraction has failed. This may require further analysis, e.g. sequencing of samples yielding negative TaqMan results (Fox *et al*., 2008). An ideal molecular tool would be able to unambiguously assign all "cod-like" eggs to species. Recent advances in rapid DNA sequencing (e.g. pyrosequencing) may provide such tools. However, the reliability of these newer methods will need to be rigorously evaluated against sample fixation protocols and compared with the TaqMan results presented here. For now, however, the TaqMan method provides a practical solution to identify early stage cod eggs in ichthyoplankton either by presorting "cod-like" eggs at sea and preserving them in ethanol (Fox *et al*., 2008) or by using formalin fixation for up to 3 weeks and accepting the need to sequence a slightly more negative TaqMan reaction. Finally, our results add to the increasing body of literature reporting successful application of molecular methods to biological samples fixed in formalin.

Acknowledgements

This work was supported by the UK's Department for Environment, Food and Rural Affairs (Defra project MF160), in collaboration with Cefas Technology Ltd (CTL). We thank Jim Treasurer, Viking Fish Farms, Michael Goddard, Mike Shaw, and Steve Milligan for their interest and support.

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doi:10.1093/icesjms/fsn180

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