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Developing an eDNA toolkit to quantify broadcast spawning events of the sea scallop *Placopecten magellanicus*: moving beyond fertilization assays

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ABSTRACT: Broadcast spawners release their gametes into the water column for 'chance' fertilization events. However, detection of such events in near real-time is extremely difficult, but needed to improve fisheries and conservation management practices. It is practically impossible to distinguish the gametes of many species by microscopy; therefore, DNA-based techniques are preferable to detect and quantify gametes from field-collected plankton samples. We developed a quantitative PCR (qPCR) approach to detect and quantify broadcast spawning events in marine environmental DNA (eDNA) samples. We applied this approach to a commercially valuable broadcast spawning bivalve species, the sea scallop *Placopecten magellanicus*. Our approach includes (1) sequencing the internal transcribed spacer (ITS) region, (2) developing a novel species-specific probe and primer set, (3) testing the probe and primer set on a dilution series of sea scallop sperm to quantify the relationship between gamete abundance and DNA copy number, and (4) conducting dockside field tests of our method on plankton samples adjacent to naturally spawning sea scallops. Quantitative PCR revealed a clear relationship between DNA copy number and *P. magellanicus* sperm cell abundance, indicating that this method is reliable for detecting sperm release by male scallops during spawning events. Plankton samples collected during the scallop spawning season revealed spikes of scallop eDNA in both the <20 µm (sperm) and >20 µm (possible eggs) particle size-fractions. This method holds great potential to provide more efficient estimates of the timing, magnitude, and spatial scale of reproductive events than conventional methods for a wide range of broadcast spawners.

KEY WORDS: Sea scallop · *Placopecten magellanicus* · Reproductive ecology · eDNA · ITS region · Gamete detection · Broadcast spawning

1. INTRODUCTION

Marine organisms spanning multiple phyla broadcast gametes into the sea where successful fertilization depends on having high concentrations of viable sperm. High-density spawning aggregations of adults can ensure high levels of fertilization success (the percentage of eggs fertilized) before gametes become diluted (Allee 1931, Levitan et al. 1992, Levitan & Petersen 1995, Levitan & Young 1995, Gaudette et al. 2006). Empirical data on fertilization success in natural broadcast spawner populations are relatively rare and generally qualitative (Levitan & Sewell 1998). To date, field studies on fertilization dynamics have been conducted on a relatively small set of experimentally tractable free spawning inver-

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tebrates and fishes: including sea urchins *Strongylocentrotus droebachiensis* (Wahle & Peckham 1999), bluehead wrasse *Thalassoma bifasciatum* (Petersen et al. 1992, Warner et al. 1995), hard corals (*Montipora digitata*, *Favites pentagona*, *Platygyra sinensis*, Oliver & Babcock 1992; *Acanthaster planci*, Babcock & Mundy 1992), soft corals *Briareum asbestinum* (Brazeau & Lasker 1992), and sea cucumbers (*Bohadschia argus, Eupta godeffroyi*, *Stichopus variegatus*, *Holothuria coluber*, *Actinopyga lecanora, Bohadschia graffei*; Babcock et al. 1992). Together they suggest that fertilization success can be quite variable and frequently well below saturating levels, especially at low adult densities and conditions promoting rapid gamete dilution.

These results underscore a long-standing concern that commercially exploited fishes and invertebrates with this mode of reproduction may be especially vulnerable to recruitment failure when populations are depleted (Myers et al. 1995, Petersen & Levitan 2001, Rowe et al. 2004). Aggregating behavior has been observed specifically during the spawning season. Sedentary or slow-moving broadcast spawners that form spawning aggregations, such as the abalone *Haliotis kamtschatkana* (Seamone & Boulding 2011) and scallop (*Chlamys asperrima* and *Chlamys bifrons*, Styan & Butler 2003; *Pecten fumatus*, Mendo et al. 2014), may be particularly vulnerable to the effects of fishing activity that target these aggregations.

Field experiments with experimentally tractable species that are relatively sedentary and easy to induce to spawn have contributed considerably to understanding the importance of sperm limitation in the wild, but they also illustrate the challenges of measuring fertilization *in situ*. Some investigators have collected naturally spawned gametes in the wild using various pumps, filters, suction samplers and large bags (e.g. Levitan 1991, Petersen et al. 1992, 2001, Warner et al. 1995, Styan 1997). These methods may bias estimates of fertilization by artificially exposing eggs to high concentrations of sperm for longer than they would normally experience (Levitan 1995). Models of fertilization kinetics predict that most sperm and egg collisions occur within the first few seconds of exposure, and that experimental artifacts biasing the measure of fertilization success may come from artificially altering the duration of exposure (Vogel et al. 1982, Styan 1998b). Several field studies have deployed freshly spawned, unfertilized eggs in synthetic mesh (e.g. nylon) chambers that retain the eggs but are permeable to sperm (Levitan et al. 1992, Levitan & Young 1995, Wahle & Peckham 1999, Gaudette et al. 2006, Bayer et al. 2016). Such chambers inevitably alter natural transport of gametes, and can produce experimental artifacts by impeding flow while keeping eggs stationary, which increases the amount of water — and therefore the amount of sperm — encountered by eggs (Levitan 1991, Levitan et al. 1992). Nonetheless, these methods have been recommended for eggs that are barely visible and are found at low concentrations (Styan 1997).

Molecular techniques to identify and quantify species-specific DNA from sea water samples through genetic markers have been applied extensively to eukaryotic microbes (Countway & Caron 2006, Moorthi et al. 2006, Garneau et al. 2011), marine viruses (Hewson et al. 2011, Matteson et al. 2013) and diverse groups of bacteria and archaea (Suzuki et al. 2000, Schwalbach & Fuhrman 2005, Ahlgren & Rocap 2012). These same molecular techniques have been used to identify and quantify metazoan DNA including that of invertebrate larvae (abalone, Vadopalas et al. 2006; sea lice, McBeath et al. 2006; decapods, Pan et al. 2008), marine fin fish embryos (Taylor et al. 2002, Fox et al. 2005) and freshwater fin fish spawning events (perch, Bylemans et al. 2017). Information gained from these studies have helped estimate larval dispersal (McBeath et al. 2006) and inform finfish stock assessments (Fox et al. 2005).

The sea scallop *Placopecten magellanicus* is a sedentary, gonochoristic, broadcast spawner found in the coastal and shelf waters of the Northwest Atlantic. Rescued from the brink of widespread depletion through a strategy of controls on fishing effort and closures of fishing grounds, the sea scallop is considered one of few existing success stories in fishery management (Hart & Rago 2006). While key components of recruitment, such as adult gamete production (Langton et al. 1987), larval transport (Tremblay et al. 1994, Tian et al. 2009), juvenile mortality (Wong & Barbeau 2003) and patchiness (Carey et al. 2013), have been subjects of intensive study, the recent literature reflects a growing recognition of how little empirical information is available on the role of spawning and fertilization dynamics as a potentially critical step in the recruitment process for scallops (Stokesbury 1999, 2012, Smith & Rago 2004). While information on the timing of spawning events can be gathered from gonad indices (Langton et al. 1987, Bonardelli et al. 1996, Thompson et al. 2014, Bayer et al. 2016), these data are limited in temporal resolution to days and weeks and can only be generalized over broad areas, thereby compromising the detection and measurement of finer-scale local spawning events. While field-deployed time-integrated fertil-

ization assays have begun to open a window on fine-scale spawning dynamics in sea scallops (Bayer et al. 2016, 2018), they are time- and labor-intensive and are prone to experimental artifacts, limiting their broad practical application.

Molecular techniques used to detect invertebrate larvae and eukaryotic plankton are well suited for the detection of sea scallop sperm presence and abundance from sea water samples. The internal transcribed spacer (ITS) region, located between the small (18S) and large (28S) subunits of the rRNA gene, is a useful locus for eDNA-based studies of marine eukaryotes. For example, previous studies have targeted the ITS region of harmful microalgae (Andree et al. 2011, Vandersea et al. 2017), fungi (Op De Beeck et al. 2014), parasites (Reece et al. 2008) and bivalves (Insua et al. 2003, Vierna et al. 2010, Lazoski et al. 2011, Salvi et al. 2014). Wang et al. (2006) found that the ITS region was a reliable genetic marker for species identification among 12 commercially harvested bivalves. The ITS region is evolutionary conserved within a species, but is diverse among species, even those closely related within a group such as Pectinidae (Insua et al. 2003). This study describes development and application of a real-time quantitative PCR assay for high-throughput detection and quantification of sea scallop sperm in seawater samples. The steps we took in developing this method included (1) sequencing a unique and reliable region, the ITS, of the sea scallop genome, (2) developing a qPCR probe and primer set to detect a diagnostic DNA fragment within this region, (3) testing the developed probe and primer set on a dilution series of scallop sperm and eggs to quantify the relationship between gamete abundance and DNA copy number, and (4) dockside field tests to evaluate the method with plankton samples collected adjacent to naturally spawning scallops. Our novel approach revealed dynamic trends in scallop eDNA abundance that coincided with the timing of an expected spawning event, and also captured finer-scale details in terms of the timing and magnitude of spawning than would have been possible using traditional approaches.

2. MATERIALS AND METHODS

2.1. DNA extraction, cultures, cloning and sequencing

We sampled *Placopecten magellanicus* mantle tissue of individuals collected from the Damariscotta River estuary using a minimally invasive approach, and stored this material at −20°C before extraction using the E.Z.N.A.® Mollusc DNA Kit (Omega Bio-Tek), following the manufacturer's protocol. DNA was quantified using a Qubit fluorometer and the Broad Range (BR) Qubit detection reagents. PCR for molecular cloning of *P. magellanicus* DNA was performed using ~10 ng of purified scallop DNA per reaction and 500 nM each of the eukaryotic primers Euk-B Forward: 5′-GTA GGT GAA CCT GCA GAA GGA TC-3′ (Medlin et al. 1988) and NLR204: 5′-ATA TGC TTA ART TCA GCG GGT-3′ (Van der Auwera et al. 1994) to amplify the entire ITS region between the small-subunit ribosomal RNA (rRNA) and large-subunit rRNA genes, including ITS1, 5.8S, and ITS2. A total of 6 PCRs were set up, using 25 µl of GoTaq® G2 Hot Start Green Master Mix (Promega), primers and DNA as noted above, and molecular grade water to achieve a total volume of 50 µl for each reaction. PCR was performed on a Bio-Rad C1000 PCR machine using a touchdown thermal protocol consisting of initial denaturation at 95°C for 2 min, followed by a touchdown phase that included 10 cycles of denaturation at 95°C for 30 s, annealing at 65 to 55°C for 30 s (decreasing by 1°C per cycle), and extension at 72°C for 90 s. An additional 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 90 s completed the amplification. The amplified DNA was run on a 1% agarose gel to isolate an ~750 bp PCR product spanning the entire ITS region of *P. magellanicus*. The ~750 bp PCR product was excised from the gel and purified using the Wizard® SV Gel and PCR Clean-Up kit (Promega). PCR products were ligated into the pCR4-TOPO® cloning vector and cloned into One Shot® TOP-10 chemically competent cells following the manufacturer's protocols included with the TOPO® TA Cloning® Kit for Sequencing (ThermoFisher Scientific). ITS clones were plated and grown overnight on LB plates with Kanamycin (50 µg ml^{-1}). Individual colonies were picked from the plates and grown overnight in Terrific Broth broth with Kanamycin (50 µg ml^{-1}) prior to extraction of plasmid DNA using the Zyppy™ Plasmid Miniprep Kit (Zymo Research). Eight of the ITS clones were selected at random and sent to Eurofins Genomics (Louisville, Kentucky) for Sanger DNA sequencing using T3 and T7 sequencing primers to obtain full-length ITS sequences. *P. magellanicus* ITS sequences were submitted to GenBank under accession numbers: MG744618-MG744625. Two micrograms of plasmid DNA from each of the clones was linearized with the *Not*I-HF® restriction enzyme (New England Biolabs) in 12 h reactions, prior to band-isolation on a

0.8% TAE gel and purification with the Wizard® SV gel kit (Promega). Linearized plasmid DNA was subsequently used as a DNA standard for the *P. magellanicus* qPCR assay.

2.2. Probe and primer design

We compared the 8 *P. magellanicus* ITS sequences (amplicons, Table 1) to all sequences in the GenBank nr database via BLAST analysis and confirmed that they were homologous to ITS sequences from other scallop species. To the best of our knowledge, these sequences are the first for *P. magellanicus* that span the entire ITS region. Using our newly sequenced ITS clones, we developed a Taqman® minor groove binding (MGB) qPCR assay (Holland et al. 1991, Heid et al. 1996) to quantify *P. magellanicus* DNA in environmental samples. The 8 clone sequences were aligned in Geneious (v.9.1) using MUSCLE (Edgar 2004) and then imported into AlleleID (v.7.83) to design a novel DNA probe, forward, and reverse qPCR primers. The resulting primers and probe were compared to all sequences in GenBank using BLAST (Altschul et al. 1997), to minimize the chance of false positives due to matches with DNA from non-target organisms in seawater samples. The analysis revealed that the primers and probe for *P. magellanicus* ITS were highly specific for the targeted species, revealing no significant matches to other marine species. The forward primer (Pmag_282F: 5′-CAG CCT GAA ATC GAG ATG-3′) was located at positions 282 through 299 and the reverse primer (Pmag_492R: 5′-CAT GCA TAC AGT TGC GAT A-3′) at positions 474 through 492 relative to our *P. magellanicus* ITS sequences, resulting in a qPCR amplicon of 211 bp in length (Table S1 in the Supplement at www.int-res.com/articles/suppl/m621p127_supp.pdf). The probe (Pmag_304F:5′-[6FAM] TAT TAT CAC TCT AAG CGG[NFQ]-3′) was located very close to the forward primer at positions 304 through 321, and was labelled on the 5′-end with the fluorescent reporter dye 6-carboxyflourescein (FAM) and on the 3′-end with a proprietary non-fluorescent quenching (NFQ) molecule (ThermoFisher Scientific) to suppress FAM fluorescence in the absence of amplification (Table 1). The probe was modified with a DNA MGB to increase its melting temperature. The FAM molecule is released from the probe during DNA amplification by the 5′-exonuclease activity of Taq DNA polymerase, causing emission of a fluorescence signal that is captured by a detector at the end of the combined annealing/extension step. The cumulative increase in fluorescence is proportional to the amount of amplified DNA, and the probe-based TaqMan assay ensures that the fluorescence is due to amplification of the specific DNA target only (Holland et al. 1991).

Table 1. TaqMan probe and primer sequences: properties including sequence, length, melting temperature (T_m) and GC base content of *Placopecten magellanicus*-specific TaqMan probe and primers

Name	Sequence (5′→3′)	Length (nucleotides)	T_m (°C)	% GC
Pmag_304F (MGB-probe)	TAT TAT CAC TCT AAG CGG	18	68	38.9
Pmag_282F (primer)	CAG CCT GAA ATC GAG ATG	18	59.1	50
Pmag_492R (primer)	CAT GCA TAC AGT TGC GAT A	19	59.2	42.1

2.3. Calibration and testing of qPCR method

Copy numbers per microliter of the cloned PCR products were calculated for each of the 8 *P. magellanicus* ITS clones based on their specific DNA concentrations and the exact nucleotide composition of each PCR product plus cloning vector. Each of the ITS clones had a slightly different DNA sequence, presumably due to the presence of multiple copies of the ITS region in the sea scallop genome and potentially due to PCR-induced errors during DNA replication. However, many of the nucleotide polymorphisms across the full-length ITS region did not appear to be random and displayed the same polymorphisms in >1 sequence, suggesting that the variation was not artifactual. Within the 211 bp region targeted by our qPCR assay, there were only 4 polymorphic nucleotides among the 8 ITS clones located at 3 polymorphic sites. Two of the sequences displayed single polymorphisms at 2 sites, while 2 sequences had the same polymorphism at a third site. None of the polymorphisms occurred within the regions where the probe or primers would bind, thus minimizing the effect of sequence variation on the qPCR assay. To account for some of the potential variability of the qPCR assay due to slight variations in sequence composition of DNA targets in natural samples, equal copy numbers of each of the 8 clones were mixed to produce a composite linear

DNA standard with a final concentration of 3.7×10^9 copies μl^{-1}. The DNA standard was serially diluted to create a standard curve spanning 7 orders of magnitude (i.e. 10^{-2} to 10^{-9}). Quantitative estimates of scallop DNA in environmental samples must be considered minimal estimates due to the potential for inhibition of the qPCR assay by natural (and unknown) inhibitor molecules and/or competition for probe and primers by non-target (and unknown) DNA templates present in our eDNA extracts. Inhibition of the qPCR assay by unknown molecules in the DNA extract should have been minimal due to our use of the proprietary Inhibitor Removal Technology within the Qiagen PowerWater extraction kit. Given the small size of our qPCR target (211 bp), ~96% of the DNA in each of the dilution-series standards was non-target DNA from the pCR4-TOPO cloning vector and could be considered 'background DNA' to approximate any 'sample matrix' effects.

All reagents, samples, and standards were prepared on ice prior to thermal cycling. Reactions of DNA standards were prepared as follows: 3 µl aliquot of each diluted ITS standard, 10 µl of 2' PrimeTime® Gene Expression Master Mix (Integrated DNA Technologies), 1 µl each of the forward and the reverse primers (500 nM final concentration of each), 0.5 µl of the probe (250 nM final concentration), and 4.5 µl of deionized water. All samples, standards and no-template controls were loaded in triplicate on a white-well PCR plate (Bio-Rad) and amplified on a Bio-Rad CFX96 real-time PCR system. Thermal cycling consisted of an initial denaturation step of 3 min at 95°C, followed by 40 cycles of 95°C for 15 s and 61°C for 30 s. Real-time relative fluorescence unit (RFU) values were collected after the combined annealing/extension step.

2.4. Cross-reactivity testing

Although our *P. magellanicus* qPCR assay was designed to be species-specific and was tested against closely related taxa *in silico*, we also conducted *in vivo* cross-reactivity testing against 2 bivalve species common to the Gulf of Maine. To test for cross-reactivity of the probe and primers, we collected DNA from the eastern oyster *Crassostrea virginica* and the blue mussel *Mytilus edulis*. We obtained an archived DNA sample from *C. virginica* that had been collected from Jones Cove (Damariscotta River, ME) and extracted by Marquis et al. (2015). Blue mussels were collected from the dock at the University of Maine's Darling Marine Center (Damariscotta River, ME, USA), and DNA was extracted from the mantle tissue of 1 individual following the same protocol used for *P. magellanicus* described above. PCR, cloning, and sequencing of the ITS region for *M. edulis* and *C. virginica* also followed the procedures described above. Triplicate qPCR reactions for each of the non-target bivalve species were conducted using 10^{-3} and 10^{-6} dilutions of each of the cloned, non-target DNA templates. Three ITS clones from each non-target species were sequenced to confirm their identity and ensure non-reactivity with the *P. magellanicus* qPCR primers and probe (Table S2, GenBank accession numbers MG744626-MG744631).

2.5. Collection of scallop gametes

Male sea scallops, that were originally collected from the Damariscotta River estuary and kept in hanging nets on docks at the Darling Marine Center, were selected for spawning during the first week of September 2016. Spawning tables (92 × 122 cm) were filled 15 cm deep with filtered (1 µm) UV-sterilized sea water and cooled to 10°C. Male scallops were transferred to spawning tables from ambient (~16°C) unfiltered seawater and induced to spawn via a cold shock as in Bayer et al. (2016). From these, 250 ml of fresh, concentrated sperm cells were collected in a 250 ml, acid-washed container and serially diluted twice before filtering onto 0.2 µm, 47 mm diameter Supor filters (Whatman) to simulate collection of a plankton-based eDNA sample. Filters were rolled gently, placed in cryo-vials and stored at −20°C until DNA extraction. This dilution series preparation and filtration was repeated 5 times, with each initial sample collected from ambient sperm concentrations within the spawning table, and subsamples collected into 1.5 ml vials, and preserved in 4% formalin for sperm cell counts by microscopy. Three subsamples of sperm cells were counted with a haemocytometer using standard counting protocols (e.g. Wahle & Peckham 1999, Andersen & Throndsen 2004, Gaudette et al. 2006).

2.6. Sperm dilution series

DNA was extracted from filtered-collected and frozen sperm cell samples using the PowerWater DNA extraction and purification kit (Qiagen) with a slight modification of the sample lysis step. Briefly, frozen Supor filters were transferred to 5 ml Power-

Water bead tubes containing pre-loaded garnet particles. One ml of heated (55°C) PW1 lysis buffer was added to the bead tubes and heated at 55°C for 3 to 5 min. The 5 ml tubes were transferred to a 5 ml sample-tube adapter (MoBio) and loaded onto a MM400 Retsch Mixer Mill for sample bead-beating. Two rounds of bead-beating were conducted for 5 min each, at an oscillation frequency of 20 s^{-1}, with 180° rotation of the tube adapter between the 5 min bead-beating sessions. Additional sample processing followed the Qiagen PowerWater protocol exactly. TaqMan qPCR was performed on the extracted samples with the *P. magellanicus* probe and primers described above using 3 µl aliquots of each purified DNA sample in triplicate, with 3 replicates of a filtered, UV-sterilized seawater control. Using the standard curve that was generated from the linearized-plasmid dilution series, we translated the Cq values (qPCR threshold cycle number) resulting from cell-based DNA samples to gene copy numbers to investigate the relationship between gene copy number and cell abundance.

2.7. Dockside experiment

All scallops used for the dockside experiment were collected by SCUBA dive surveys in July 2017 from the Damariscotta River estuary near Bigelow Laboratory for Ocean Sciences. Specimens were kept in tanks at the University of Maine's Darling Marine Center until the start of the experiment at Bigelow Laboratory. To independently monitor spawning activity over the duration of our dockside experiments, we tracked gonadosomatic indices (GSI) in a small (<50 individuals) population (i.e. Bayer et al. 2016, 2018) maintained in flowing seawater tanks at the Darling Marine Center, located ~8 km upriver from the Bigelow dock. GSIs were monitored as a general indicator of the onset and end of the spawning season. We sampled between 6 and 10 individuals (with equal sex ratios) 4 times during the spawning season with two 3 wk intervals between sampling events.

Two lantern nets were suspended ~1 m apart under the dock at Bigelow Laboratory for Ocean Sciences, on the lower portion of the Damariscotta River estuary. One net contained 2 females and 9 males, and the other contained 5 males and 5 females, for a total population of 21 individuals. The scallops were spread out over 4 levels of net as in Bayer et al. (2016).

We collected seawater samples with a suction hose pumping at a rate of 15 l min^{-1} adjacent to the nets. The end of the sampling hose was deployed <10 cm from the edge of a lantern net, approximately halfway down (~0.5 m) the length of the net. We let the plankton pump run for at least 60 s to rinse the sample hose, then rinsed each of three 6 l carboys before filling them completely. We sampled daily during different phases of the lunar cycle: 1 to 2 times per week during non-target weeks, and more intensively during the week between the full and third quarter moons, based on our previous observations of spawning during that time period (Bayer pers. obs.). On each sampling day, we collected water within 2 h of low or high slack tide to maximize uptake of potential cells downstream from the lantern nets. Water samples were size-fractioned to test for scallop eDNA in particles > 20 µm (inclusive of scallop eggs) and those ranging from <20 to 0.2 µm (inclusive of scallop sperm cells). The 6 l samples were gravity-filtered through 20 µm Nylon mesh filters installed in 47 mm, in-line filter holders (Pall Laboratory) for extraction of DNA from particles > 20 µm. One liter of the <20 µm filtrate was subsequently filtered through a 0.2 µm Supor filter for DNA extraction of particles <20 um, the size-fraction that included scallop sperm cells. All filters were frozen, and DNA was extracted using the PowerWater DNA kit (Qiagen) as described above.

After running samples through the scallop qPCR protocol, we examined the time-series for noticeable increases (peaks) in gene copy number. For samples < 20 um, we assumed that all scallop DNA was attributed to sperm and converted gene copy number to sperm cells based on the sperm dilution series described above. In the laboratory, we were unable to develop a linear relationship between cell counts and gene copy numbers for eggs despite sampling freshly spawned scallop eggs that were well-mixed prior to dilutions, filtering, and subsequent DNA extraction. We suspect that the method we developed for sperm cell counts and DNA extraction may not be appropriate for eggs.

2.8. Phylogenetic reconstruction

A total of 14 full-length bivalve species ITS sequences were generated for target (*P. magellanicus*, n = 8) and non-target species (*C. virginica*, n = 3, and *M. edulis*, n = 3). These sequences were combined with other bivalve ITS sequences from GenBank (Benson et al. 2013) to generate a phylogenetic tree within the Geneious v9 software package, using the MUSCLE sequence aligner (Edgar 2004) and the

Bayesian phylogenetic inference program, MrBayes v3.2 (Huelsenbeck & Ronquist 2001). The initial MUSCLE alignment was refined by manually trimming the non-overlapping regions at the 5′ and 3′ ends, and was subsequently realigned, resulting in an alignment spanning 1635 nucleotide positions (see the Supplement Data file, www.int-res.com/articles/suppl/m621p127_supp/). The aligned sequences were imported into PAUP* v4.0a164 (Swofford 2003) and processed with the MrModelblock file (available from https://github.com/nylander/MrModeltest2/) to generate a 'mrmodel.scores' input file for MrModeltest v2.4 (Nylander 2004). The result of MrModeltest indicated that the best nucleotide substitution model for our DNA alignment was the 'general time reversible' (GTR) nucleotide substitution model with rate variation set to 'invgamma' (+I +G), indicating a gamma distribution of nucleotide substitution rates with a proportion of invariable nucleotide sites. An ITS sequence from the marine gastropod *Coralliophila meyendorfii* (Oliverio & Mariottini 2001) served as the outgroup for this phylogenetic tree.

3. RESULTS

3.1. *Placopecten magellanicus* qPCR calibration and cross-reactivity tests

Real-time qPCR showed a clear logistical relationship between Cq values from cell extracts and gene copy numbers using the probe and primers designed from the *P. magellanicus* ITS region (Fig. 1). The lowest limit of quantification (LOQ) with reproducible amplification was ~11 gene copies per reaction, using 3 µl of the most dilute DNA standard (10^{-9}) in a 20 µl reaction volume. The limit of detection, which accounts for all of the factors that might impact the detection of a qPCR target (Forootan et al. 2017), was not determined empirically, given that a single scallop gamete (the basic ecological unit in our study) likely has more copies of the ITS region than the observed LOQ for this assay. The efficiency of the *P. magellanicus* TaqMan qPCR assay was generally in the range of 92 to 96% for all sample runs. No-template control Cq values were >38, indicating very little background contamination, suggesting that the vast majority of the signal from the cell-based samples was due to the extracted DNA from *P. magellanicus* sperm cells. The UV sterilized seawater sample, serving as a background sample to accompany the sperm dilution series, had a Cq value of ~31 (~100 gene copies). All samples in the sperm dilution series had Cq values ≤20 (e.g. ≥760400 gene copies), indicating background DNA signals of ~100 gene copies impacted the dilution series samples only minimally. After translating Cq values from the standard curve to gene copy numbers, the sperm dilution series indicated a strong relationship between gene copy number and cell counts by microscopy (Fig. 2). We observed ~1 ITS gene copy per 4 to 5 sperm cells consistently across samples.

We tested our *P. magellanicus* qPCR assay against eastern oyster *Crassostrea virginica* and blue mussel

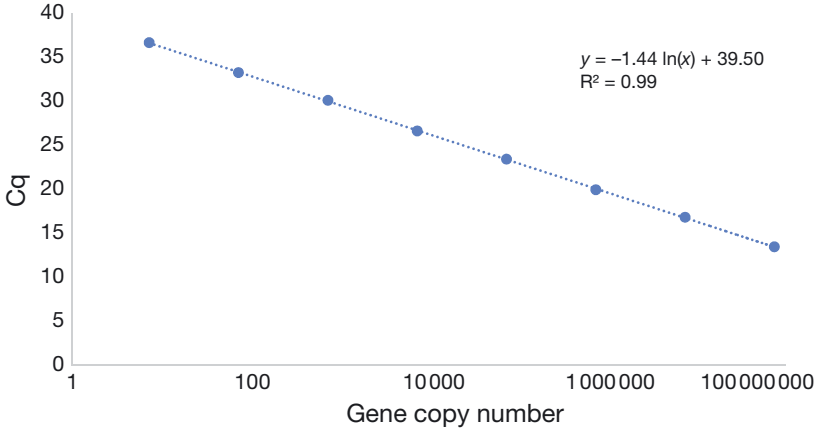

Fig. 1. Real-time PCR cycle number (Cq) and gene copy number for dilution series of standard clonal *P. magellanicus* ITS regions

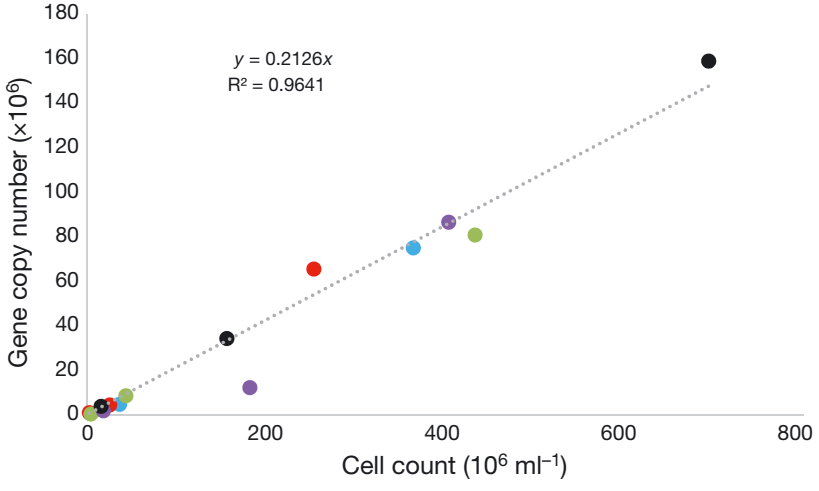

Fig. 2. Cell count of *P. magellanicus* sperm and resulting gene copy number from 5 dilution series (red, blue, green, purple and black filled circles)

Mytilus edulis ITS clones that were generated from Damariscotta River specimens. These 2 bivalve species were selected as co-occurring broadcast spawners that could potentially contribute DNA to our samples. Real-time qPCR demonstrated no cross-reactivity of the *P. magellanicus* probe and primers to eastern oyster or blue mussel ITS clones.

3.2. Dockside experiment

In 6 l of pumped seawater, we detected millions of scallop gene copies from samples of cells < 20 µm and thousands of gene copies from cells > 20 µm. Our dockside time-series, consisting of the 1 l, <20 µm samples, indicated possible spawning events on 28 and 29 August and 27 September (Fig. 3a). The scallop eDNA signal in the >20 µm size-fraction was high on 28 and 29 August, and 1 additional sample was very high on 16 September (Fig. 3b). A nearby *P. magellanicus* population at the Darling Marine Center that we monitored for changes in GSI revealed a reduction in male and female gonad size between 19 August and 9 October (Fig. 3c), consistent with spawning activity over the period that we conducted dockside sampling.

3.3. Phylogenetic reconstruction

P. magellanicus ITS sequences varied from 758 to 765 nucleotides in length, and none of the 8 sequences were identical, suggesting a high degree of intragenomic variability and multiple copies of the rRNA operon within the scallop genome. Despite this high variability, the *P. magellanicus* primers and probe were designed to target a conserved region that would amplify all of the known sequence variants that we identified. We found a single, non-annotated *P. magellanicus* cDNA sequence in GenBank (accession: GADG01000430.1) that overlapped partially with our full-length scallop ITS sequences (Pairett & Serb 2013). This cDNA sequence was too short to include in the phylogenetic reconstruction but contained the entire region targeted by our scallop qPCR assay, revealing 100% sequence identity to our newly designed probe and primers (see Fig. S1 in the supplement at www.int-res.com/articles/suppl/m621p127_supp.pdf). All *P. magellanicus* sequences were most similar to ITS sequences from other species of scallops (Fig. 4). Blue mussel and Eastern oyster ITS sequences were most similar to other ITS sequences

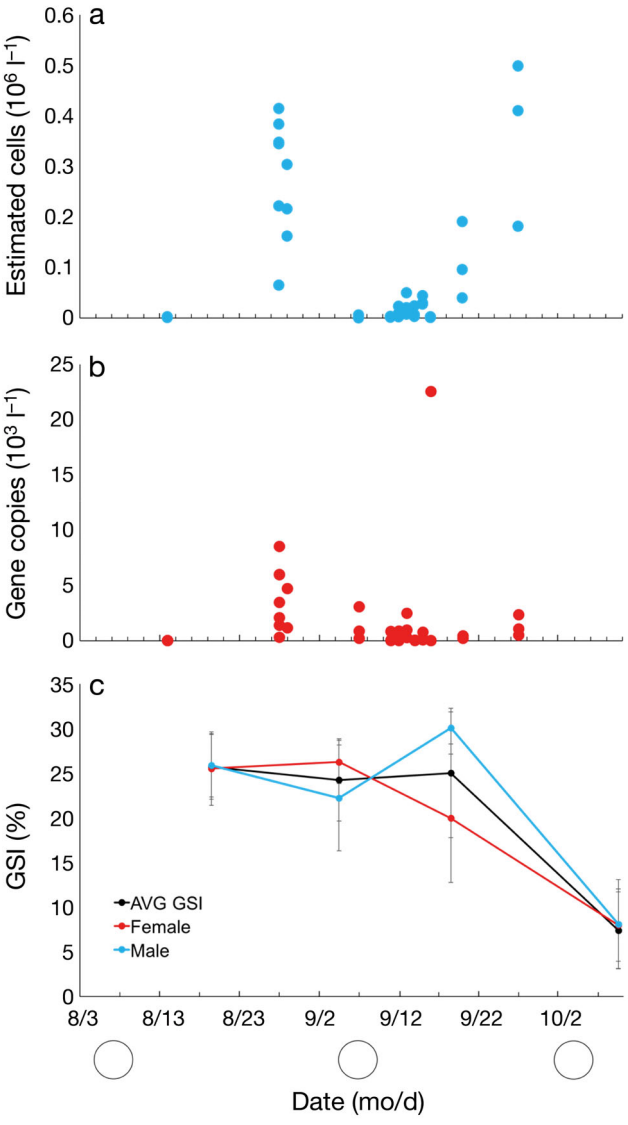

Fig. 3. Time series of (a) estimated sperm (cells < 20 µm) abundance and (b) gene copy number from cells > 20 µm from plankton pump samples collected <0.5 m from dockhung scallops during the spawning season, and (c) gonad index (GSI, gonad weight/soft tissue weight) from a nearby population during spawning season. Error bars are ±1 SD. Full moons are indicated by open circles. Only gene copy numbers are reported in (b) due to the unknown cell concentration of the samples

that have been reported for these 2 species in GenBank (e.g. Wang et al. 2006). Overall, our phylogenetic reconstruction establishes the homology of the ITS sequences we generated with publicly available molluscan sequences. It suggests that the ITS region may serve as an informative locus for the development of eDNA assays for several species of commercially important bivalves in Maine coastal waters.

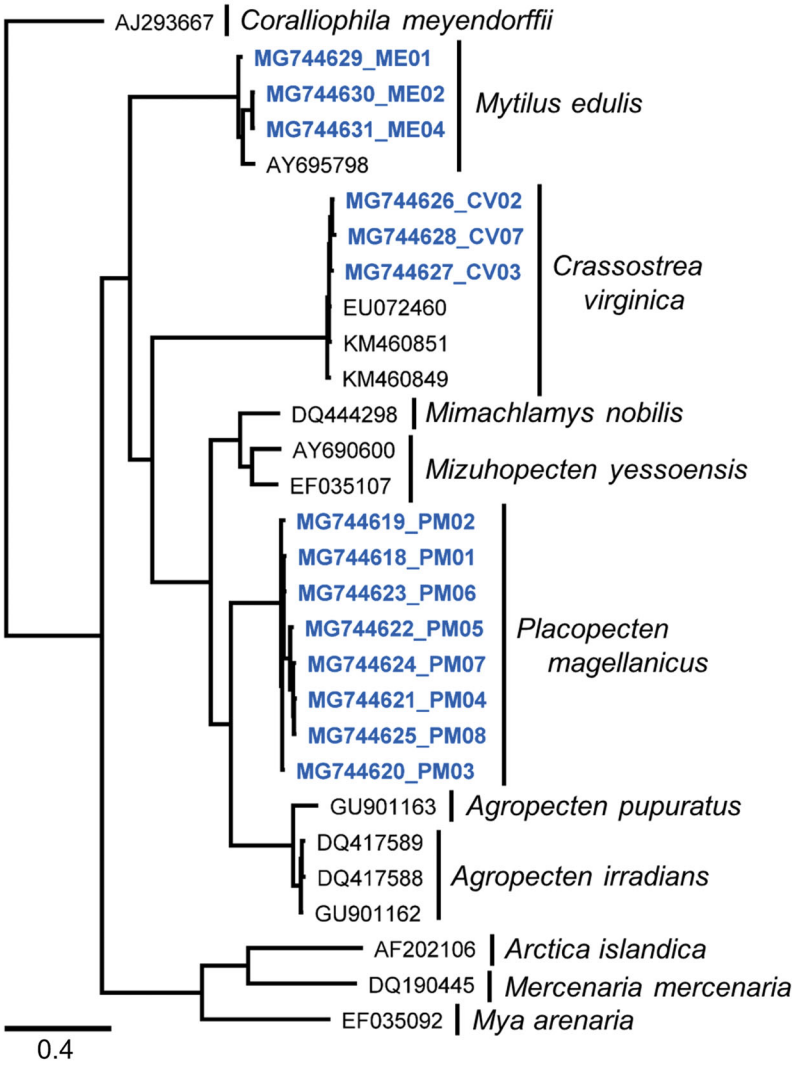

Fig. 4. Bayesian phylogram with proportional branch lengths inferred from ITS sequences of scallops, mussels and oysters. Full length ITS sequences from this study are highlighted in blue and include GenBank accession number plus clone ID. The scale bar represents 0.4 substitutions per site in the underlying DNA sequence alignment

4. DISCUSSION

Environmental DNA samples from aquatic ecosystems can contain genetic material from tens of thousands of species, and have the potential to inform ecological studies across multiple trophic levels. As eDNA science rapidly emerges as an area of interdisciplinary research, it is proving useful in identifying and quantifying a spectrum of biota, from microbes (e.g. Countway & Caron 2006) to metazoans, including life-stages from larvae to adults (e.g. Fox et al. 2005, Thomsen et al. 2012, Pikitch 2018). These data can be used to infer reproductive events through aggregation activity (Tillotson et al. 2018), behavior (Erickson et al. 2016, de Souza et al. 2016), and community structure (Rees et al. 2014).

Size-fractionation has been used to detect an eDNA signal (via qPCR) for the extremely small (~0.8 µm) microbial eukaryote, *Ostreococcus*, in 2 different size-classes of plankton (i.e. 0.7–80 µm vs. 80–200 µm), revealing the overwhelming presence of *Ostreococcus* in the smaller size-class (Countway & Caron 2006). In a situation analogous to the present study, the work by Countway & Caron (2006) demonstrated a dramatic increase in gene copy numbers with the smaller size-class of plankton, indicating a large bloom of *Ostreococcus*. Size-fractionation of plankton samples has proved to be a useful technique to explore functional differences among marine organisms in certain size-classes. To our knowledge, this is the first time size-fractionation combined with a quantitative eDNA assay has been applied to the detection of broadcast spawning events in the marine environment.

Our DNA dilution series of ITS clones indicates a reliable relationship between Cq values from scallop sperm cell extracts and ITS copy numbers using the TaqMan probe and primer set that we developed for *Placopecten magellanicus* (Table 1). The application of the dilution series to our scallop sperm samples suggests that not all of the scallop sperm cells were lysed during the DNA extraction process, or conversely the cells may have been undercounted by our hemocytometer estimates. Another possibility is that our TaqMan probe and primers do not bind as well to potential (but unknown) variations of the *P. magellanicus* ITS region. It is likely that there are other variants of the ITS region in the *P. magellanicus* genome, given that all 8 of the sequences in our study were unique. In fact, the genome of *P. magellanicus* is thought to be among the largest of the scallop species (Krause & von Brand 2016, and references therein). It is generally accepted that species with larger genomes have more copies of the rRNA operon (Prokopowich et al. 2003) with potentially greater numbers of ITS sequence variants. We attempted to control for this unknown

sequence variability by designing the scallop qPCR assay in a region that was conserved for all of the ITS sequences in our study. Specifically, the 211 bp region that we targeted for qPCR amplification showed very little DNA sequence variation among the 8 *P. magellanicus* ITS clones and the single *P. magellanicus* transcriptome sequence (Pairett & Serb 2013) from GenBank. Notably, the GenBank sequence displayed 100% homology to the consensus sequence of our 8 ITS clones within the qPCR region. Only 3 single-nucleotide variants were observed among 4 of the *P. magellanicus* ITS sequences between the forward and reverse qPCR primers, and none of these variations were found within the probe or primer regions. Our work to characterize the *P. magellanicus* ITS region via low-throughput cloning and sequencing has generated the first full-length ITS sequences for this species. Although these sequences likely do not capture all of the diversity in the ITS region of *P. magellanicus*, they were extremely useful for development of our novel quantitative eDNA application. It is probable that high-throughput DNA sequencing could be used to address the question of ITS sequence variation, given the recent advances in long-read, next generation sequencing technology for DNA barcoding (Hebert et al. 2018).

The GoTaq® G2 Hot Start DNA polymerase that was used to amplify the ITS region has an error rate that is consistent with values reported previously for Taq DNA polymerase (Promega technical support pers. comm.) ranging from 20×10^{-6} to 100×10^{-6} errors per nucleotide incorporated (Sambrook & Russell 2001). Although it is likely that some of the variable positions in the ITS region were the result of PCR error, a large proportion of the variants were repeated in multiple sequences (Fig. S1), suggesting that much of the variability across the full-length ITS region (~750 bp) reflected true genetic differences. Errors in the ITS sequences caused by PCR might have contributed to slight variations in the topology of the phylogenetic tree, but they were unlikely to have impacted the qPCR assay due to the high degree of sequence similarity that was observed in the targeted region for the 8 clone sequences and 1 transcriptome sequence. Despite the observed offset between cell counts and gene copy numbers, the strong linear relationship (Fig. 2) suggests that any possible errors related to cell lysis, cell abundance estimates, or undetected variation in the ITS regions were consistent across all samples. It is possible that some sea scallop sperm cells released during spawns are anucleated, as observed in the protandric hermaphroditic bivalves *Tellimya ferruginosa*, *T. tenella*, and *Montacuta percompressa* (Fox et al. 2007). However, anucleated paraspermatazoa have not been observed in scallops to the best of our knowledge.

The results from our dockside experiment suggest that we detected at least 2 male spawning events in the field, both during periods of time when decreases in GSI occurred (Fig. 4). While particles >20 µm showed a spike in the abundance of scallop eDNA at the same time as the first spike in scallop eDNA for particles <20 µm, this parallel response did not happen during the second spike in scallop eDNA for particles <20 µm. We cannot confirm whether the larger particles were eggs or were attributed to some other source of scallop eDNA. It is possible that scallop eDNA in the larger size-fraction could have originated from other scallop tissue due to decaying adult biomass. However, we did not observe any scallop mortality during the dockside experiment, so it is unlikely that cellular debris could have originated from such an event. Cell buoyancy could also impact our eDNA signal, causing gametes to sink to greater depths or float to the surface. While sperm are usually neutrally buoyant, they have been found to swim downwards across multiple phyla (Falkenberg et al. 2016) which may impact eDNA signals. Additionally, sea scallop eggs are negatively buoyant (S. R. Bayer & R. A. Wahle unpubl. data) but have been observed to travel >3 m in unidirectional flumes without sinking (S. R. Bayer pers. obs.). This last observation suggests that the interaction between gametes and the surrounding physical dynamics, including flow rates, turbulence, and shear (i.e. Denny & Shibata 1989, Riffell & Zimmer 2007), deserves greater study and attention when evaluating eDNA data.

A factor to consider in our observations is persistence of the spawning signal. Within the Damariscotta River tidal estuary, we predict that an eDNA signal from spawning would be impacted the most during maximum ebb and flood tides, showing up many kilometers downstream from the adult source, similar to the results of the O'Donnell et al. (2017) study. During these periods of high flow, we would expect to have challenges detecting accurate spatiotemporal eDNA signals similar to that of river and stream studies (i.e. Jerde et al. 2016, Shogren et al. 2017). We sampled specifically at slack tides to avoid these high-flow conditions that may sweep away scallop gametes rapidly. This ensured, to the best of our ability, observation of a signal that was as physically close to our source population as possible.

Both of our observed spikes in sperm coincided with the first quarter of the moon (see Fig. 3). Lunar cues have been documented in sea scallops (Bonardelli et al. 1996). In the Damariscotta River, we have consistently observed female scallop spawning on the third quarter of the moon (S. R. Bayer pers. obs.). Our preliminary data suggest that the temporal spawning patterns in males and females may be worthy of closer investigation given the possible mismatch of male and female spawning events observed in our time-series. Given that lunar cycles strongly influence tides, tidally induced variability in flow may be an important spawning trigger for sea scallops, but the proximate mechanism is not understood. High-flow environments can reduce gamete concentration and wash a majority of sperm and eggs away from spawning adults, while low-flow conditions can allow for higher fertilization success (e.g. Denny & Shibata 1989, Coma & Lasker 1997, Marshall 2002, Riffell & Zimmer 2007).

We do not know how quickly *P. magellanicus* gametes degrade in natural marine conditions. However, we do know that sea scallop eggs remain viable for fertilization between 8 and 24 h, while sperm may be viable for several hours (Bayer et al. 2016). It is assumed sperm DNA persists for several hours while a sperm swims and searches for an egg to fertilize, but this may vary with acidity, salinity and temperature of their environment (Desrosiers et al. 1996). Very few studies investigating eDNA dynamics have accounted for eDNA degradation and the persistence of macrobial eDNA ranges from 1 d to >2 wk in aquatic environments (Barnes et al. 2014). Degradation and persistence should be given serious consideration when using eDNA data in fishery and conservation management decisions.

Accurate identification of invertebrate gametes and larvae can be extraordinarily difficult if not impossible by microscopy. Creating a species-specific qPCR assay to detect scallop eDNA allowed us to monitor and quantify sea scallop spawning events and could eventually lead to estimates of the size of subsequent larval output from water samples taken *in situ*. Application of our probe and primers to plankton samples collected near scallop beds would help indicate the presence, magnitude, and timing of scallop spawning events. Collection of size-fractionated samples would be a necessary step to help distinguish sperm cells from eggs, embryos and larvae (~10-fold the size of sperm; Naidu 1970). Expanding beyond scallops, this gamete detection assay could help identify the presence of rarer species through spawning events. Collected eDNA has been used to identify the presence of rare or elusive species in aquatic environments, like sturgeon Acipenseridae (Pfleger et al. 2016). However, Erickson et al. (2016) found no relationship between eDNA and spawning activity of bigheaded carps (*Hypophthalmichthys* spp.) in rivers. This is further evidence that hydrography is to be carefully considered and studied when studying spawning events.

The role of sperm limitation in population dynamics of free-spawning species has been a long-standing question in marine ecology (Petersen & Levitan 2001), particularly for species whose populations have been commercially exploited or experienced high levels of natural mortality (Yund 2000, Petersen & Levitan 2001, Gascoigne & Lipcius 2004). The challenge has been to detect and quantify gametes in wild populations as they are spawned. One approach has been to use time-integrated fertilization assays (Levitan et al. 1992, Levitan & Young 1995, Wahle & Peckham 1999, Gaudette et al. 2006, Bayer et al. 2016, 2018), a labor- and time-intensive method that requires the induced spawning in the laboratory of viable unfertilized eggs prior to their deployment *in situ* within artificial mesh containers that are permeable to sperm. The method is tractable for a small number of species, but is prone to experimental artifacts (e.g. Levitan 1991, Levitan et al. 1992, Styan 1998a, Bayer et al. 2016, 2018). Development of a molecular assay could dramatically enhance the measurement of spawning events and aid our understanding of spawning and fertilization dynamics in nature.

Our probe and primers are species-specific for *P. magellanicus,* and could aid in quantifying other water-borne life stages in addition to sperm, such as eggs, embryos and larvae. Our field experiment demonstrated that the scallop qPCR assay was able to detect sperm *in situ*. In future studies, we would like to rigorously develop methods for reliably detecting and quantifying eggs and embryos, accounting for physical properties like degradation and buoyancy. Another way to augment this kind of study would be to determine relative mitochondrial eDNA and nuclear eDNA abundances of sea scallops to detect gametes. This method has been demonstrated as reliable in the endangered Macquearie perch *Macquaria australasica* (Bylemans et al. 2017) but not necessarily invertebrates to the best of our knowledge. Identifying gamete and embryo presence and abundance has influenced stock assessments for cod (*Gadus morhua*) in the Irish Sea using similar molecular methods (Fox et al. 2008). Several larval parasitic copepod species, which are difficult to differentiate

by eye, were accurately identified and quantified in plankton samples by a TaqMan probe and primer set (McBeath et al. 2006). Similarly, larval stages of bivalve species can appear almost identical in appearance during early development (Garland & Zimmer 2002). Given that gametes and larvae of marine organisms can be difficult to distinguish, qPCR analysis could revolutionize the identification and quantification of species-specific spawning events in the field. This analysis may also determine if larval cohorts are found within the boundaries of critical fishing grounds and could greatly influence fishery management decisions and the designation of area closures. This method has the potential to be far more practical and less artifact-prone than the use of time-integrated fertilization assays. It could also improve detection of spawning events in remote and hard to access locations that do not lend themselves to fertilization assays, such as offshore and deep-water populations. To build on the specific scallop qPCR assay we developed with this study, future investigations with this novel eDNA methodology should involve the development of probes and primers for other broadcast spawning invertebrates and involve more rigorous field tests around naturally spawning populations.

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