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TWO METHODS FOR DETERMINING THE FERTILITY STATUS OF EARLY-STAGE AMERICAN LOBSTER, *HOMARUS AMERICANUS*, EGGS

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ABSTRACT

The American lobster (*Homarus americanus* Milne Edwards, 1837) is the focus of the most important commercial fishery in New England, which relies on a variety of biological monitoring programs and surveys to guide the development of appropriate management plans. One key piece of information provided by these surveys is the number of females that are carrying eggs (ovigerous) that will subsequently contribute new recruits to the fishery. A major assumption is that all eggs carried by ovigerous females are fertilized and will thus result in viable recruits. However, because some lobsters extrude, and briefly carry, unfertilized eggs, this assumption needs to be re-evaluated. In particular, it is important to determine the approximate proportion of newly extruded eggs that are either fertilized, or not. The major goal of this project was to develop reliable methods for determining if early-stage lobster eggs (live and preserved) were in fact fertilized. One method involved using a nucleic acid stain to visualize egg DNA, after pretreatment of eggs with a proteolytic and collagenolytic enzyme solution to facilitate stain penetration through the egg membrane. With this method multi-nucleated (fertilized) eggs could be clearly distinguished from unfertilized eggs. A total of 20 egg clutches were tested to determine their fertility status using this method. Of these, 16 clutches (80%) were fertilized while 4 were not fertilized (20%). Of the 16 clutches with fertilized eggs, two had a mix of both fertilized and unfertilized eggs. A second method, using fluorometry to obtain measurements of total egg DNA, was also developed. There was a significant difference between the total DNA concentration in unfertilized control oöcytes and early-stage fertilized eggs ($P < 0.001$), and the total amount of DNA gradually increased as eggs developed ($r = 0.961$, $P < 0.0001$). Both of these methods will make it possible to make a more accurate assessment of the proportion of female lobsters that will actually contribute new recruits to the fishery.

KEY WORDS: American lobster, DNA, egg development, Hoechst stain, *Homarus americanus*, lobster eggs, proteolytic and collagenolytic enzymes, ovigerous

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INTRODUCTION

The American lobster, *Homarus americanus* H. Milne-Edwards, 1837, is one of the most valuable commercial fisheries in the North Atlantic and supports the economy of many New England coastal communities (> $372 million in 2007; FAO Stat, 2009). As a result, fisheries scientists and managers spend a considerable amount of time and effort monitoring the fishery so they can make informed decisions and effectively manage this resource. Along with data on growth, mortality, and reproduction (fecundity, spawning stock biomass), some surveys assess the abundance of egg-bearing (ovigerous) females that will contribute new recruits to the fishery. Estimates of the reproductive capacity and future recruitment rates of the stock are then based, in part, on the number of egg-bearing females caught during these surveys (ASMFC, 2009).

There are a number of models and indices used in American lobster stock assessment, two of which are the spawning stock biomass (SSB) index of abundance, and the egg per recruit (EPR) model (Fogarty, 1995; ASMFC, 2009). SSB indices are used to estimate the total reproductive potential of a population and can be done on a statewide or regional basis (ASMFC, 2009). EPR models have been modified from finfish models and generally use the number of mature lobsters on the bottom, their carapace length, and the probability of surviving and producing eggs, as a means of calculating the number of eggs that will be produced in the near future. In the case of the EPR model used in a recent lobster stock assessment (ASMFC, 2009), the model assumes that sexually mature females, provided they survive, will mate and extrude a quantity of eggs based on their total fecundity, which is dependent on their carapace length (CL) (Herrick, 1909). The assumption of these EPR models is that female lobsters fertilize 100% of the eggs that they carry. While some crustacean fisheries (spiny lobster and crab) tend to be quite resilient to heavy exploitation (Pollock, 1993; Hankin et al., 1997), it is unclear how fishing pressure might influence the reproductive dynamics of American lobsters. For example, slight shifts in size-at-maturity schedules and sex ratios (Landers et al., 2001; Little and Watson, 2005) could reduce mating success in some areas, and this might be manifested in a decline in fertilization rates and, eventually, new recruits.

Although the length and timing has been debated (see Waddy and Aiken, 2005), the female American lobster reproductive cycle typically involves molting and mating in

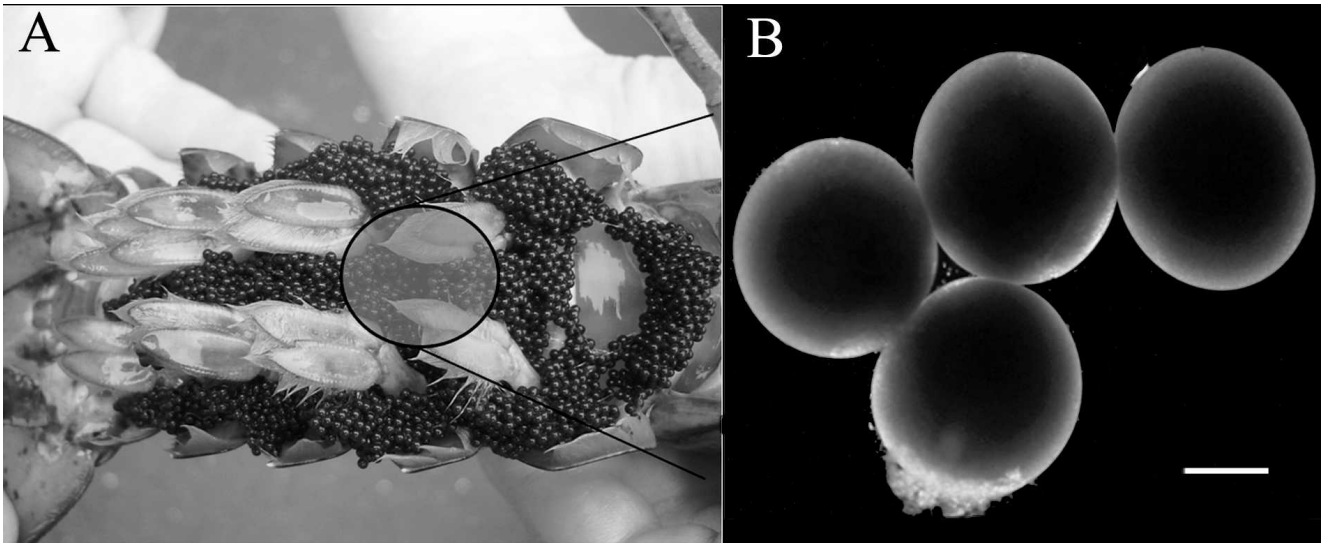

Fig. 1. A, typical clutch of lobster eggs. At 20 days through at least one month old, (green eggs, size range = 1.6-1.7 mm ± 0.4 mm in diameter), there are no discernible features that indicate fertilization status. The photograph of the egg was taken under a dissecting microscope at a total magnification of 40× (scale bar = 50 μm).

summer, the storage of sperm in the spermatophore, the extrusion and presumed fertilization of the egg clutch, and the incubation of eggs for 9-12 months until they hatch into larvae one to two summers later (Bumpus, 1891; Herrick, 1895). Throughout egg development, growth measurements of the size of the prominent eyespot, along with egg color and other morphological and physiological features, are often used to stage eggs and determine if they are fertilized (Bumpus, 1891; Herrick, 1909; Templeman, 1940; Perkins, 1972; Helluy and Beltz, 1991). However, with newly extruded egg clutches, it is virtually impossible to visually determine if eggs are fertilized, especially in the field. Early-stage eggs (< 2 months old) are characterized as featureless, solid, and dark green, with no discernible differences from eggs that are unfertilized (Fig. 1). Growing evidence from both lab and field studies suggest that female lobsters may extrude unfertilized egg masses that are a result of unsuccessful mating attempts or perhaps inadequate sperm stores, i.e., sperm limitation (Knight, 1918; Talbot and Harper, 1984; Aiken and Waddy, 1982; MacDiarmid and Butler, 1999; Gosselin et al., 2003; Pugh et. al., unpub. data). In at least two studies, females of *H. americanus* have been observed extruding unfertilized eggs that they subsequently carried for varying amounts of time (Talbot and Harper, 1984; Talbot et al., 1984). This has also been observed in spiny lobsters, e.g., *Panulirus cygnus* George, 1962 (Chittleborough, 1976). Other studies suggest significant egg attrition (early on) from lobsters in both lab and field studies stemming from a variety of causes including disease, trap handling, faulty egg-attachment, and sub-optimal environmental conditions, e.g., increased temperatures (Perkins, 1971; Aiken and Waddy, 1980; Hedgecock, 1983; Talbot and Harper, 1984). However, discerning the origin of these losses early on, especially for those egg clutches that could be unfertile, remains elusive and largely uninvestigated. Therefore, if some of the egg-bearing females observed during assessments are carrying unfertilized eggs, managers could be overestimating the number of new recruits to the fishery. A major goal of this project was to develop a technique for determining if early stage eggs are fertilized. Eventually, we hope to use the methods developed to estimate the percentage of egg-bearing females in a given population that are carrying unfertilized eggs and thus not contributing recruits to the population that year.

Lab-based methods that have been developed to assess fertilization of early developing eggs using nucleic acid stains, e.g., DAPI and Hoechst, have become common amongst a diverse range of terrestrial and aquatic invertebrates (Buttino et al., 2003; Masci and Monteiro, 2005; Zirbel et al., 2007). Because of the strong affinity of DNA-binding proteins and their specificity to the major groove of DNA and its A-T rich region, nuclei can be readily visualized with these DNA specific stains, especially if there are multiple nuclei and cells that are actively dividing (Dervan, 1983). However, staining techniques for lobster eggs have not been very successful due, in part, to their complex morphology and the nature of their fertilization membranes compared with other decapod crustaceans (Cheung, 1966; Talbot and Goudeau, 1988). For example, during the extrusion process, lobster eggs develop two prominent, thick, outer envelopes that help protect the developing embryo. The mechanism by which these membranes are formed has been debated, however in a study by Talbot and Goudeau (1988), it was concluded that the outer coat of the oöcyte is formed in the ovary and the inner coat originates from a complex cortical reaction that occurs during fertilization. Together, these tightly bonded coats comprise the fertilization envelope of the developing egg and, due to its impermeable nature, make typical DNA staining extremely challenging.

In this study, we modified existing nuclear staining methods so they would work consistently with American lobster eggs. Specifically, we used an enzyme solution to breakdown the outer egg membranes so that a nuclear staining agent was able to penetrate into the egg and bind with the DNA. We also demonstrated that this method will

work with fixed eggs, making it possible to obtain numerous egg samples from field sampling surveys and then store them, prior to subsequent analyses in the laboratory. In addition, a secondary method of fertility testing was utilized to quantify the amount of DNA within individual lobster eggs using fluorescence spectroscopy, i.e., fluorometry. This method allowed us to quantify and compare the amount of DNA present in unfertilized control oöcytes and early- and late-staged fertilized eggs. Like the DNA staining method, this technique made it possible to reliably determine if eggs were fertilized and contained a large amount of DNA due to multiple nuclei, or were unfertilized. In the future either method will make it possible to obtain data that could improve the accuracy of programs designed to predict the number of new recruits that will be added to the lobster fishery in a given year.

Materials and Methods

Animals

A total of 14 female lobsters were caught in standard traps along the New Hampshire seacoast near Rye, New Hampshire, U.S.A., by permitted commercial lobstermen and transported to the University of New Hampshire (UNH) Coastal Marine Laboratory in Newcastle, New Hampshire. All animals were held in floating totes (81.3 cm × 50.8 cm × 38 cm) at ambient light and temperature levels (16.3 ± 1.6°C; mean ± SD) in an outside impoundment until they extruded their egg clutches. Lobsters were fed twice weekly with fresh herring and rock crabs (*Cancer* spp.) and checked for egg extrusion three times per week.

In order to monitor the appearance of eyespot formation (and confirm fertilization status), after egg clutches were extruded, small batches of eggs (n = 10/lobster) were removed from each lobster's clutch with forceps at weekly intervals (from 1 June to 15 August) and photographed. All sampled eggs were disinfected for 5-10 minutes by dipping them in a 10% solution of medical-grade iodine and sterile seawater at a concentration of ~ 150 mgL^{-1} (Uglem et al., 1996) to clean them (externally) of epibiotic bacteria. Digital images of a subset of eggs from each clutch were taken with an Olympus SZH-5 stereomicroscope equipped with a color digital Olympus DP-20 camera system (Olympus America, Center Valley, Pennsylvania) to monitor the appearance of eyespot formation. In some cases (later-developed eggs), developmental stage was determined using staging tables described in Helluy and Beltz (1991).

Egg samples were collected from an additional six ovigerous females during sea sampling efforts in the same area (n = 10 eggs/lobster). However, these eggs were first photographed and then placed into 1.5 mL sample vials containing the following fixative: 97% glucamine-acetate buffer, 2% formalin, and 1% Triton-X (Sainte-Marie and Carriere, 1995). Therefore, a total of 20 clutches of eggs (4 fixed and 16 live) were used for testing the two methods developed for this study.

Method I: Nuclear Staining of Lobster Eggs

Fertilization status was determined by sampling a single subset of eggs from each lobster within 1-2 weeks after egg extrusion. Nuclear staining was performed on both the fixed and live eggs that had been photographed earlier. For live eggs, a total of five eggs were removed from each clutch (one time) and stained, for a total of 70 eggs (n = 5 eggs × 14 clutches). For fixed eggs, a total of five eggs/clutch were stained from the samples collected from the six lobsters sampled at sea, yielding a total of 30 fixed eggs. Both fixed and live eggs (5 eggs/sample) were first placed into 1.5 mL plastic conical tubes and rinsed 3X in a PTA buffer solution (phosphate buffered saline, 0.4% Triton X-100, 0.1% sodium azide). Eggs were then set in 100 µL of Accutase™ enzyme solution (A6964, Sigma-Aldrich, Inc., St. Louis, Missouri) and left on a rotating plate (Nutator model 421105) at room temperature for 24 h. Samples were then rinsed 3X in PTA, placed in 100 µL of Hoechst nucleic stain (H6024, Sigma-Aldrich, Inc.) and placed back on the rotating plate for 24 h. Finally, eggs were rinsed 3X in PTA and placed on silica glass depression slides with a few drops of sterile seawater (32 psu) for viewing.

Stained eggs (live and fixed) were observed and photographed using a Zeiss Axioplan-2 imaging compound microscope (Carl Zeiss IMT Corp., Thornwood, New York) using a DAPI filter cube (excitation = 358 nm; emission = 463 nm). These filter cubes are typically inserted into the fluorescence filter revolver of the microscope and reflect UV excitation while transmitting DAPI emission (see http://www.zeiss.com, for details).

Successive digital images were taken using AxioVision v.4.7 software and the multi-dimensional acquisition routine (z-stacking) through an Axiocam MRm/MRc5 camera (Carl Zeiss IMT Corp.) connected to a PC-based computer (Dell Optiplex G2410T). Eggs with multiple stained nuclei were considered fertilized, while those with either one or no nuclei visible, were considered not fertilized.

Extraction of Unfertilized Oöcytes (control)

Pre-extruded oöcytes were removed from the intact lobster ovaries of eight females (CL range: 86-98 mm; n = 5 eggs/lobster) (n_{total} = 40 eggs) according to methods described in Little and Watson (2005). Briefly, a small square was cut in the carapace behind the eyes, and a section of ovarian tissue was removed with blunt forceps. Pressure was then applied to the wound to stop the blood flow and the incision was sealed with the use of cyanoacrylate glue, gauze, and adhesive tape (lobsters typically survived this procedure). The dissected ovaries were then placed in a small glass petri dish with sterile seawater, and the ova were gently teased away and separated from their connective tissue (Talbot, 1981). The same nucleic acid staining protocol used for fertilized eggs was then followed. Typically, staining of one nucleus was observed under UV excitation as opposed to the visualization of multiple nuclei in fertilized eggs.

Method II: Egg DNA Measurements (fluorometry)

In tandem with staining, total DNA concentration was measured in unfertilized and fertilized eggs (each measurement was made using a total of 5 eggs), as well as some that were at more advanced stages of egg development. Samples (n = 10 eggs/female) from two control females (oöcytes extracted from the ovaries) yielded a total of 20 unfertilized ova. Samples of fertilized eggs (n = 10 eggs/lobster) were obtained from three females (n_{total} = 30 eggs) when eggs were 5-10 days old and again when their eggs were 20-30 days old. An additional subset of eggs (n = 10 eggs/female) was removed from two additional lobsters (n_{total} = 20 eggs) that were developmentally staged as advanced (50-60% developed; Perkins, 1972). All egg samples were set in 200 mL of SDS buffer solution and carefully homogenized in separate 1.5 mL plastic conical tubes. Next, the tubes were vortexed (Vortex-Genie 2, model G-560) for ~ 5 seconds and placed onto a rotating plate (Nutator model 421105) at room temperature for 24 h. Egg samples were again homogenized and vortexed and then allowed to sit and settle for 30 min before measurements were obtained.

The fluorometer unit (Hoefer DyNA Quant 200, Hoefer Inc., Holliston, MA) was calibrated with a known 100 µL standard (calf thymus DNA, D3664, Sigma-Aldrich, Inc.) before each trial. A 2 mL aliquot of reagent solution (100 µL Hoechst with 100 mL 1XTNE) was mixed with 2 µL of homogenized egg solution in a 5 mL cuvette and gently shaken. The cuvette was then placed into the fluorometer and the concentration of DNA in the sample was measured in ng/mL. Each sample was measured in triplicate and the values averaged. A correlation analysis was conducted using JMP v. 8.0.2 (SAS Institute, Cary, NC, U.S.A.) to examine the relationship between days after egg extrusion and the total amount of DNA per egg, with the expectation that DNA content increased as eggs developed.

Results

Method I: Nucleic Acid Staining

A total of 20 lobster egg clutches were tested to determine their fertility status (14 from females kept in holding tanks and 6 from lobsters captured while sea sampling); a total of 16 clutches (80%) were fertilized and 4 were not (20%). Of the 16 clutches with fertilized eggs two had a mix of both fertile and unfertilized eggs (Table 1).

Eggs that were fertile typically displayed multiple nuclei that were readily visualized when exposed to UV illumina-

Table 1. Lobsters that served as a source of eggs for this study. A total of 14 egg-bearing lobsters (two-digit ID numbers) were held in totes at ambient seawater temperatures (16.3 ± 1.6 °C; mean ± SD) and photoperiod until they extruded their clutches from 28 June-2 August 2010. We continued to hold these lobsters, remove eggs weekly (n = 10/lobster), and examine the eggs to determine if they developed normally or not. An additional 6 egg-bearing lobsters were collected during sea-sampling trips (ID: 100-105) and their eggs were fixed and also examined (n = 20 lobsters total). For staining purposes, we collected a total of 70 live eggs (n = 5 eggs × 14 clutches) and 30 fixed eggs (n = 5 eggs × 6 clutches). Lobsters were sized (carapace length, CL), and in some cases egg extrusion date was noted.

ID	CL (mm)	Extrusion date	Staining result (fertilized)?	Egg development (eyespot formed)?
05	86	28-June	yes	yes
17	98	30-June	yes	yes
01	85	10-July	mixed	some
26	87	11-July	yes	yes
14	87	17-July	yes	yes
43	86	17-July	yes	yes
48	85	17-July	yes	yes
53	93	17-July	yes	yes
66	92	17-July	yes	yes
89	88	21-July	yes	yes
04	100	28-July	no	no
10	82	28-July	mixed	some
12	83	2-August	yes	yes
42	86	2-August	no	no
100	88	unknown	yes	N/A
101	78	unknown	no	N/A
102	91	unknown	yes	N/A
103	86	unknown	yes	N/A
104	82	unknown	no	N/A
105	87	unknown	yes	N/A

tion (Fig. 2). Conversely, stained unfertile eggs emitted a hazy blue halo around the outer egg membrane, and occasionally a single nucleus was also visible. Thus, it was simple to distinguish between fertilized and unfertilized eggs using this staining procedure. While fertilized eggs of all different developmental stages were successfully stained, the most important finding was that this method made it possible to determine if eggs as early as 7 days old, expressing no other discerning biological indicators of their fertility status, were fertilized or not (Fig. 3).

In order to confirm that DNA staining was yielding an accurate assessment of egg fertilization status, after we removed eggs for staining, we retained the 14 egg-bearing females and held them at ambient conditions so that their eggs could continue to develop. The eggs carried by these females were observed weekly to determine if they were fertilized or not. Appearance of an eyespot after ~ 25 days indicated they were fertilized, while unfertilized eggs either fell off the females, turned a yellowish-orange color, or did not develop an eyespot after approximately 30 days. In all cases, if the staining method indicated that a female was carrying fertile eggs, these eggs continued to grow until the eyespot stage of development (Perkins, 1972; Table 1). In addition, in the few cases where staining yielded mixed results, a female was carrying a clutch of eggs that contained some eggs that developed eyespots and some infertile eggs that never developed, started to deteriorate, and often changed color. Thus, while only 14 clutches (lobsters in holding) were tested, this method indicated their fertilization status with an accuracy of 100%.

Method II: DNA Fluorometric Measurements

Measurements of total DNA in unfertilized eggs and eggs at various stages of development were obtained to determine if, as cells divided, DNA levels would increase and thus serve as another proxy of fertilization status. There were significant differences in DNA concentrations between eggs that were: 1) unfertilized (n = 4, 5 eggs/sample, 20 eggs total); 2) 5-10 days old (n = 6); 3) 20-30 days old (n = 6)

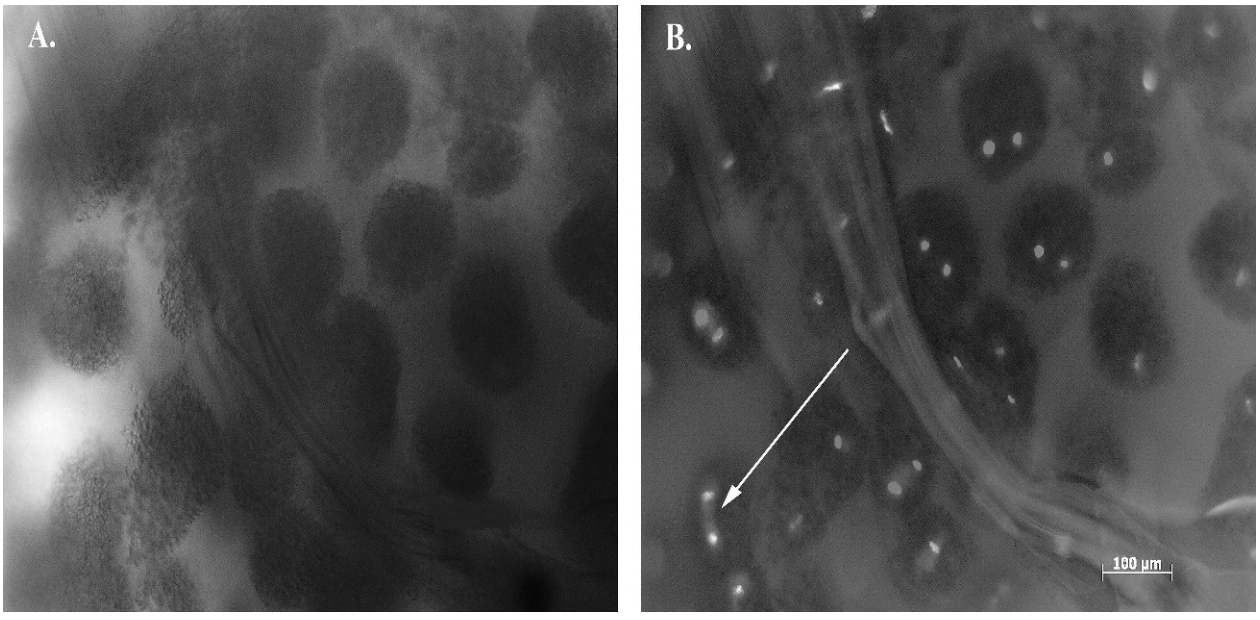

Fig. 2. A, Dividing cells in a fertilized lobster egg visible under bright field illumination; B, The same dividing cells with nuclei visible (arrow) after being treated with Accutase™ and Hoechst stain and viewed under UV light (λ = 463 nm). Note the appearance of dividing nuclei resulting in the visualization of two clusters of DNA present within some cells, (total magnification = 100×).

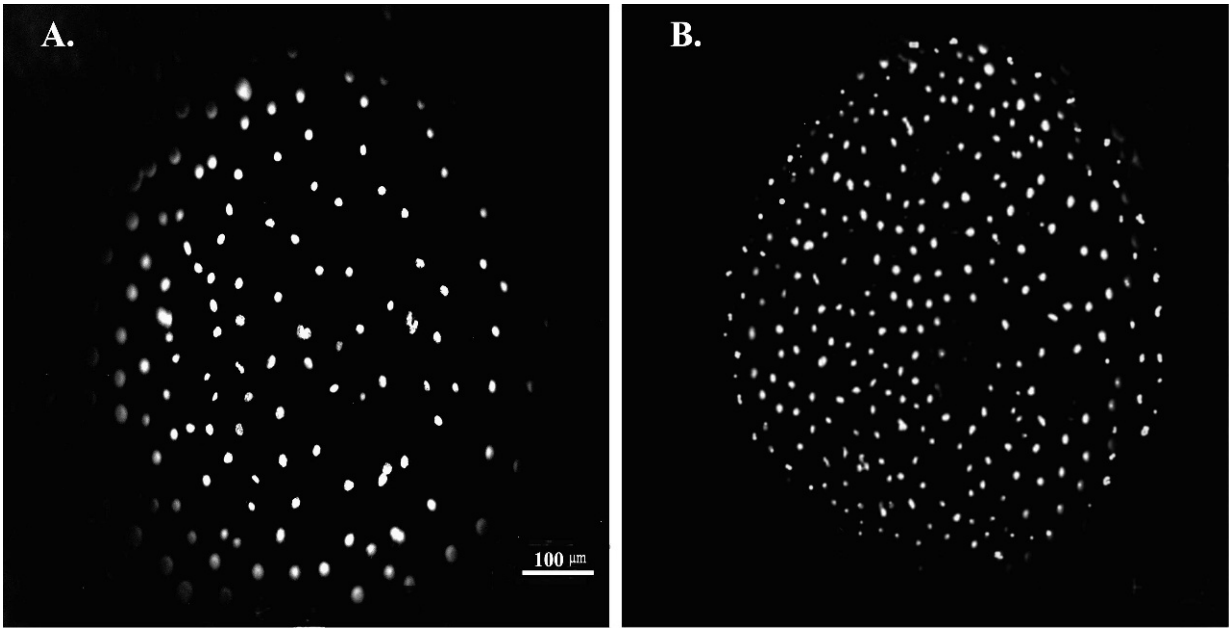

Fig. 3. Appearance of lobster eggs following DNA staining. A, Stained nuclei are visible in an early-stage egg (7 days after extrusion, DAE) exposed to fluorescent excitation and created as a z-stack image; B, Stained nuclei in an egg taken from the same clutch 14 DAE. Notice the increased number of nuclei present due to continued mitotic divisions, (total magnification = 100×). (scale bar = 100 μm).

and; 4) 60-80 days old (50-60% developed) ($n = 4$) ($r = 0.961$, $P < 0.0001$; Fig. 4). Importantly, for our purposes, there was also a difference in the total DNA concentration between unfertilized control oöcytes and early stage fertilized eggs that did not have eyespots (unpaired t test, $t = 8.581$, $P < 0.001$). The average concentration of DNA in unfertilized oöcytes was 28.6 ± 16.1 ng/mL (range = 10-50 ng/mL), while it was 80.5 ± 5.86 ng/mL (range = 55-131 ng/mL) in fertilized eggs (Fig. 4). Therefore, it appears as if this fluorometric assay could also be used effectively to determine if young eggs had been fertilized or not.

Discussion

We have described two simple procedures for determining if early-stage lobster eggs have been fertilized. The first method, nucleic staining of DNA with the use of the enzyme (Accutase™) and Hoechst stain, enabled us to visualize nuclei and distinguish fertilized eggs with multiple nuclei from unfertilized, haploid eggs. Fluorometry was also used to demonstrate that the amount of DNA differed in a predictable manner between unfertilized, early fertilized, and more advanced-staged eggs. Although our results from both egg nuclear staining and fluorometric methods complement each other, nucleic acid staining should be considered the preferred method to test for lobster egg fertility status because it is: 1) more cost-effective ($US 50.00/~ 300 eggs), 2) less time consuming, and 3) a more consistent and reliable procedure that allows for a clear cut determination based on the presence or absence of multiple stained nuclei.

The major modification that made it possible to attain consistent results with Hoechst stain was the use of the Accutase™ enzyme solution to degrade egg membranes enough so that the stain could penetrate into the egg. Talbot (1981) reported that hydrolytic enzymes, such as collagenase, appear to weaken lobster egg cell membranes and allow in-vitro fertilization to occur. While our initial attempts with collagenase and other enzymes were only moderately successful, we found that Accutase™, a cell detachment solution consisting of a mixture of proteolytic and collagenolytic enzymes, was the most successful at degrading egg membranes and allowing the Hoechst stain to penetrate and bind to egg DNA. Eggs that were first preserved with fixative and then set in Accutase™ and Hoechst stain also showed successful staining of nuclei. Fixing eggs makes it possible to collect eggs in the field and store them for subsequent examination in the laboratory. Therefore, for example, eggs could be collected and preserved by offshore lobstermen who are often at sea for up to 10 consecutive days.

In early development, following fertilization, lobster eggs go through superficial cleavage and rapid cellular division before reaching the 16-cell morula stage. The nuclei of dividing cells, each surrounded by an amoeboid mass of protoplasm, divide within the yolk and approach the periphery (Bumpus, 1891). As development continues, constant cellular division results in the formation of a blastula and eventually leads to gastrulation. This growth and increase in cellular density can be visualized as eggs develop based on the amount or concentration of stained nuclei present in the egg. Thus, while very young eggs can be seen dividing using a light microscope, eggs that are days to weeks old are difficult to stage, especially due to the high degree of yolk reserves present that often occlude developmental features (Sasaki et al., 1986). These are the kinds of eggs that this method was designed to examine.

Unfertilized haploid oöcytes contain one nucleus and one set of DNA within that nucleus. Ideally, upon staining, one

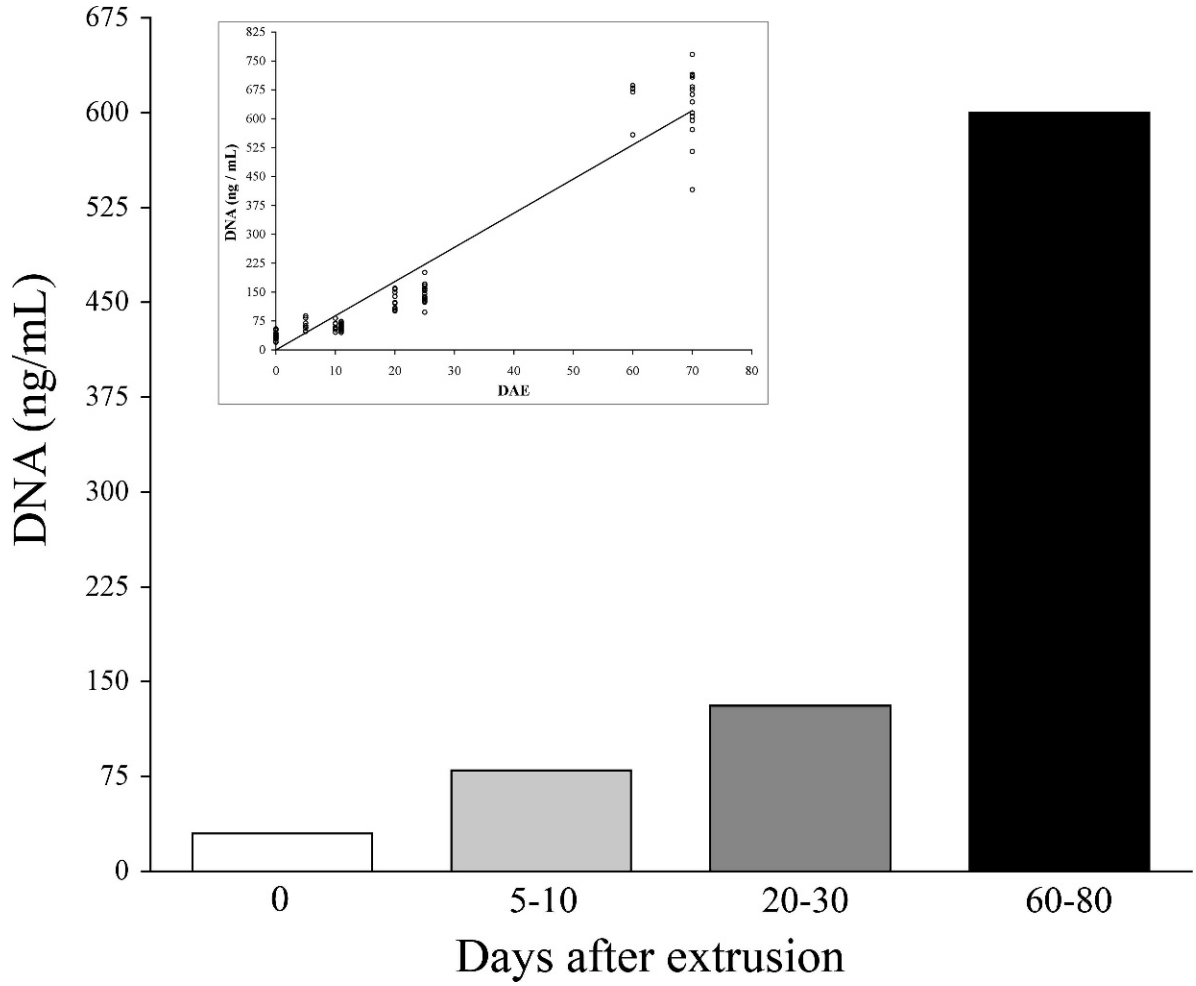

Fig. 4. Total DNA (ng/mL) per egg determined by fluometry (see text for sampling details). Eggs: unfertilized (5 eggs/sample; $n = 20$ eggs, 2 females $\times$ 10 eggs each), early-stage (5-10 days old, $n = 30$ eggs, 3 females $\times$ 10 eggs each), early-stage (20-30 days old, $n = 30$ eggs, 3 females $\times$ 10 eggs each), and advanced-stage (60-80 days old, $n = 20$ eggs, 2 females $\times$ 10 eggs each). Inset: correlation between DAE and total DNA concentration ($r = 0.961$, $P < 0.0001$).

strand of DNA should be visible. However, we found that the single haploid nucleus was difficult to locate within the oöcyte. Rather, stained unfertilized eggs had a hazy-blue overall stain and no evidence of the individually stained nuclei seen in fertilized eggs. Therefore, while it is not easy to identify one haploid nucleus in an unfertilized egg, it is easy to determine if an egg is fertilized or not.

Our staining assay allowed us to collect data on the fertility status of 20 different clutches of lobster eggs (Table 1). Egg clutches that had not been fertilized ($n = 4$ lobsters, size range = 78-100 mm CL) were likely the result of females that molted and then failed to mate (lobster 04; Table 1). At the present time, it is not clear how common this phenomenon is in natural populations. In a separate study, female lobsters ($n = 6$) were held in isolation after they had molted, so that they did not have a chance to mate. Four of these lobsters did not extrude eggs and the remaining two extruded eggs that were unfertilized; if a spermatophore is present, it is lost when they molt (Aiken and Waddy, 1980; Goldstein, unpublished data). Sato et al. (2006) reported that on occasion female king crabs, *Paralithodes brevipes* (Milne Edwards and Lucas, 1841), extrude eggs that are not fertile due to insufficient sperm allocation from males. We also found two cases where lobsters (CL = 82, 85 mm) had a mixed clutch of eggs. This situation is likely caused by a female attempting to fertilize a clutch of eggs using a spermatophore that does not contain sufficient sperm for all the eggs.

Both of the aforementioned situations suggest that some sexually mature females are not obtaining sufficient sperm to fertilize all their eggs. Possible causes for this situation may include: 1) females molting and failing to find a mate during the time period when they are most receptive; 2) females mating with a male that produces a smaller than normal spermatophore due, for example, to an effort to allocate sperm to many different females or; 3) females using a single spermatophore to fertilize more than one clutch of eggs (but see Gosselin et al., 2005). Male sperm depletion has been confirmed in both spiny and clawed lobsters (Gosselin et al., 2003; MacDiarmid and Stewart, 2005), and sperm supply is now considered a potential factor limiting the reproductive output in some lobster and crab populations (MacDiarmid and Butler, 1999; Rondeau and Sainte-Marie, 2001; Kendall et al., 2002; Gosselin et al., 2003; Hines et al., 2003; Sato and

Goshima, 2007). Although sperm limitation has not been formally documented in American lobster populations, several observations suggest that in some heavily exploited areas where there is a highly skewed sex ratio, the reproductive dynamics could be altered, e.g., evidence for multiple paternity in some females (Gosselin et al., 2005; ASMFC, 2009). Sperm limitation has not been well documented or quantified in American lobsters because either pre-extruded, unfertilized eggs are resorbed (Waddy et al., 1995) or extruded, unfertilized eggs tend to fall off females within a month and, during this first month, before they develop eyespots, unfertilized eggs look very similar to fertilized eggs. We anticipate that the methods reported in this paper will make it possible to examine these early eggs and develop a much better understanding of the reproductive dynamics of different American lobster populations. In particular, it will be useful to know if some type of sperm limitation is occurring and, if so, why?

The potential now exists for fishery biologists and managers alike to use these methods when conducting biological surveys to help determine the fertilization status of early-staged eggs before the development of eyespots. Combined, these methods alongside seasonally-timed surveys, e.g., fall egg-bearing lobster surveys, would improve measurements of the reproductive potential of ovigerous populations of lobsters, especially in areas where differences in reproductive dynamics may exist, e.g., sex ratios, mating structure, male size differential. The ability to quantify those females that fall into a potential 'sperm limitation' category will make it possible to improve models that predict the number of new recruits to the fishery and also better understand how the fishery may, or may not, be influencing the reproductive dynamics of this very valuable marine resource.

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