Fluorochrome Marking of Out-planted Green Sea Urchins, *Strongylocentrotus droebachiensis*, for Sea Ranching and Restocking Programs in the Gulf of Maine, USA

Pamelia Fraungruber[*1], Stephen D. Eddy[*1], Nicholas P. Brown[*1], and Larry Harris[*2]

Abstract: Marking calcified structures with fluorochromes is done in a variety of vertebrate and invertebrate species to tag individuals for growth, population, and ecological studies. Here, we describe the use of the fluorochrome tetracycline to identify hatchery reared green sea urchins released on-bottom onto two aquaculture leases known as Job and Sloop, located in the Gulf of Maine, USA. This was done to examine the viability of sea ranching and stock enhancement by looking at recovery rates and growth of reseeded juveniles over the course of two years. 21,000 hatchery reared green sea urchin juveniles *(Strongylocentrotus droebachiensis)* were marked with the fluorochrome tetracycline when they were at 10 – 20 mm test diameter, and released onto 400m^2 study areas located at each lease. Juveniles from the same hatchery cohort were simultaneously reared in a land-based recirculating aquaculture system so that sea ranching could be compared with tank farming. The release areas were surveyed by SCUBA divers at 3–5 month intervals for over two years. Urchins were collected from the field, measured, and dissected to remove the jaw structures, which were then examined with fluorescence microscopy. Tetracycline fluorescence was detected for up to 27 months post-release in recaptured urchins. Numbers of recaptured marked urchins fluctuated over time, causing large variability in population survival estimates for each site at each sample interval. Size measurements of recaptured urchins showed a decline in average test diameter at the Job site, but at the Sloop site average test diameter increased during the two year study. Green sea urchins from the same hatchery cohort reared in a land-based tank system had significantly better growth than those recaptured from either lease site. Environmental factors, rather than genetic factors (hatchery source), were likely the cause of the size differences observed between hatchery seed recaptured from the lease sites and those reared in tank culture. Site factors may have resulted in size dependant mortality and/or out-migration of larger urchins. One of the limitations of the mark/recapture approach with sea urchins is that dive surveys need to expand over time to account for urchin movement away from the release area. Given the high cost of such efforts, this may not be practical or cost effective. Because the marked jaw structures were internally located, it was not possible to identify marked sea urchins in the field, and the animals had to besacrificed for laboratory analysis. Recent advances in fluorochrome marking and visualization could allow field identification of marked urchins. This would enhance the ability of resource managers to evaluate restocking programs in the Gulf of Maine, as well as to assign provenance or ownership of sea ranched urchins.

Key Words: green sea urchins, fluorochromes, sea-ranching

The green sea urchin *Strongylocentrotus droebachiensis* has been an economically important fisheries species in the Gulf of Maine, USA (GOM) since the 1980's. Catch levels peaked in 1993 at 19,050 metric tons, and the fishery value peaked in 1995 at $35,604,275. However, these large annual harvests couldn't be sustained, and ecological changes have contributed to a steep decline in wild stocks (Steneck *et al.*, 2004). Since 2008 the annual catch in Maine has averaged about 1,300 mt, with an average value of about $5.5 million.Although the fishery may not return to 1990 levels, resource managers and fishermen believe that wild stocks can and should be rebuilt to allow for increased harvest levels. Several management approaches have been taken to help rebuild natural stocks, mostly based on fishing restrictions. Stock enhancement through release of hatchery seed has also been discussed, but uncertainty regarding its ecological and economic viability has discouraged public funding of any sustained stock enhancement programs in the GOM.

Evaluating the effectiveness and benefits of sea urchin reseeding programs is an essential but complex task. The economic return will be a function of seed production costs, growth and survival of out-planted seed, and market prices at the time of recapture. In Japan, sea urchin stock enhancement, known as reseeding, has been done at large scale (>50 million seed annually) for over 20 years (Agatsuma *et al.*, 2004; Sakai *et al.*, 2004). In Hokkaido it cost 4–10 JPY (4–8 US cents) to produce one seed of 5 mm test diameter, and nearly 3x that for 20 mm seed (Sakai *et al.*, 2004). The economic benefits of reseeding to the Japanese fishery remain uncertain; in some cases catches have declined or remained static despite widespread reseeding (e.g. *Strongylocentrotus intermedius* in Hokkaido) , whereas in other cases reseeding is correlated with improved catch levels (e.g. *S. nudus* at Esan near Hakodate City) (Agatsuma 2014, in publication). Ultimately, the costs of seed production versus the economic return to the fishery must be considered in the context of cultural values and ecological consequences.

Although increased catch levels might imply that restocking has been successful, it is not evidence of a cause and effect relationship. Ecological changes, increased recruitment, or intensified fishing effort can also lead to improved stocks or increased catch levels. Measures of survival, growth, and return to the fishery are needed to assess the cost/benefit of stock enhancement. This can only be done if released stock can be differentiated from wild stock, but there are no discernible external differences between hatchery and wild urchins (Agatsuma *et al.*, 2004). However, sea urchins can be internally marked with fluorochromes, either through injection or bath immersion. Kobayashi and Taki (1969) were the first to use tetracycline to mark the sea urchin S. *intermedius* for growth studies. Since that time, a variety of studies have used fluorescent markers to identify sea urchins in the lab or in the field (Ellers and Johnson, 2009). This paper describes the use of fluorochrome marking to identify hatchery seed of *S. droebachiensis* released onto ocean bottom leases in the GOM in order to evaluate sea ranching. Sea ranching is similar to reseeding, but in this case the juveniles were released onto privately held aquaculture leases. The project was carried out by the Center for Cooperative Aquaculture Research (CCAR), working with industry partner Friendship International (FI), a sea urchin trading company based in Maine. We were interested in ascertaining whether this privatized mode of reseeding could be a viable model for the fishery. To do this we needed to determine if released seed would remain within lease site boundaries, and whether growth and recovery rates would be sufficient to realize a return to the lease site operator or to the fishery.

Materials and Methods

Hatchery: The CCAR is a multi-species aquaculture research and development facility operated by the University of Maine (http://www.ccar.um.maine.edu/index.html). Hatchery production for the project was carried out at the CCAR in the spring of 2009 (Feb.–June). Green sea urchinswere induced to spawn (N=39 females and 30 males) to provide gametes that were fertilized for larval rearing. Laboratory spawning, fertilization and larval rearing methods for *S. droebachiensis* are similar to those described for many other sea urchin species (McBride, 2005). Larvae were reared in conical bottom 230 L clear

fiberglass vats continuously supplied with fresh seawater at about 12 ℃. *Dunaliella tertiolecta and Rhodomonas salina* were the primary algal feeds. The larvae were competent for settlement at 24–30 days post-fertilization. Following settlement, juveniles were reared for an additional period of 8–10 months in a land based nursery system. During the nursery period they were held in plastic hydroponic plant baskets in shallow fiberglass raceways, and fed *ad libitum* with freshly harvested *Saccharina latissima*.

Fluorochrome marking and visualization: The juveniles were marked (tagged) with tetracycline about four months before release onto the lease sites, using methods adapted from Ellers and Johnson (2009). Juvenile urchins were graded into perforated baskets, and immersed for 24 hours in tanks filled with 0.2 μm filtered seawater and 37.5 mg per L^{-1} tetracycline (Sigma-Aldrich Tetracycline T3258). Urchins were fed to satiation before and during tagging to ensure active growth and uptake of the fluorochrome into the calcareous exoskeleton. Two weeks following marking twenty-five urchins were examined using a fluorescence microscope. The jaws of each individual were removed and placed in a sodium hypochlorite solution to dissolve all organic material, leaving only the calcareous jaws behind. These structures were then examinedthrough a GIB filter using a Zeiss Axio Imager Z1 fluorescence microscope. Oxytetracycline goes through excitation at 390nm and emission at 560nm. Tags appear as a bright line of fluorescence spanning the jaw horizontally, and for the most part were easily identifiable (Fig. 1). 100% of those examined directly after tagging had clearly visible tags.

Sea ranching: The tagged juveniles were released at two aquaculture leases located in Penobscot Bay, Maine. Site 1 (Sloop) was located off of Northaven, Maine near Sloop Island (44° 12.2'N 68° 50.1'W) and Site 2 (Job) was off of Camden, Maine near Job Island (44° 13.5'N 68° 50'W). Each site comprised two acres (0.81 ha) of sea bottom, with a mean water depth of about 2–5 m. The leases were marked with buoys to indicate that harvesting urchins by dragging nets across the bottom was prohibited. In February of 2010, 10,500 juveniles were released at each site onto a small study area located approximately within the middle of each lease. The
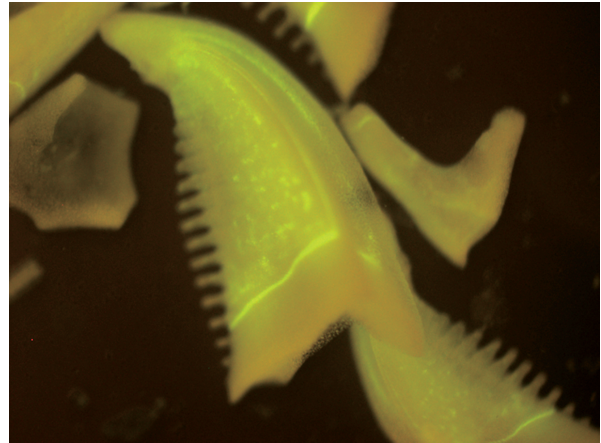

Fig. 1. A tetracycline marked green sea urchin *Strongylocentrotus droebachiensis* demipyramid viewed using a WIB filter on a fluorescence compound microscope.

juveniles were transferred in plastic bags by divers onto the bottom and distributed along transect lines laid out to 15 m in all four compass directions, encompassing a total area of 400 m^2. Between 1,000 and 1,500 juveniles were released at 5 m and 10 m markers along the transects to ensure an even distribution. The juveniles were not enclosed and therefore were free to move. No feeding or any other husbandry activity was conducted during the two years following the release.

Site surveys: The sites were characterized in a previous study (Kirchhoff *et al*., 2008), but prior to out-planting an initial transect dive was done to estimate the extent of existing sea urchins, predators, and bottom cover. At each release area a baseline was laid out in a North-South orientation and five transect lines were laid out on a perpendicular (East-West) bearing extending to 10 m. Sample quadrats consisting of a 1 m2 PVC frame were placed at the 10 m marker in each direction, at the center of the transect, and just over the baseline (0 m on transect), for a total of 15 quadrats per site. During the pre-release survey the bottom substrate was characterized and the numbers of predators (crabs, sea starts, etc.) and naturally occurring (pre-existing) urchins were counted. The extent and composition of algal feed was also observed for each site. The out-planted areas were then dive surveyed on six more occasions at 3–5 month intervals over the course of 27 months. All urchins within each

sample quadrat were enumerated and those between 4–30 mm TD were collected in numbered mesh tubes to be taken to the laboratory for measurement and identification (absence/presence of fluorochrome marker). Urchins smaller or much larger than the original release size were not collected in early surveys, but during later surveys larger urchins were collected to account for any growth.

Tank culture: During the two year sea ranching study 9,500 green sea urchins from the same hatchery cohort as the lease site urchins were reared in a tank system at the CCAR, to compare growth and survival of lease site urchins with juveniles reared on land. The juveniles were stocked into raceways assembled to form a slanted V interior profile (V-trough), with a perforated bottom plate to remove wastes. The V-troughs were plumbed into a recirculating seawater aquaculture system equipped with a parabolic filter for solids removal, moving bed biofilter, foam fractionator, oxygen injection, 3 hp chiller, and UV sterilizer. Rearing temperatures were held between 6–16 ℃ year round and the juveniles were fed high quality formulated diets (Nofima diet from Norway). It was anticipated that sea urchins reared under these conditions would have good growth and survival, to provide a benchmark by which the lease site urchins could be compared.

Specimen analysis: Specimen bags containing urchins from the sample quadrats were brought back to the lab, drained and frozen until analysis. These were later (within 2–6 weeks) thawed in seawater, and all individuals were blotted dry and weighed to the nearest 0.1g. Test diameter (TD) was measured to the nearest 0.1mm with digital calipers (model CD-6PMX Mitutoyo Corporation, Kawasaki, Japan). Each sea urchin collected from the lease sites was analyzed for the presence of oxytetracycline marking, as described above. In some cases multiple or single bands of auto-fluorescence were seen that appeared atypical or ambiguous (e.g. diffuse). Sources of ambiguity and therefore error in identification included size of the jaws, intensity of the light used to make the tags fluoresce, and ambient light from the surrounding room. If the results were uncertain, then the jaws were either reevaluated or marked as "untagged". Urchins that were clearly tagged were considered as recaptured (hatchery origin).

Urchins reared in the land-based tank system were sampled at intervals coinciding with the lease site surveys. Thirty urchins from each tank were randomly removed and measured for weight to the nearest 0.1 g, and TD to the nearest 0.1 mm using digital calipers.

Data analysis: The average number of total urchins (tagged and untagged) per square meter was calculated for each study area and survey date as the total number of urchins collected per site divided by the number of sample quadrats (usually 15). The number of released seed remaining at each site and survey date was estimated as the average number of recaptured (tagged) urchins per sample quadrat (m^2) x 400 m^2 (the size of the release area as a whole). The mean, minimum and maximum test diameter of recaptured urchins was calculated for each site and survey date. Chi squared tests were used to determine whether or not the numbers of tagged and untagged urchins were significantly different from each other. The standard deviation of the mean test diameter was determined to see if the average size of recaptured urchins significantly differed (± 1 SD) between the two sites and from the tank reared urchins. Data were plotted to display trends in numbers, average TD, and maximum TD of recaptured urchins at each site over time, and the TD of lease site urchins was compared with that of tank reared urchins.

Results

Site characteristics: The two sites were less than six nautical miles apart and of comparable depth (2–6 m mean water), but they differed in terms of exposure, current, bottom substrate, and population density of naturally occurring (pre-existing) urchins. The Job Island site had relatively uniform depth, but was subject to periods of extreme slack tide and periods of strong current. The bottom substrate at Job was 80% rock cobble with several small boulders throughout, which were populated with macroalgae, but relatively little drift algae was found. Predators were not found in abundance, with only one large Jonah crab (*Cancer borealis*) observed, and the initial population density of pre-existing urchins at the Job site was 2.25 animals/m^2. At the Sloop site,

the bottom substrate was 80% shell hash, which provided abundant refuges for small and medium-sized urchins. The study area was on a sloped ledge, so the depth varied across the area compared with Job, which was more flat. A few small boulders were found on the Sloop site with macroalgae growing on them, and drift algae, mostly kelp, were abundant. The Sloop Island site had an abundance of large urchins and sea stars present on it at out-planting. The initial population density of pre-existing urchins at the Sloop site was 4.5 animals/m^2.

Recapture rates: Sea urchins were found on both study areas at every survey for over two years. The total number (tagged and untagged) found at each survey ranged from 4 – 674 at the Job site and from 194 – 397 at the Sloop site. Tagged urchins (hatchery origin) were recaptured at both sites and at every dive survey up to the last, 27 months post-release. Recapture rates declined in the first year but then significantly spiked in the summer of the following year at both sites, before again declining in subsequent surveys (Fig. 2). At the Job site 10% to 100% of the urchins collected during each dive survey were determined to be of hatchery origin, and at Sloop 35% to 71% of collected urchins were of hatchery origin. It's important to note that on the one occasion when 100% of the animals collected at Job were tagged, the entire sample population consisted of just four animals, all very small (<7 mm TD). At the final survey a total of 107 urchins were collected from Job and about 30% of these were tagged. At the final Sloop survey the urchin population showed a significant decline from previous levels, and there was evidence (disturbed grounds, gear tracks, and broken tests) that the site had been recently fished by a dragger boat.

Population estimates: Population estimates of hatchery origin urchins remaining within the 400m^2 release areas at each survey varied in direct proportion with the recapture rates (Fig. 3). Originally, 10,500 urchins were released at each study area. Extrapolation from dive surveys indicated that the number of hatchery origin urchins remaining within the Job release area at each survey ranged from 45 to 36,894; with 3,306 projected as still remaining at 27 months post-release. At the Sloop site, population estimates of hatchery urchins remaining at each survey ranged from 3,680 to 18,165; with 7,360 projected as still remaining at the final survey, 27 months post-release (Fig. 3).

Average and maximum size: The average test diameter (TD) of hatchery origin urchins recaptured at the Job site declined to 5.1 mm over the course of the study, which was the minimal release size, but TD increased at the Sloop site (Fig. 4). At Job, the average TD declined from 10.6 mm at release to 5.1 mm 27 months post-release, whereas at Sloop the average TD increased from 11.3 mm at release to 18.3 mm at 27 months. The largest marked urchin recaptured from any of the surveys at Job during the course of the study was 19.7 mm (Aug 2010), and at Sloop it was 49.3 mm TD (Sept 2011, 19 months post-release) (Fig. 5). The Job site had a disproportionate number of small urchins remaining on it at every survey throughout the course of the
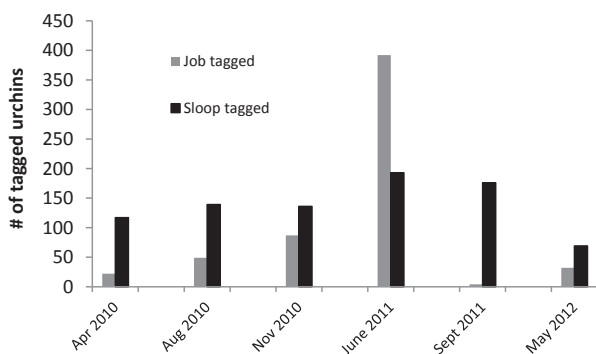

Fig. 2. Total numbers of hatchery origin *S. droebachiensis* recapturedfrom two release sites in Penobscot Bay, Maine at each survey.
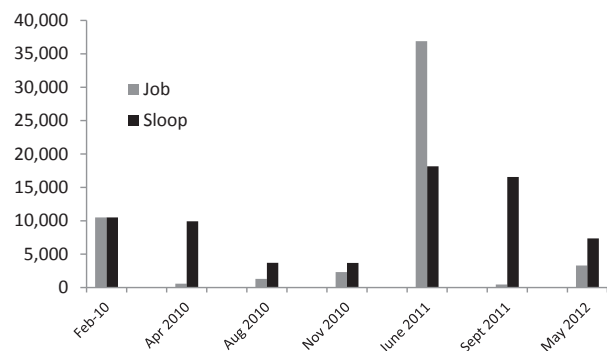

Fig. 3. Estimated population of hatchery origin *S. droebachiensis* remaining within the study area at each lease site and sample date. Calculated as average number of recaptured urchins per sample quadrat (m2) x 400 m2 (total study area).
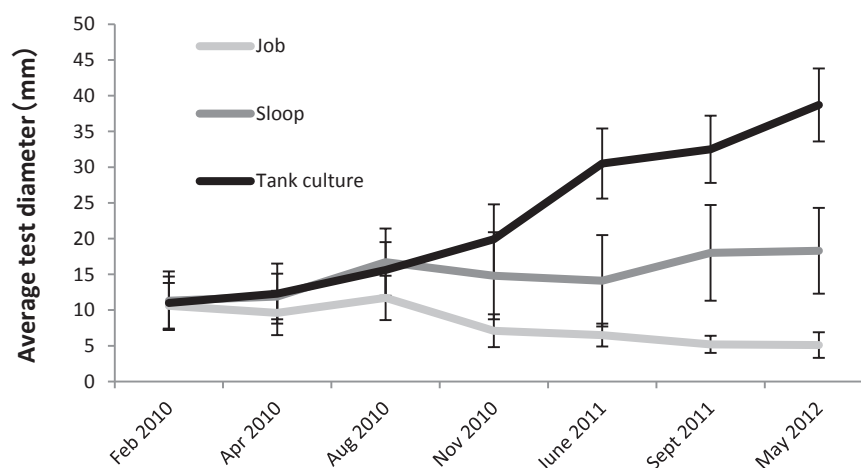
Fig. 4. Average test diameter of hatchery origin *S. droebachiensis* recaptured at two release sites in Penobscot Bay, Maine at each dive survey, and in tank culture at the CCAR. Error bars = ±1 standard deviation from the mean.
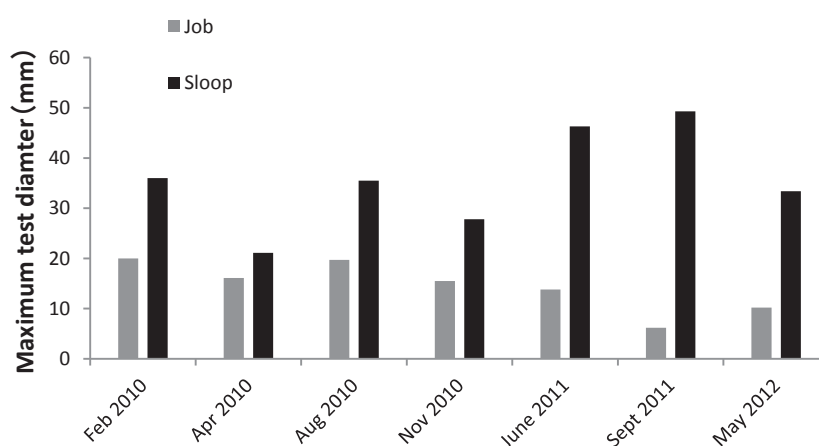

Fig. 5. Maximum test diameter of hatchery origin *S. droebachiensis* recaptured at two release sites in Penobscot Bay, Maine at each dive survey.

study. Of the total number of urchins (sum of six surveys) recaptured from Job, 84% were ≤6mm. At the Sloop Island site, only 1% of the total recaptured urchins were ≤6mm.

Growth rates diverged between land and sea based hatchery urchins within the first year of the study. Sea urchins reared in the land-based culture system were much larger on average at the end of the two year study than those recaptured from either ocean lease site (Fig. 4). After 27 months the largest urchin sampled in the tank culture system was 53.4 mm, and ≈ 1/3 of the tank reared urchins were ≥40 mm.

Discussion

Few previous studies in North America have monitored survival and growth of tagged sea urchins released into the field. Dumont *et al.* (2004) released three size groups of green sea urchins tagged with tetracycline onto a small study area. Similar to the present study, they found that recapture rates were size and time specific: 69% for <10 mm and 2% for >15 mm urchins after nine days, and 25% and 0% respectively after forty days. In a study by Rogers-Bennett *et al.* (1994), red urchin juveniles (*Strongylocentrotus franciscanus*) were tagged and

released onto study areas that varied in depth. Recovery rates after 12 months were 21% from shallow habitats and 11% from deep habitats.

The present study provides evidence that hatchery reared green sea urchins can be successfully out-planted for reseeding or sea ranching in the Gulf of Maine. Success is defined as the ability of seed to survive and grow to legal harvest size (52 mm) within 5 years of release. We saw that released juveniles survived and remained for an extended period (27 months) within each release area. However, recaptured juveniles were disproportionately smaller at one site (Job) than at the other (Sloop). This suggests that site factors modified the size distribution of surviving or remaining out-plants in different ways at the two sites. The Sloop Island site may have had a habitat more favorable for sea urchins, with more and larger refuges, and greater feed abundance.

Following settlement, juvenile green sea urchins take refuge under rocks, in crevices, or under debris as an adaptation to escape predation (Cameron and Schroeter, 1980; Dumont *et al.*, 2004). Here they graze on diatoms, coralline algae and detritus (Raymond and Scheibling, 1987). While both sites in the present study supported urchins, the shell hash at the Sloop Island site was full of cracks, holes and larger spaces, providing refuges for a broader size range of juveniles. The rock cobble at the Job Island site was, for the most part, flat against the sediment, with fewer and smaller hiding places for juveniles. The rock cobble had small interstices that were well suited for juveniles at or below 5–6 mm, but too small for larger urchins. Most of the urchins recovered from the Job site surveys, whether wild or tagged, were < 15 mm TD.

The Job site generally had lower recapture rates of tagged seed than the Sloop site, indicating that it was less hospitable for out-planted sea urchins. The notable exception occurred in the summer of the second year, when a large and significant number of small urchins were captured at the June 2011 Job site survey, and subsequently identified as hatchery origin due to presence of the fluorochrome mark. This spike in recapture numbers could have been due to misidentification (e.g. detecting auto or pseudo-fluorescence and attributing it to the tag), or

it could have been a sampling artifact. Presumably, misidentification would have occurred equally at Sloop at this survey date, and it did not. Although Sloop had higher recapture numbers (per quadrat and total) at this survey date than at other surveys, they were not significantly different from the other Sloop surveys. Also, we were concerned about this issue and any specimens with atypical fluorescence patterns were considered as unmarked. For these reasons, we believe that the spike in recapture numbers observed at Job during the June 2011 survey was a sample artifact. Every sample quadrat had to be thoroughly and equally searched, often by overturning rocks and shells to find hidden urchins. This effort had to be consistent between sites and survey dates, which in practice was difficult to accomplish. Under varying field conditions of bottom substrate, current, turbidity, ambient light, and temperature, it's likely that the success rate for finding urchins would vary between sites and dates. In addition, random movement patterns of urchins onto and off of the study areas probably occurred, because urchins move in response to food availability and the presence/absence of predators (Dumont *et al.*, 2007). At about 15mm TD sea urchin juveniles are less vulnerable to predation, and a shift from cryptic to active foraging occurs (Dumont *et al.*, 2004). Migration of urchins larger than 10 mm TD away from the release area in search of feed or refuge might explain the disproportionate numbers of small tagged urchins seen at the Job site.

Active foraging enhances the availability and quality of macroalgae, increasing the growth rate. When there is abundant food sea urchins will aggregate in high densities, and they can remain stationary for several months or longer (Dumont *et al.*, 2007). In the present study, both sites provided feed in the form of encrusting algae and particulate macro-algae. However, the Sloop site was more exposed and had greater currents (Kirchhoff *et al.*, 2008), and urchins at this site thus had access to large pieces of drift algae, mostly kelp, that were carried onto the site by the current. This greater feed availability might explain why recaptured urchins had a larger average and maximum TD at Sloop than at Job. The lack of a substantial food source at the Job site might have encouraged

more of the larger urchins to leave the site, while also causing slow growth of the small urchins that remained, due to low food intake. Green sea urchin growth rates can be highly variable in the natural environment, primarily in response to feed availability and type (Nestler and Harris, 1994; Brady and Scheibling, 2006). Growth can be very slow and rates of ≤ 0.25 mm per year have been documented for urchins found in tide pools (Russell, 1998). We observed that green sea urchins from the same hatchery cohort reared in the land-based culture system had significantly better growth than those recaptured at either lease site. This is further evidence that growth potential at the lease sites was limited more by environmental factors than by genetics or by the fact that the urchins were of hatchery origin.

In the present study we were able to differentiate hatchery origin from wild urchins for up to 27 months in the field. Johnson *et al.* (2013) reported that tetracycline fluorescence could be detected for at least two years in green sea urchins held in the lab, when tetracycline was administered via injection. The fact that fluorochromes can persist for such extended periods makes this marking/tagging method invaluable for long term lab and field studies of sea urchins, and was essential to carrying out the research described above. Recent advances in the application and visualization of fluorochromes offer further advantages, which could bring down costs and improve the effectiveness of sea urchin mark/recapture studies. Ellers and Johnson (2009) describe methods to create multiple marks on the demipyramids (e.g. at intervals or with multiple fluorochromes), which would allow for differentiation of multiple year classes released into the field. The same authors also describe visualization of fluorochromes on external structures such as the skeletal plates (test) and spines, which allows tagged individuals to be identified without sacrifice (Johnson *et al.*, 2013). Ultimately, development of a field portable device for visualizing fluorochromes seems feasible, to allow reliable identification of stocks *in situ* while minimizing adverse impact on the population (Johnson *et al.*, 2013). These methods provide powerful tools for evaluating the results of future restocking and sea ranching programs for green sea urchins in the Gulf of Maine.

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