

Does calcein affect estimates of growth rates in sea urchins?

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ABSTRACT: Skeletal-binding fluorescent markers are used to quantify whole organism growth in a variety of taxa including echinoderms. When using these markers to measure growth it is necessary to demonstrate that the marker itself does not affect growth either positively or negatively. One fluorescent marker, calcein, has been used to measure growth in several marine species, including sea urchins. We tested whether calcein affects growth in juvenile green sea urchins, *Strongylocentrotus droebachiensis*, by monitoring average size of tagged sea urchins relative to a control group over a 15 week period. Initially we divided the samples so that replicates of 15 sea urchins each had approximately the same mean size and variance. During the initial five weeks the sea urchins acclimated to laboratory conditions and we observed measurable growth increments among all replicates. For the next ten weeks, we measured growth in the control group relative to the group marked with calcein (N = 6 replicates each group). The first week following the application of calcein, the experimental group showed a temporary reduction in growth rate compared to the control group. This difference disappeared in the subsequent weeks of the experiment. Our results show that calcein does not interfere with growth in juvenile green sea urchins and can be used confidently in growth studies in echinoderms.

1 INTRODUCTION

The use of ossicle-staining fluorescent markers has advanced our understanding of echinoderm growth dynamics (e.g., Johnson et al. 2001, Lamare & Mladenov 2000, Russell & Meredith 2000, Ebert 1980b, Taki 1978, Pearse & Pearse 1975, Märkel 1975, Taki 1972b, a, 1971, Kobayashi & Taki 1969). Estimates of size-specific growth rates play a key role in quantifying life histories and these data are essential for gaining an appreciation of basic population biology. In addition, reliable estimates of growth are critical to fisheries and other natural resource managers (Ebert & Southon 2003, Pfister & Bradbury 1996, Hilborn & Walters 1992).

Kobayashi and Taki (1969) were the first to employ fluorescent markers in growth studies of echinoids. They used tetracycline to quantify growth dynamics of test plates in *Strongylocentrotus intermedius*. Since their study, several authors have used tetracycline to quantify growth rates in the field (Rogers-Bennett et al. 2003, Ebert & Southon 2003, Russell 2001, Ebert et al. 1999, Russell et al. 1998, Ebert & Russell 1993, Ebert & Russell 1992, Gage 1992b, a, 1991, Kenner 1991, Ebert 1988, Russell 1987, Ebert 1982, Ebert 1980a, Ebert 1977). The technique is straightforward – tetracycline is injected through the peristomal membrane and it combines with the calcium that is incorporated into the growing edges of all ossicles. The samples

are left in the field and collected some period of time later (typically 1 yr). Once the samples are recovered, the skeletal elements are cleaned of all soft tissue. Usually the demipyrmaid of Aristotle's lantern is isolated and examined under UV-light, which reveals the fluorescent mark and the original size at the time the animal was tagged. These data (size at time of tagging and size one year later) are then used to estimate parameters in a growth model (see Ebert 1999). In controlled laboratory studies Ebert (1988) demonstrated that tetracycline neither enhances nor interferes with growth. These controlled experiments are necessary to validate the field estimates of growth using this method.

The major disadvantage of tetracycline is the lower limit on the size of the animal that can be tagged. It is very difficult to mark sea urchins <15 mm test diameter without damaging them with the syringe. More recently, some workers have used another fluorescent marker, calcein, to tag a variety of organisms with calcified skeletons, including sea urchins (Rogers-Bennett et al. 2003, Russell et al. 1998, Rowley & Mackinnon 1995, Wilson et al. 1987). For larger sea urchins, calcein is administered the same way as tetracycline. However, it is easier and less injurious to mark smaller animals because these individuals are simply soaked in a calcein solution for 24 hours.

Calcein is clearly both an effective and efficient fluorescent marker and offers the advantage of tagging a wider size range of individuals. Unfortunately,

the effect, if any, of calcein on growth in echinoderms has not been established. Here we report the results of a controlled laboratory experiment designed to assess what effect calcein has on growth in the green sea urchin, *Strongylocentrotus droebachiensis*.

2 METHODS AND MATERIALS

We collected juvenile green sea urchins (<10 mm – this and all subsequent sizes refer to test diameter) from subtidal locations (2–5 m) at Cape Neddick (43°08'N, 70°38'W) in the Gulf of Maine, USA between September 20–21, 1999 (see Lambert & Harris 2000 for map of the site). The next day we transported the samples to Villanova University where they were kept in a 1200 L recirculating seawater system that is housed in a temperature-controlled environmental chamber. The water was maintained at 10°C and the salinity at 31–33‰ for the duration of the experiment. The lights in the chamber were kept off and the windows darkened because feeding rates in other species of *Strongylocentrotus* increase with decreased light levels (Fuji 1967). The only illumination occurred when light filtered into the chamber during feedings and weekly measurements. Once we assigned the urchins to individual replicates (see below) we fed them an overabundance of the brown kelps, *Laminaria sp.* and *Alaria esculenta*. The kelp was collected fresh and then frozen in sea water for storage. The kelps were thawed before feeding.

Initially we established 14 replicates (in a sea table fitted with a standpipe) of 15 urchins each such that each replicate had approximately the same mean size and variance. Each replicate unit was a 15 cm segment of PVC pipe with three, 4 cm sections, cut out of the bottom to form a tripod support (see “Downweller” in Deming & Russell 1999 for illustration). Each replicate was lined with 400 µm plastic mesh to keep the urchins and food inside while allowing the fecal material to pass through the bottom. We provided each replicate with an airstone and filtered sea water hose adjusted to a flow rate to 2 L/min.

Starting on September 25, 1999, and continuing once each week for 15 weeks, all urchins in the replicates were measured with knife-edge digital calipers. After the measurements on October 30 (week 5) the replicates were divided into two groups so that the mean sizes and variances of the groups were approximately equal. The groups were then randomly assigned to control and calcein treatments. Two identical 55 L aquaria were prepared and equipped with recirculating pumps and airstones. The only difference between the aquaria was that a stock solution (2.5 g calcein and 0.5 g NaCO₃ dissolved in 400 ml of tap water) was added to the calcein aquarium. The urchins from each replicate were placed in 400 ml plastic cups (bottoms

replaced with 400 µm plastic mesh). No food was allowed in the cups which were then suspended in one of the two aquaria for 24 h (each cup supplied with an airstone for circulation). After application of the treatment all urchins were then thoroughly rinsed in filtered sea water and returned to their appropriate replicate in the sea table. Following the last measurement on January 9, 2000, all soft tissue was removed from the surviving urchins with sodium hypochlorite. The skeletal elements were then examined under a UV light for the presence/absence of the fluorescent calcein tag.

The data for the analyses are mean values for each replicate. We calculated growth rates for one week before, and two weeks after, the application of the treatment by taking the difference between mean sizes for each replicate between consecutive weeks. We used t-tests (Zar 1999) to compare the slopes and elevations of the linear regressions of mean sizes vs. time as well as to compare the mean sizes at the end of the experiment. We did not use the regression method with replication (Zar 1999) because the mean values for each replicate are not independent.

3 RESULTS

After the application of the treatment, two of the replicates (one from each group) were mistakenly mixed in a single container. The appropriate group for these individual urchins could not be distinguished and so the following results and analyses are based on the remaining 12 replicates (6 calcein and 6 control). Of the original 180 urchins, 140 survived to week 15 (Fig. 1). There were a total of 22 mortalities in the control group (18 before and 4 after the treatment) and 18 mortalities in the calcein group (17 before and 1 after the treatment). At the end of the experiment all surviving urchins in the calcein group were tagged whereas no control group urchins were tagged.

Figure 2 plots the average sizes of urchins in the replicates over the course of the experiment. The linear regressions describing growth after application of the treatment (weeks 6–15) are:

$$S_{\text{calcein}} = 3.58 + 0.43t \quad (r^2 = .995, p < .0001) \quad (1)$$

and for the control:

$$S_{\text{control}} = 3.59 + 0.45t \quad (r^2 = .999, p < .0001) \quad (2)$$

where S is size and t is time.

There is no significant difference in the slopes of these regression lines ($t_{(2),16} = 2.12, p = .09$), however there is a significant difference in the elevations ($t_{(2),16} = 6.24, p < 0.001$). Comparing the mean sizes in the replicates at the end of the experiment on week

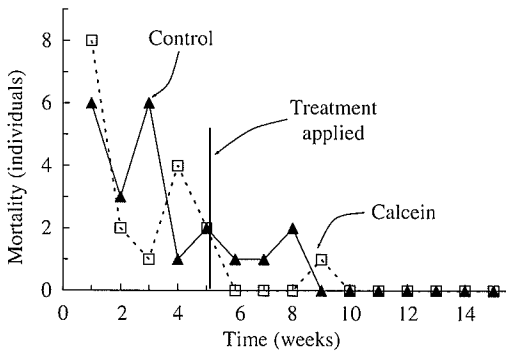


Figure 1. Number of mortalities in the control and calcein groups among all replicates throughout the experiment. Filled triangles = control and open squares = calcein.

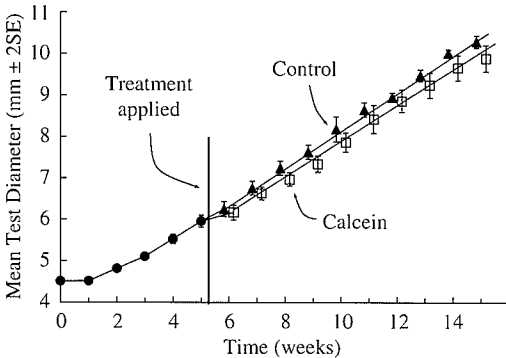


Figure 2. Weekly mean size (± 2 standard errors) among the replicates throughout the experiment. Closed circles ($n = 12$) = mean of all replicates before tagging on week 5 (treatment applied). Filled triangles = control ($n = 6$) and open squares = calcein ($n = 6$). There is no significant difference in slopes, but there is a significant difference in elevations, between the regression lines for the control and calcein groups.

15 reveals no significant difference ($t_{(2),10} = 1.96$, $p = .079$) between the two groups.

Figure 3 plots the mean growth rate in the two groups the week before the application of the treatment and the following two weeks. The week after the application of the treatment there is a significant difference in growth rate between the two groups ($t_{(2),10} = 2.39$, $p = .038$). With the exception of the first week (Fig. 2), the week after the treatment is the only time we observed zero growth in any of the replicates (two replicates in the calcein group).

4 DISCUSSION

The process of collecting the samples in the field combined with transporting them to the lab undoubtedly

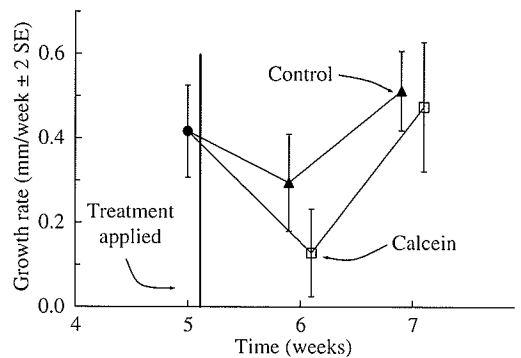


Figure 3. Weekly growth rates (± 2 standard errors) one week before and two weeks after application of the treatment. Although there was a significant reduction of growth in the calcein group relative to the control group the week after the treatment, growth rate immediately rebounded to pre-treatment levels the following week.

stressed the urchins. This stress resulted in the highest mortality (Fig. 1) and slowest growth rates (Fig. 2) during the first week of the experiment. Although we selected urchins that appeared healthy to stock the replicates at the start of the experiment, it is possible that some of these animals were damaged from the collecting process.

Growth rates (slope of the regression of size vs. time) following the application of the treatment were not significantly different between the calcein and control groups. However, for the 10 weekly measurements after the application of the treatment, the mean size of the control group was always greater than the mean size of the calcein group (Fig. 2). The largest difference in weekly growth rate between the two groups (0.17 mm/week) occurred immediately after the application of the treatment (Fig. 3). The low growth rates observed during this week in both groups was likely due to isolation in the aquaria and the absence of food during this 24 hour period. However, the growth rate in the calcein group was significantly lower than the control group during this week and is likely due to the presence of calcein. This difference in weekly growth rate in week 5 is the reason that all the mean sizes in the control group are greater than the mean sizes in the calcein group post-treatment.

Weekly growth rates were identical (0.38 mm/week) in both groups between weeks 8 and 9. Between weeks 9 and 12 weekly growth rates in the calcein group were actually greater than growth rates in the control group. On week 12 the difference in mean size between the groups was only 0.09 mm (control = 8.95 and calcein = 8.86 mm). Clearly any residual negative effect that calcein had on growth had completely disappeared just 3 weeks after the application of the treatment (and probably just one week later, Fig. 3).

The difference in mean size was 0.40 mm at the end of the experiment (control = 10.29 and calcein = 9.89 mm) and weekly growth rate in the control group (equation 2, Fig. 2) was 0.45 mm. Therefore the difference in mean size at the end of the experiment represents less than one week's worth of growth. On week 12 the difference between the two groups is less than two days of growth.

All of the urchins in the calcein group were marked and calcein is clearly an efficient and effective method of tagging sea urchins. Any negative effect that calcein has on growth is short term and does not appear to last beyond one week after the application of the treatment. Calcein neither enhances nor significantly interferes with growth in green sea urchins and we have confidence in growth parameters of other echinoid species based on this method as well.

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REFERENCES

Deming, C.J. & Russell, M.P. 1999. Assessing manipulations of larval density and culling in hatchery production of the hard clam, *Mercenaria mercenaria*. *Journal of Shellfish Research*. 18: 99–105.

Ebert, T.A. 1977. An experimental analysis of sea urchin dynamics and community interactions on a rock jetty. *Journal of Experimental Marine Biology and Ecology*. 27: 1–22.

Ebert, T.A. 1980a. Estimating parameters in a flexible growth equation, the Richards function. *Canadian Journal of Fisheries and Aquatic Sciences*. 37: 687–692.

Ebert, T.A. 1980b. Relative growth of sea urchin jaws: An example of plastic resource allocation. *Bulletin of Marine Science*. 30: 467–474.

Ebert, T.A. 1982. Longevity, life history, and relative body wall size in sea urchins. *Ecological Monographs*. 54: 352–394.

Ebert, T.A. 1988. Calibration of natural growth lines in ossicles of two sea urchins, *Strongylocentrotus purpuratus* and *Echinometra mathaei*, using tetracycline. In R.D. Burke, P. Mladenov, P. Lambert & R.L. Parsley (eds.), *Echinoderms: Proceedings of the Sixth International*

Echinoderm Conference: pp. 435–444. Rotterdam: Balkema.

Ebert, T.A. 1999. *Plant and Animal Populations: Methods in Demography*. San Diego: Academic Press.

Ebert, T.A. & Russell, M.P. 1992. Growth and mortality estimates for red sea urchin *Strongylocentrotus franciscanus* from San Nicolas Island, California. *Marine Ecology Progress Series*. 81: 31–41.

Ebert, T.A. & Russell, M.P. 1993. Growth and mortality of subtidal red sea urchins (*Strongylocentrotus franciscanus*) at San Nicolas Island, California, USA: problems with models. *Marine Biology*. 117: 79–89.

Ebert, T.A., Schroeter, S.C., Dixon, J.D., Kalvass, P., Richmond, N., Bradbury, A. & Woodby, D. 1999. Growth and mortality of red sea urchins *Strongylocentrotus franciscanus* across a latitudinal gradient. *Marine Ecology Progress Series*. 190: 189–209.

Ebert, T.A. & Southon, J.R. 2003. Red sea urchins (*Strongylocentrotus franciscanus*) can live over 100 years: confirmation with A-bomb ¹⁴carbon. *Fisheries Bulletin*. 101: 915–922.

Fuji, A. 1967. Ecological studies on the growth and food consumption of Japanese common littoral sea urchin, *Strongylocentrotus intermedius* (A. Agassiz). *Memoirs of the Faculty of Fisheries Hokkaido University*. 15: 83–160.

Gage, J.D. 1991. Skeletal growth zones as age-markers in the sea urchin *Psammechinus miliaris*. *Marine Biology*. 110: 217–228.

Gage, J.D. 1992a. Growth bands in the sea urchin *Echinus esculentus*: Results from tetracycline-mark/ recapture. *Journal of the Marine Biological Association United Kingdom*. 72: 257–260.

Gage, J.D. 1992b. Natural growth bands and growth variability in the sea urchin *Echinus esculentus*: results from tetracycline tagging. *Marine Biology*. 114: 607–616.

Hilborn, R. & Walters, C.J. 1992. *Quantitative Fisheries Stock Assessment: Choice, dynamics, and uncertainty*. New York: Chapman and Hall.

Johnson, A.S., Ellers, O., Lemire, J., Minor, M. & Leddy, H. 2001. Sutural loosening and skeletal flexibility during growth: determination of drop-like shapes in sea urchins. *Proceedings of the Royal Society of London*. 269: 215–220.

Kenner, M.C. 1991. Population dynamics of the sea urchin *Strongylocentrotus purpuratus* in a central California kelp forest – recruitment, mortality, growth and diet. *Marine Biology*. 112: 107–118.

Kobayashi, S. & Taki, J. 1969. Calcification in sea urchins. I. A tetracycline investigation of growth of the mature test in *Strongylocentrotus intermedius*. *Calcified Tissue Research*. 4: 210–223.

Lamare, M.D. & Mladenov, P.V. 2000. Modelling somatic growth in the sea urchin *Evechinus chloroticus* (Echinoidea: Echinometridae). *Journal of Experimental Marine Biology and Ecology*. 243: 17–43.

Lambert, D.M. & Harris, L.G. 2000. Larval settlement of the green sea urchin, *Strongylocentrotus droebachiensis*, in the southern Gulf of Maine. *Invertebrate Biology*. 119: 403–409.

Märkel, K. 1975. Wachstum des Coronarskelettes von *Paracentrotus lividus* Lmk. (Echinodermata, Echinoidea). *Zoomorphologie*. 82: 259–280.

- Pearse, J.S. & Pearse, V.B. 1975. Growth zones in the echinoid skeleton. *American Zoologist*. 15: 731–753.
- Pfister, C.A. & Bradbury, A. 1996. Harvesting red sea urchins: Recent effects and future predictions. *Ecological Applications*. 6: 298–310.
- Rogers-Bennett, L., Rogers, D.W., Bennett, W.A. & Ebert, T.A. 2003. Modeling red sea urchin (*Strongylocentrotus franciscanus*) growth using six growth functions. *Fishery Bulletin*. 101: 614–626.
- Rowley, R.J. & Mackinnon, D.I. 1995. Use of the fluorescent marker calcein in biomineralisation studies of brachiopods and other marine organisms. *Bulletin de l'Institut Océanographique (Monaco)*. Special Issue 14(2): 111–120.
- Russell, M.P. 1987. Life history traits and resource allocation in the purple sea urchin, *Strongylocentrotus purpuratus*. *Journal of Experimental Marine Biology and Ecology*. 108: 199–216.
- Russell, M.P. 2001. Spatial and temporal variation in growth of the green sea urchin, *Strongylocentrotus droebachiensis*, in the Gulf of Maine, USA. In M. Barker (eds.), *Echinoderms 2000: Proceedings of the Tenth International Echinoderm Conference*: pp. 533–537. Rotterdam: Balkema.
- Russell, M.P., Ebert, T.A. & Petraitis, P.S. 1998. Field estimates of growth and mortality of the green sea urchin, *Strongylocentrotus droebachiensis*. *Ophelia*. 48: 137–153.
- Russell, M.P. & Meredith, R.W. 2000. Natural growth lines in echinoid ossicles are not reliable indicators of age: A test using *Strongylocentrotus droebachiensis*. *Invertebrate Zoology*. 119: 410–420.
- Taki, J. 1971. Tetracycline labelling of test plates in *Strongylocentrotus intermedius*. *Scientific Reports of Hokkaido Fisheries Experimental Station*. 13: 19–29.
- Taki, J. 1972a. A tetracycline labelling observation of growth zones in the jaw apparatus of *Strongylocentrotus intermedius*. *Bulletin of the Japanese Society of Scientific Fisheries*. 38: 181–188.
- Taki, J. 1972b. A tetracycline labelling observation of growth zones in the test plate of *Strongylocentrotus intermedius*. *Bulletin of the Japanese Society of Scientific Fisheries*. 38: 117–121.
- Taki, J. 1978. Formation of growth lines in test plates of the sea urchin, *Strongylocentrotus intermedius*, reared with different algae. *Bulletin of the Japanese Society of Scientific Fisheries*. 44: 955–960.
- Wilson, C.A., Beckman, D.W. & Dean, J.M. 1987. Calcein as a fluorescent marker of otoliths of larval and juvenile fish. *Transactions of the American Fisheries Society*. 116: 668–670.
- Zar, J.H. 1999. *Biostatistical Analysis*. Fourth ed. Upper Saddle River: Prentice Hall.

