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In situ fluorochrome calcein marking of deep-sea molluscs using a new growth chamber

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Abstract A new instrument designed for in situ chemical marking experiments was developed and applied to the deep-sea seep clams *Calyptogena soyoae* and *Calyptogena okutanii*. Fluorochrome calcein was used for vital staining of four living clams kept in a specially designed in situ growth chamber that was placed on the seafloor at a cold-seep site off Hatsushima Island, Sagami Bay, central Japan. The shell margins of both *C. soyoae* and *C. okutanii* were clearly stained, forming a thin fluorescent band. This method will be useful for age and growth-rate determinations of many other marine organisms with accretionary growing skeletons.

Keywords In situ growth chamber · Fluorescent marking · *Calyptogena soyoae* · *Calyptogena okutanii* · Shell growth

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Introduction

Molluscs record daily environmental changes in chronological order in their shell microgrowth sequence. Since most bivalves living in intertidal settings secrete a pair of two etch-sensitive increments and two etch-resistant lines every lunar day, age and shell growth rate have been investigated in many species using sclerochronological technique (e.g., Dettman et al. 2004; Schöne et al. 2002a). In recent studies, environmental controls on daily shell growth have been examined in selected species by comparing the shell microgrowth patterns of specimens collected monthly with environmental data on the nearby location (e.g., Schöne et al. 2002b). In deep water environments, however, several technical difficulties prevent the estimation of the growth rates of deep-sea benthic animals in their natural habitats (see Cailliet and Andrews 2008 for a recent review).

Several methods have been devised to estimate the shell growth rate of intertidal bivalve molluscs, for example, cohort analysis (Schöne et al. 2002b), mark and recovery experiments (Peterson et al. 1983; Tanabe 1988), and chemical marking experiments (Fujikura et al. 2003; Thebault et al. 2006). Chemical marking experiments with in situ growth chambers can minimize stress to the organisms caused by excessive handling, for example, moving from their habitats, pressure changes, and human contact (Thebault et al. 2006). In this study, an enclosing, nonelectronic, mechanically simple in situ growth

chamber was developed. Using this new growth chamber and a remote-operated vehicle (ROV), an in situ chemical marking method for deep-sea marine organisms with accretionary growing skeletons was established. The first advantage of this method is that physical stress is limited to the minimum level because the specimens are not touched or moved during the experiment. Second, this method has been widely applied to shallow-water molluscs as a useful method to estimate growth rates and longevity (Kaehler and MacQuaid 1999; Moran 2000).

Materials and methods

Large-scale chemosynthesis-based communities characterized by abundant vesicomyid bivalves *Calyptogena soyoae* Okutani 1957 and *Calyptogena okutanii* Kojima and Ohta 1997 are patchily distributed at several deep-sea seep sites off Hatsushima Island, in the western part of Sagami Bay, central Japan. In situ shell growth experiments on *Calyptogena* clams were carried out at one of the cold-seep sites, at a depth of 1,174 m (latitude 35°00.1′N, longitude 139°13.5′E) on three occasions (March 13, 15 and 25, 2006), as a part of cruises NT06-04 and -05 of the Research Vessel (R/V) *Natsushima*, of the Japan Agency for Marine-Earth Science and Technology (JAMSTEC). All of the operations on the seafloor were performed by the ROV *Hyper-Dolphin*.

For the in situ shell growth experiment, a specially designed in situ growth chamber (Fig. 1), modified from the feeding chamber for benthic foraminifera designed by Nomaki et al. (2006), was placed on a site with numerous *Calyptogena* spp., and four specimens were enclosed in the chamber on March 13, 2006. The growth chamber, measuring 12.5 cm in inner diameter, consists of a head and a polycarbonate tube (Fig. 1a). The head is divided into a hand grip, containing two pistons, and a base unit, containing two syringes just beneath the pistons (Fig. 1b). The base unit has two holes for the syringes and six holes for seawater flow (Fig. 1c). The holes are covered by a polycarbonate plate with a rubber cover, which is held in place by a spring. When the growth chamber settles into the bottom sediments, seawater escapes through the flow holes. During the staining experiment, the inner side of the polycarbonate tube is enclosed by the polycarbonate and rubber covers. The head contains two stainless steel needles, which are attached to pistons with two syringes (Fig. 1b). Two polycarbonate stoppers compress the springs and maintain this position (see Fig. 1b) as the trigger system. When ROV pulls the trigger and removes the stoppers, the pistons attached to the needles are depressed by the freed springs. A water balloon filled with staining solution is placed on the drilled Teflon plate in the tube section beneath the two needles (Fig. 1a). On the seafloor, specimens are enclosed in the space beneath the Teflon plate. Remote operation of the ROV *Hyper-Dolphin* depressed the needles and punctured the water balloon, allowing the staining solution to diffuse into the tube.

Nontoxic calcein (3,3′-*Bis*[*N*,*N*-*bis* (carboxymethyl) aminomethyl] fluorescein, cat. no. 348-00434, Wako Pure Chemical Industries, Osaka, Japan), a Ca^{2+}-binding fluorochrome, was used for vital staining. The fluorescence emission peak was at 515 nm when excited by irradiation of 490 nm. Based on the results of a staining experiment on the Japanese littleneck clam *Ruditapes philippinarum* by Fujikura et al. (2003), the volume and concentration of the calcein solution injected via the water balloon were calculated to be 7.0 g l^{-1}, which was diluted to 0.7 g l^{-1} by ambient seawater when the water balloon was punctured. In the present experiment, the *Calyptogena* clams were stained by exposure to calcein for 48 h. During the experiment, the specimens remained in the growth chamber filled with the calcein solution. Subsequently, they were released from the calcein solution, trapped, and kept in an aerated cage. After exposure to calcein, the growth chamber was replaced with a weighted polypropylene cage on March 15, 2006. Ten days later, the cage was removed and the stained specimens were recovered using a scoop sampler on March 25, 2006.

The four recovered specimens measured 73.6, 73.6, 76.9 and 87.9 mm in maximum shell length, respectively. In Sagami Bay, two species with shell shapes similar to those of the vesicomyid clams *C. soyoae* and *C. okutanii* were described from cold-seep sites. The shells of *C. okutanii* are slightly narrower than those of *C. soyoae*, and the length–height ratios are 2.19 ± 0.10 and 1.98 ± 0.11, respectively (Kojima and Ohta 1997). Based on these morphologic descriptions, the first three specimens were identified as *C. soyoae* and the fourth specimen

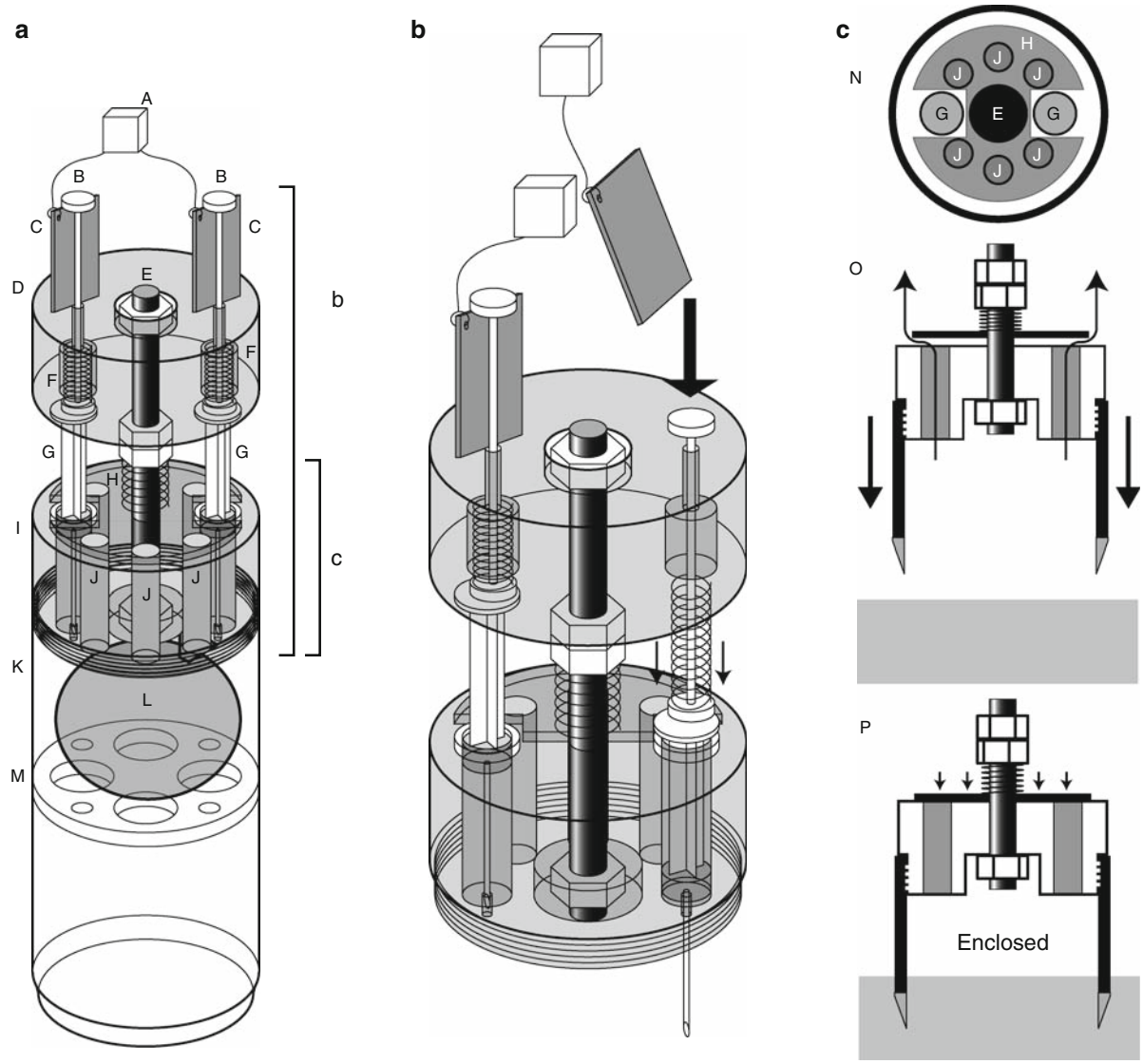

Fig. 1 **a** Schematic drawing of the in situ growth chamber used in this study: A, float to be pulled up by the ROV; B, pistons to depress the needles; C, stoppers for the pistons joined to the float; D, hand grip for the ROV; E, main strut; F, springs to hold the pistons in place; G, stainless steel needles with 10-ml syringes; H, cover for aeration holes; I, base unit; J, drilled aeration holes; K, polycarbonate tube; L, water balloon filled with staining solution; M, Teflon plate with drilled aeration holes. **b** The trigger system. The three aeration holes (J) and half of the aeration hole cover (H) on this side are omitted. When the ROV pulls up the float (A) and removes the stoppers (B), the springs (F) are recovered and depress the stainless steel needles (G). **c** Schematic drawing of the base unit (I): N, vertical image of the base unit [a polycarbonate plate with a rubber sheet (H) avoids the main strut (E) and the syringes (G) and covers the aeration holes (J) only]; O, when the growth chamber is placed on the seafloor, seawater escapes through the aeration holes; P, after placing the growth chamber on the seafloor, the aeration holes are covered and the polycarbonate tube is enclosed completely

as *C. okutanii*. Immediately after recovery, the soft tissues of the specimens were removed and the shells were soft-brushed, air-dried, and frozen at −80°C onboard the R/V *Natsushima*.

The stained shells were observed under a scanning laser microscope (SCM, FV300 scanning laser unit and BX51 fluorescent microscope, Olympus, Tokyo, Japan) installed in the Department of Earth and Planetary Science, University of Tokyo. The left valves of the stained shells were embedded in unsaturated polyester resin (P-resin, Nichika Inc., Kyoto, Japan) at 50°C and cut along the maximum

growth axis with a low-speed saw. The sectioned shell margins were observed under the SCM using a 10× objective lens. To identify the characteristic fluorescence of calcein, images of the shells were scanned for fluorescent signals of calcein and compared with images showing no fluorescence after excitation with an Ar (488-nm wavelength) and HeNe (635-nm wavelength) laser, respectively. Shell microgrowth increments between the upper margin of the detected fluorescent band and the shell margin were measured sequentially on the SCM images using ImageJ freeware (available from http://rsb.ino.nih.gov/).

Confirmation of calcein fluorescence in bivalve shells

All shell sections of the four stained specimens of *C. soyoae* and *C. okutanii* revealed characteristic fluorescent images. Representative SCM images of the shell margins of *C. soyoae* after irradiation with the Ar and HeNe lasers are shown in Fig. 2a, b, respectively, and an image with the fluorescence of Fig. 2b subtracted from that of Fig. 2a is shown in Fig. 2c. A schematic illustration of Fig. 2a is shown in Fig. 2d. On each Ar laser-irradiated image (Fig. 2a), a narrow bright zone is distributed along the outer and inner shell surfaces. On each HeNe laser-irradiated image (Fig. 2b), a bright zone is distributed along the outer shell surface, but the internal bright band observed in Fig. 2a is not detected. In this experiment, the Ar laser with a wavelength of 488 nm strongly excited calcein fluorescence, but HeNe laser irradiation with a wavelength of 635 nm is not absorbed by calcein. Thus, the bright fluorescent bands detected on the Ar laser-irradiated images (Fig. 2a) indicate calcein fluorescence. The bright areas on the outer shell surfaces (Fig. 2a, b) possibly originated from the autofluorescence of periostracal chitin. Since the autofluorescent area can be eliminated by comparing the two fluorescent images irradiated by lasers with different wavelengths (Fig. 2a, b), only the calcein-stained areas are seen on the fluorescence-subtracted image (Fig. 2c).

The magnified image of part of the inner shell margin (Fig. 2e) shows a narrow zone between the bright band and the resin. The bright band represents a shell layer precipitated by the supply of fluorochrome calcein. The nonfluorescent shell area, precipitated after the calcein-fluorescent area, was calcified after removing calcein from the soft tissues of the specimen. Thus, the bright band and the narrow nonfluorescent shell area grew during the in situ growth experiment, in the interval between March 13 and March 25, 2006 (12 days).

The growth chamber utilized in this study stained the shells efficiently. The simple mechanical combination of needles attached to pistons and a water balloon decreases the error rate of operation. The use of fluorochrome calcein seems not to affect the shell growth of *C. soyoae* and *C. okutanii*. Barry et al. (2007) reported a high mortality rate in a calcein-staining experiment using *Calyptogena kilmeri* when the clams were brought onboard their research vessel and then returned to the seabed after injecting calcein solution into the soft tissues. In this study, the *Calyptogena* clams remained on the seafloor in their natural habitats while in the in situ growth chamber, and the specimens were not touched physically during the experiment. The lowering of physiologic stress on specimens using our system decreases the mortality rate in staining experiments using *Calyptogena* clams.

The measured shell microgrowth increments precipitated during the 12-day culture of *C. soyoae* (73.6, 73.6, 76.9 mm in maximum shell length) and *C. okutanii* (87.9 mm in maximum shell length) were 66, 71, 46 and 59 μm, respectively. Unfortunately, the number of specimens examined was not sufficient to discuss the differences in the observed growth rates of *C. soyoae* and *C. okutanii*. In addition, the short-term culture was not sufficient to estimate the annual shell growth rate. Since no specimen experienced any shell growth breaks during this experiment, we plan to perform longer in situ growth experiments with *C. soyoae* and *C. okutanii* using this method to obtain shell growth data comparable with that of *Calyptogena* species living at hydrothermal vent sites and clod-seep areas.

Conclusions

A clear growth band stained with fluorochrome calcein was detected on the shells of the deep-sea chemosynthesis-based clams *C. soyoae* and *C. okutanii* in an

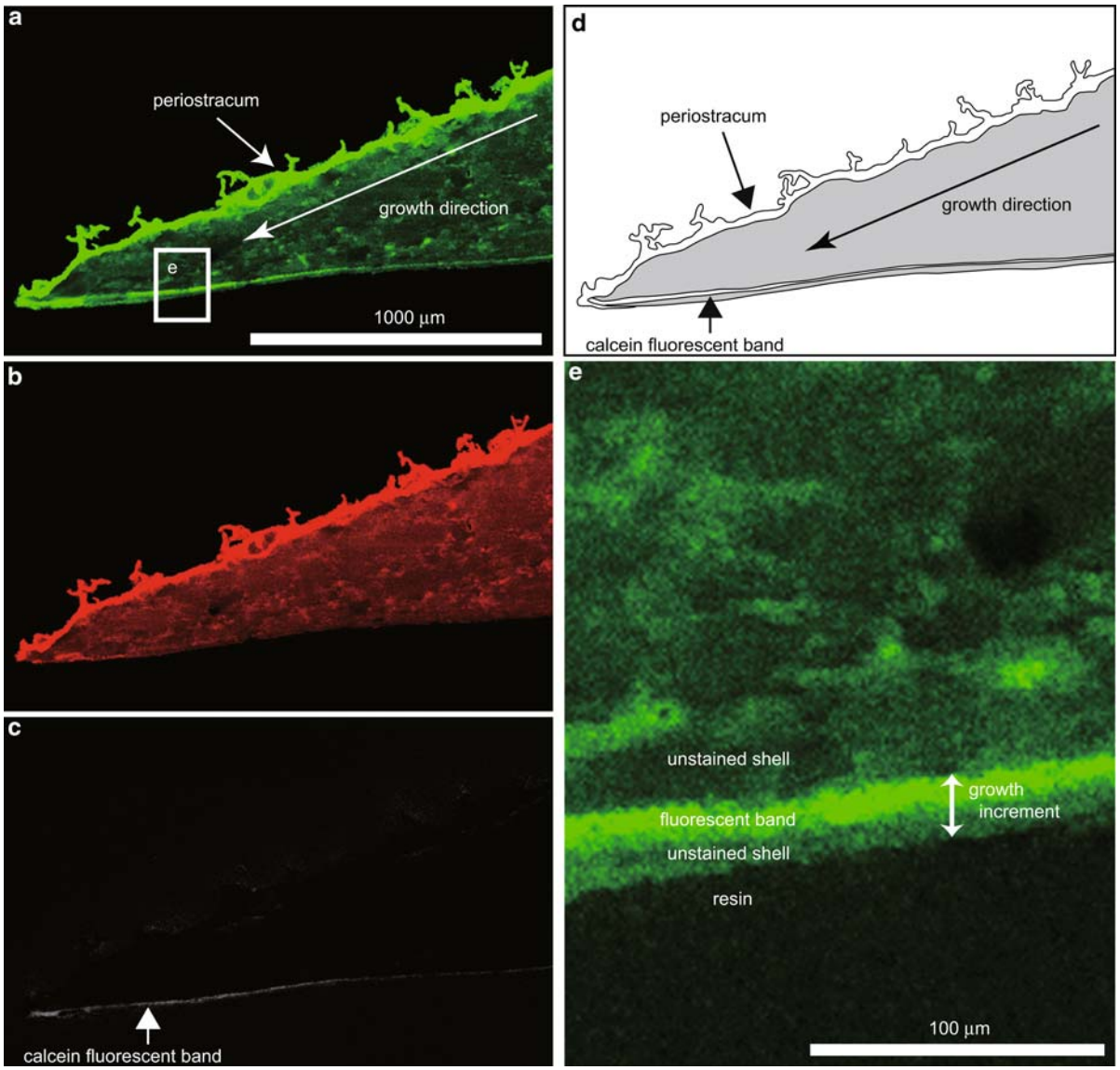

Fig. 2 Laser-irradiated shell section images and illustration of *Calyptogena soyoae*. **a** Ar laser-irradiated image. A bright excited area is observed on the inner and outer shell surfaces. **b** HeNe laser-irradiated image. Only the outer shell surface is excited. **c** Fluorescent image with the HeNe laser-irradiated fluorescence subtracted from that of the Ar laser-irradiated image. The excited area on the outer shell surface is clearly eliminated and only the inner shell surface remains as a bright band. **d** Illustration of Fig. 2a. **e** Expanded image of the square in Fig. 2a. A narrow shell space is observed between the fluorescent band and resin space. The "growth increment" area is precipitated shell space during the in situ growth experiment from March 13 to 25, 2006

in situ staining experiment using a newly designed growth chamber. Since the staining solution is compatible with other chemical solutions, the growth chamber can be used in various marking and labeling experiments. Conductivity temperature depth-profiling instruments and/or other sensors can also be attached to the growth chamber and aerated cage to monitor ambient seawater conditions. The present method can be applied to many other invertebrates with calcareous skeletons exhibiting accretionary growth and contribute to the assessment of their age, growth rate and other ecological information.

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