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— Report —

In situ vital staining for chasing the galatheid crab *Shinkaia crosnieri* on deep-sea floor

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Shinkaia crosnieri, a galatheid crab, has ectosymbiotic bacteria on its ventral setae, and forms very dense crowds in hydrothermally active regions and seep areas. They feed on the symbiotic bacteria and do not chase other animals for predation. To study how they move and behave in jostling crowds, we developed a vital staining to mark their individuals and trace them by using a camera on a remotely operated vehicle (ROV). Among the various dyes examined, Coomasie Brilliant Blue R250 (CBB) stained the galatheid crab the darkest, and its color lasted for more than 5 months in the laboratory at 4–5°C. The ventral setae were strongly stained, while the dorsal shell was weakly stained. The stained galatheid crab survived for more than 8 months. For the *in situ* staining of *S. crosnieri* at the Iheya North hydrothermal field in the Okinawa Trough, Japan, we applied a dye solution mixture (20 L) containing CBB and Acid Blue 161 to the galatheid crab population through a funnel equipped on the ROV *Hyper-Dolphin*. After staining for approximately 5 minutes, more than 18 individuals of *S. crosnieri* were dyed blue. They were disturbed by the staining process but seemed to be unharmed. The dyed galatheid crabs were identified by the ROV one and two days post staining. They seemed to remain at the place where they were stained.

The present vital-staining marking method may present a new way to analyze the behavior and changing habitable range of deep-sea animals like *S. crosnieri*, and may give us a deeper insight into how these animals behave in a very dense population and explore newer habitats.

Keywords : galatheid crab, *Shinkaia crosnieri*, deep-sea, vital staining, behavior, ROV

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1. Introduction

In deep-sea ecosystems at hydrothermal vents and seeps, high-density animal communities are often found (Van Dover, 2000). Two dominant animal groups in the communities, the tubeworms and the giant deep-sea *Calyptogena* clams, are known to have intracellular symbionts, which provide chemosynthetic products to the host animals (Van Dover, 2000). The galatheid crab *Shinkaia crosnieri*, which has been originally described in the Edison Seamount near Papua New Guinea, forms very dense populations, jostling each other in crowds at deep-sea hydrothermal vent fields and methane seep sites in the Okinawa Trough and South China Sea (Chan et al., 2000; Baba and Williams, 1998; Tokeshi, 2011; Tsuchida et al., 2003; Yang et al., 2016). Although it has been observed that *S. crosnieri* feeds on dead shrimp or some other meat such as tuna flesh under cultivation in aquaria (Kitajima et al., 2012), no predatory behavior has been observed during *in situ* observations. *S. crosnieri* harbors ectosymbiotic bacterial communities including thioautotrophs and methane-oxidizing bacteria on their ventral setae (Tsuchida et al., 2011; Watsuji et al., 2010; Watsuji et al., 2012), and feeds on the bacteria by combing and harvesting them with its third maxllipeds (Tsuchida et al., 2011; Watsuji et al., 2015). *S. crosnieri* does not feed on other animals; it does not seem to move around to chase other prey animals in the habitat. It typically does not move unless disturbed, but it has recently been reported to migrate from large colonies to areas where new hydrothermal vents had been artificially created on the seafloor (Nakajima et al., 2015). While it appears to be able to traverse large distances over the time scale of months, information regarding its local motion is not available. It is not clear how far *S. crosnieri* could travel and communicate with other individuals of the species in high-density populations in their natural habitats. However, individuals of *S. crosnieri* are difficult to distinguish in a crowd and need to be marked to overcome this problem.

Marking methods using catch and release processes are powerful tools to analyze how animals move in the natural habitats (Haddaway et al., 2011). However, this strategy seems to be unsuitable for monitoring the *in situ* behavior of deep-sea animals because of the stresses caused by environmental changes, including temperature and pressure shifts during the procedures. Therefore, to analyze their behavior and movements on the deep-sea floor near hydrothermal vents, we aimed to develop a simple, inexpensive, and easily practicable method to mark them *in situ* without causing them noticeable stress. Crystal Violet has been used to stain the ectosymbiotic bacteria of live *S. crosnieri*, and to prove the digestion of the symbionts in the digestive tracts of galatheid crabs (Watsuji et al., 2015). The carapace of crustaceans (cuticle) contains calcified chitin (Nagasawa, 2012). Uncalcified chitin has been reported to be stained with Acid Blue dyes for analyzing the growth of the tubeworm sheaths in deep-sea environments (Bergquist et al., 2000, 2002). In the present study, we compared the efficiency of dyes for vital staining in a laboratory experiment, and developed a new *in situ* staining method for deep-sea crustaceans. This is the first report about marking *S. crosnieri* populations and tracking them in the natural habitat using an ROV (Remotely Operated Vehicle) in a deep-sea vent field. Vital staining for studying the behaviors of deep-sea animals which form very dense jostling crowds is discussed.

2. Materials and Methods

To identify a suitable dye for the vital staining, we used a live *S. crosnieri*, which had been captured from a hydrothermal field at the Iheya North in the Okinawa Trough, Japan during dive #1618 on 29 January, 2014 (27°47.45'N, 126°53.81'E, at a depth of 988 m) using the remotely operated vehicle (ROV) *Hyper-Dolphin* during the R/V *Kaiyo* cruise (KY14-01), and cultured in 100 L of artificial sea water (ASW, pH7.6; (3.5% Rohto Marine [Rei-Sea Ltd., Tokyo, Japan] in tap-water)) at 4–5°C with methane (final concentration = 34 μM; Watsuji et al., 2017) in a tank (length × width × height = 80 × 45 × 45 cm). One small individual, with a carapace approximately 2 cm in width, was selected and used for staining with a series of various dyes. We examined Acid Blue 161 (AB161; Sigma-Aldrich, 0.5% in ASW, once for 4 minutes and the other time, for one hour), Rose Bengal (Wako Pure Chemicals, 0.5% in ASW, 13 minutes), Alizarin Red-S (Wako Pure Chemicals, 1.66 mg/L in ASW, 24 hours), Aniline Blue (Wako Pure Chemical; 0.14% in ASW, 10 minutes), and Coomassie Brilliant Blue R250 (CBB; Wako chemical, 0.25% with 2.5% DMSO [Wako Pure Chemicals, Co. Ltd] in ASW, 15 minutes). This individual of *S. crosnieri* was

stained in 30–60 mL of ASW containing various dyes at 4–5°C for a few minutes to one day. The period of staining was desirable to be a few minutes for the *in situ* staining, but it was prolonged when the staining was weak (e.g., Alizarin Red-S). The stained animal was then washed in a 1-L beaker by changing approximately 1-L of ASW 5–8 times, and incubated at 4–5°C in approximately 800 mL ASW in the 1-L beaker. After the CBB-staining, it was kept under this condition for 5 months; the ASW was changed 2–3 times a week. After 5 months of culturing in the beaker, the galatheid crab was transferred to a tank (l × w × h = 50 × 50 × 50 cm) for culturing *S. crosnieri* in the Enoshima Aquarium. Sand-filtered natural sea water was replenished daily (200 L/day; 4°C; sulfide concentration was 400–1200 μM; pH was 6.8–8.0), and the galatheid crab was kept in this tank for an additional 3 months.

In situ staining of *S. crosnieri* was performed at the Iheya North field (position of 27-47.484N; 126-53.804E, Jan. 03, 2016. 09:33-09:38) at a depth of 1,001 m using the ROV *Hyper-Dolphin* during the R/V *Natsushima* NT16-01 cruise. The dye solution (CBB-AB161; 100 g of Acid Blue 161 [Sigma Aldrich, final concentration of 0.5% w/v] and 25 g of Coomassie Brilliant Blue R250 [Wako Pure Chemicals. 0.125% w/v] and 250 mL DMSO [Wako Pure Chemicals. 1.25% v/v] was dissolved in 20 L of natural sea water) was stored in a 20-L plastic (Polyethylene) tank, which was connected to a specially designed staining funnel with a square opening of 30 cm × 40 cm through a tubing equipped with an impeller water pump (Fig. 1). This staining facility had been made by the operation team of the ROV. Using a manipulator of the ROV *Hyper-Dolphin*, the funnel covered a small population of *S. crosnieri* in the hydrothermal vent field. The dye solution was then poured on this population, and staining was performed for a few minutes. The funnel was removed after the *in situ* staining, and the stained individuals were left in their habitat.

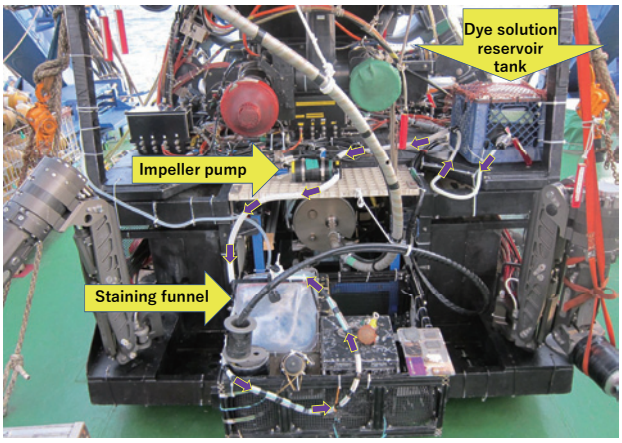

Fig. 1. Staining devices equipped on the payload of the ROV *Hyper-Dorphin*. Dye solution (CBB-AB161 mixture, 20 L) was stored in the resevoir tank. At the place, where galatheid crabs dwell, it was driven by the impeller pump to flow through a tubing and was finally poured on the galatheid crabs via the staining funnel. Violet arrows indicate the direction of the flow of the dye solution in the tubing.

This location of the staining was revisited by the ROV *Hyper-Dolphin* the next day (24 h post staining; day 2) and 2 days after the staining (48 h post staining; day 3). Images of the *S. crosnieri* population were captured with a high-definition video camera installed on the ROV *Hyper-Dolphin*, and analyzed in the laboratory after recovery by using softwares Adobe Photoshop CS4 version 11.0 and ImageJ version 1.51j8 (http://imagej.nih.gov/ij/).

3. Results

3.1 Screening of dyes for *in situ* vital staining

In the screening of dyes for the *in situ* vital staining, we tested Acid Blue 161 (AB161), Aniline Blue, Rose Bengal, Alizarin-Red S and Coomassie Brilliant Blue R250 (CBB). AB161 was expected to stain the calcified chitin of crustaceans. The galatheid crab was stained with this dye at a concentraion of 0.5% w/v at 5°C for 4 minutes. However, the staining was weak and the color almost completely faded away on the next day. The galatheid crab was restained with the same dye at the identical concentration and temperature for 1 hour. The ventral setae were densely stained, while the carapace was only faintly stained (Fig. 2a, b). After 2 days, the color of the carapace faded but fainter blue color still remained on the setae (Fig. 2c, d). The carapaces and setae were also stained with another chitin-staining dye, Aniline Blue (0.14%), for 10 minutes at 5°C. The staining profile was similar to that of AB161, and the color faded within a few days and almost disappeared 7 days post staining. Rose Bengal has been used for staining the members of the phylum, Foraminifera (Bernhard et al., 2006). Although the ventral setae were stained bright red with 0.5% Rose Bengal in ASW for 13 minutes at 5°C, the carapace was only faintly stained at its edge. The color was not stable; even the color of the setae faded the next day and disappeared after 3 days. After incubation in Alizarin-Red S in ASW (1.66 mg/L) for a day, the setae of the galatheid crab were only slightly stained,

while the carapace was not stained.

Coomassie Brilliant Blue R250 (CBB), which is usually used for staining proteins in electrophoreses, stained *S. crosnieri* in blue more densely than the other dyes examined; not only the ventral setae, but also the carapaces (Fig. 3a and b) were stained. The color faded very slowly, and remained even after 5 months (the color after 55 days is shown in Fig. 3c and d). It is also noteworthy that the galatheid crab voided blue feces even on 39 days post staining (Appendix 1). After the staining processes of any dyes, the animal did not show any wriggling or squirming behavior; however, the behavior could not be observed during the staining process. This suggested that it was not very perturbed by any of the dye solutions.

After the staining, the stained *S. crosnieri* was kept in approximately 800 mL of ASW in a 1-L beaker for 5 months at 4–5°C, without a supply of sulfide or methane; the blue color of CBB was recognizable even after 5 months.

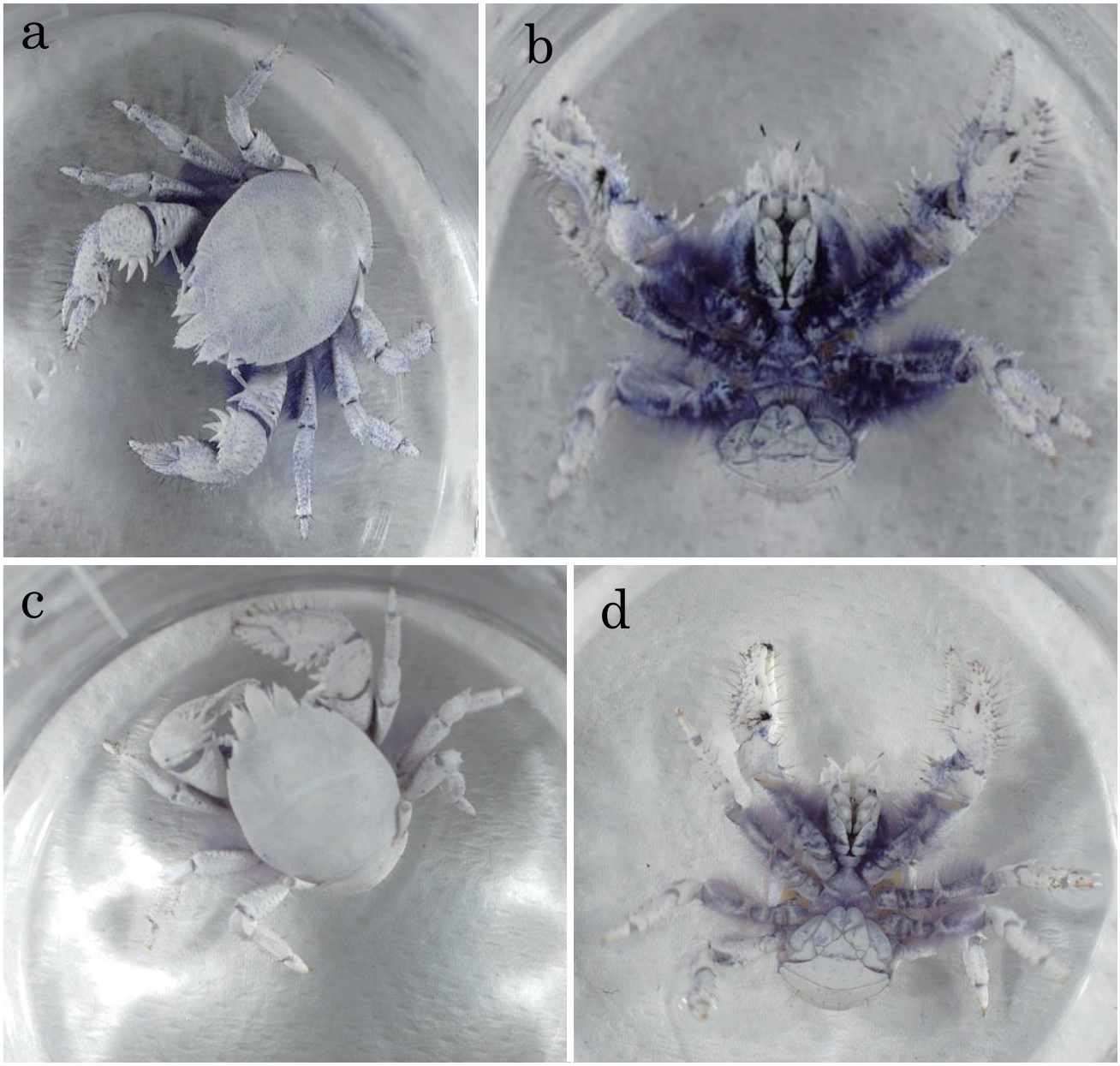

Fig. 2. *Shinkaia crosnieri* stained with acid blue 161. **a** and **b**, *Shinkaia crosnieri* was stained with 0.5% w/v acid blue 161 (AB161) in artificial sea water (ASW) for 1 hour at 4–5°C, and washed 7 times with ASW. **c** and **d**, The blue color of *S. crosnieri* faded from the carpace but fainter blue remained on the ventral setae two days after the staining. **a** and **c**, Dorsal side. **b** and **d**, Ventral side.

After the transfer to a tank in the Enoshima Aquarium, the color gradually faded, and the ectosymbiotic bacterial community on the setae seemed to grow. The galatheid crab exuviated its shell after 8 months of cultivation post staining; however, it died a month after the molting. The molt was white and looked just like an intact living galatheid crab having ventral setae with ectosymbionts, but no blue color was recognizable on the molt (data not shown), indicating that the color was lost before molting.

3.2 *In situ* staining of *S. crosnieri* at the Iheya North hydrothermal field.

A small group of *S. crosnieri* individuals in a wild population were stained with the CBB-AB161 solution at the Iheya North hydrothermal field (Fig. 4). After staining with 20 L of the dye solution, at least 18 individuals were recognizably well-stained (Fig. 5a). The appearance of the stained galatheid crab *in situ* through a video camera was slightly different from that of the laboratory-stained

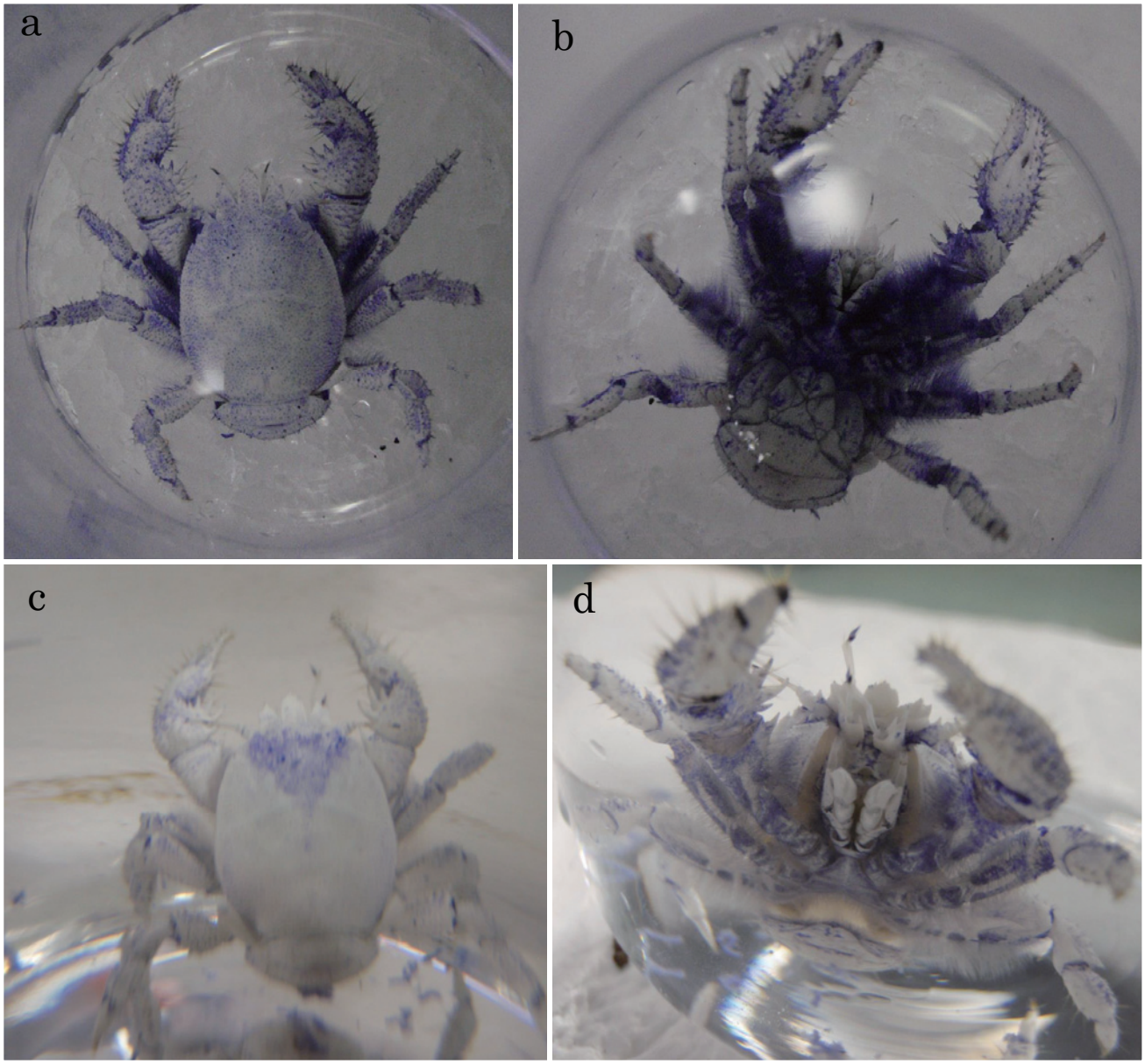

Fig. 3. *Shinkaia crosnieri* stained with 0.25% Coomassie Brilliant Blue R250 (CBB). *Shinkaia crosnieri* was bathed in 60 mL of 0.25% CBB in 2.5% dimethylsulfoxide (DMSO) in ASW for 15 min on ice. It was then washed with ASW 4 times (**a, b**). The ventral setae were stained dark blue (**b**) and the carapace was also stained (**a**). The color slowly faded, but persisted for more than 5 months. **c** and **d** indicate that the color remained on the dorsal side (**c**) and the ventral side (**d**) after 55 days.

galatheid crab observed directly. The whole body of the *in situ*-stained galatheid crabs seemed to be dyed blue (Yellow arrowheads in Fig. 5b). While the ventral setae were stained much darker than the carapace in the laboratory experiment (Fig. 3a and b), the carapaces and the setae were similarly stained after the *in situ* staining procedure (Yellow arrowheads in Fig. 5b). The galatheid crabs seemed to be a little disturbed by the moving of the staining funnel, but most individuals became calm soon after. No avoiding response was observed in the stained galatheid crabs and in those in the surrounding area. This suggested that the staining process did not greatly disturb their distribution.

This place was revisited 24 and 48 hours after the staining (Fig. 5c and d). The dyed galatheid crabs were recognizable in the *in situ* video images taken by the ROV *Hyper-Dolphin*, although the blue color was not very dark. Compared to the color observed immediately after the staining, there appeared to be no change in color even a period of 48 h (Fig. 5d). However, the stained individuals were more easily recognizable after changing the color and contrast parameters of the captured video images (Appendix 2). Twenty-four and 48 h after the staining, most of the stained galatheid crabs were found in approximately the same location that they were originally stained at (Fig. 5c and d).

4. Discussion

In the laboratory staining, color of *S. crosnieri* stained by AB161 did not last long, but faded away within a few days (Fig. 2). Color of the acid blue (including AB161) that stains the chitin in the sheath of vestimentiferan tubeworms has been reported to last stably for more than a year (Bergquist et al., 2000, 2002; Fujiwara et al., unpublished data). Acid blue stainability of calcified chitin

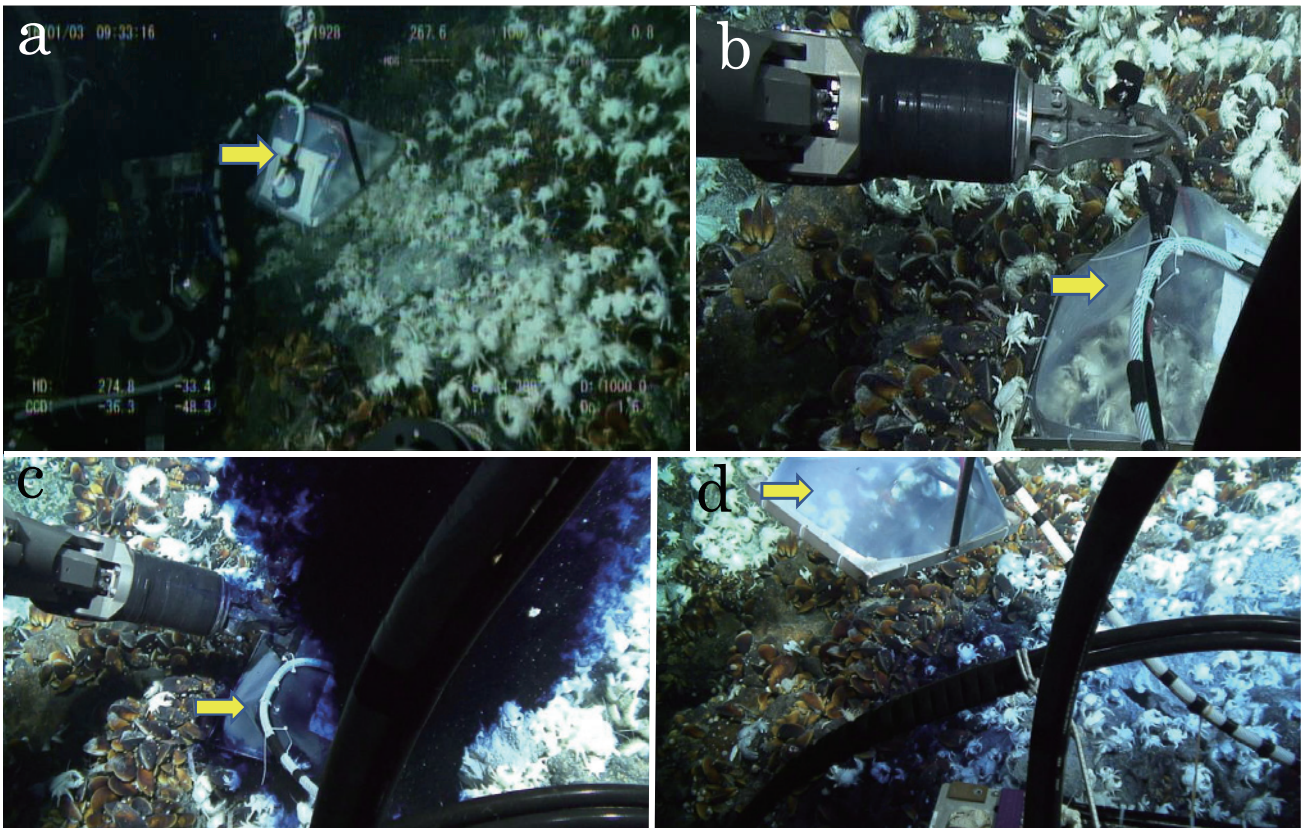

Fig. 4. *In situ* staining of a wild population of *Shinkaia crosnieri* at the Iheya-North hydrothermal vent area on January 3, 2016. The staining funnel was placed over the *S. crosnieri* population (**a**). After placing the funnel on the population, the trapped galatheid crabs were observed through the transparent plastic funnel (**b**). During the staining process, the leaked dye solution rose from the funnel in the form of smoke. Initially, the trapped galatheid crabs were hardly observable because of the dye solution (**c**). After the funnel was lifted, the dye was quickly diluted out, and the stained galatheid crabs remained in place (**d**).

may be much weaker than that of uncalcified chitin.

The blue color of the CBB-stained *S. crosnieri* was the darkest among the examined dyes and lasted for the longest period, which was more than 5 months in the laboratory (Fig. 3). Galatheid crab often wipe the back portion of the carapace by using its pereiopods, and may feed on the bacteria growing there (Miyake, personal communication). On the back portion of carapace, blue color of CBB staining remained in the inverse triangle region, where might be difficult to be scraped with the pereiopods (Fig. 3c). CBB was probably the most effective dye in the dye mixture (CBB-AB161) for the *in situ* staining of *S. crosnieri* (Fig. 5). However, the appearances of the *in situ*-stained galatheid crabs were different from that of the laboratory-stained galatheid crab. There are two possibilities for this difference: 1) differences between the illuminations (including light path length) in the laboratory and deep-sea, and 2) differences between the culture conditions of *S. crosnieri* in the laboratory and natural habitat. In the natural habitat, there must be some flows of sea water including woozing water movement from the bottom sediments. In addition, *S. crosnieri* has been shown to produce water flow, which plays an important role for the symbiotic bacterial growth (Watsuji et al., 2017). These water movements in addition to the water disturbance by the funnel movement, probably diluted the dye concentration during the staining. Wild *S. crosnieri* seemed to have a denser ectosymbiontic bacterial community on the setae than its laboratory-grown

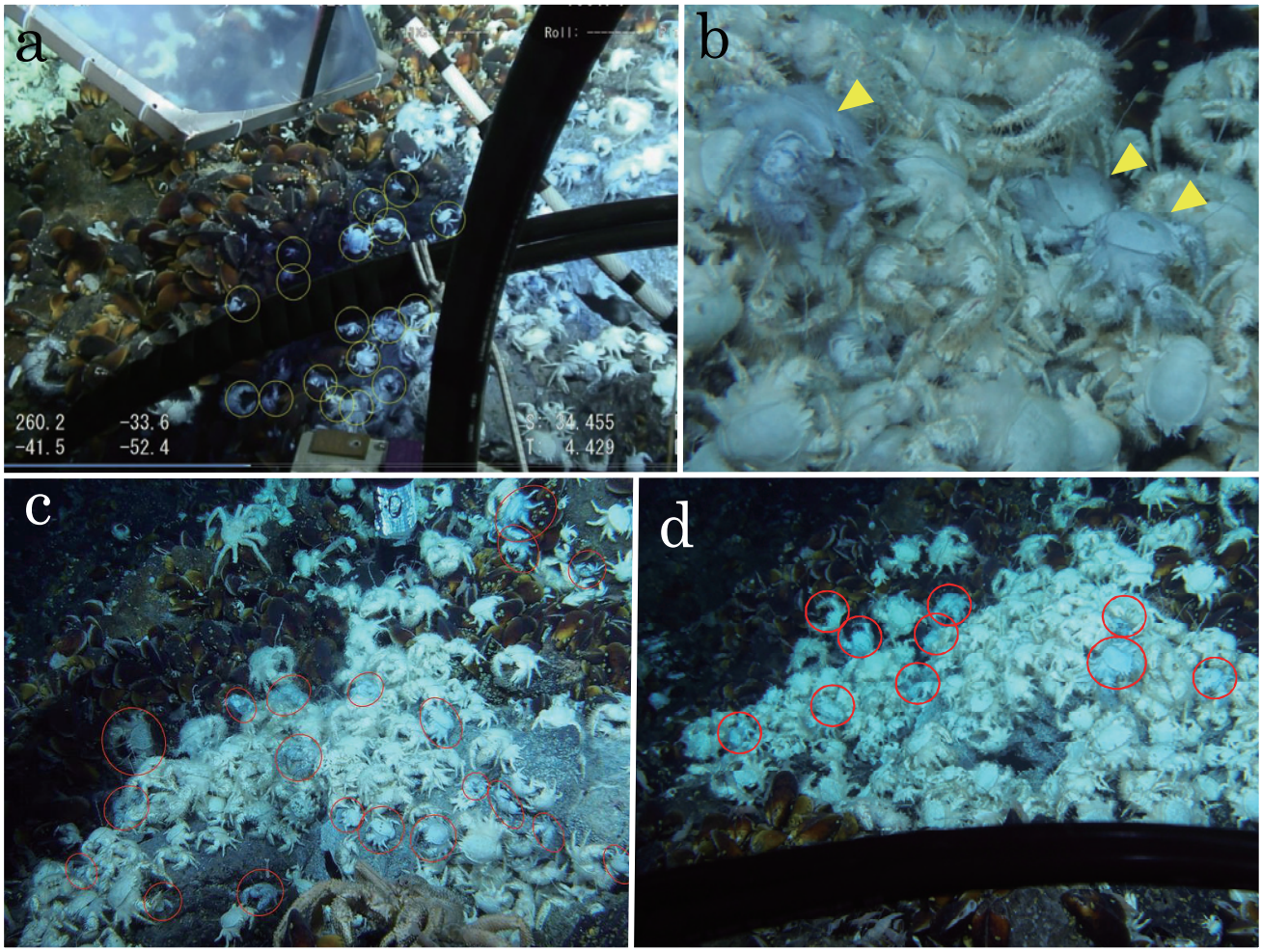

Fig. 5. Wild *Shinkaia crosnieri* stained with CBB-AB161 at the Iheya-North hydrothermal field. **a**, Blue-colored *Shinkaia crosnieri* just after the staining. 18 galatheid crabs (yellow circles) were found to be stained blue in this picture. **b**, A digitally enlarged picture of the stained galatheid crabs. The carapaces and the ventral setae with thick bacterial films were found to be stained blue to almost equal extents. **c**, 24 h after staining, the blue-dyed galatheid crabs were found at the same place. 20 individuals were observed in this picture (red circles). **d**, Blue-colored galatheid crabs (red circles) were still recognizable 2 days post staining (48 hours after staining).

counterpart (Figs. 2d and 5b). The setae covered with a thicker bacterial layer might be less stainable by the CBB-AB161 than one covered with a thinner bacterial layer. It has been reported that crystal violet-stained ectosymbionts on the setae were traced and detected in the intestines of *S. crosnieri* (Watsuji et al., 2015). The feces of the laboratory-stained galatheid crab were blue, (Appendix 1), suggesting that the blue color of the feces was derived from the stained bacteria, and/or that inside of the digestive tract was stained and discharged after. Therefore, the CBB-AB161 mixture may also be used for detecting how ectosymbionts are digested by the host animal, *S. crosnieri*.

In the field, the stained galatheid crabs were recognizable through the video observations recorded on the ROV. Apparently, the darkness of the color did not change for 2 days. We found the stained galatheid crabs in the same place on day 3 post staining. This indicates that this method is useful to observe the behavior and movements of the galatheid crabs for at least a few days.

In the deep-sea environment outside of the Iheya North hydrothermal vent area, very few or no *S. crosnieri* are usually observed. (Nakajima et al., 2015). When a new artificial hydrothermal vent, which was 264 m away from the nearest natural vent site, was established, *S. crosnieri* appeared 11 months after the drilling (Nakajima et al., 2015). It is deduced that they migrated from their comfortable original habitat to the newly made vent area (Nakajima et al., 2015). It is speculated that they have a chemotactic behavior towards the new vent site (Nakajima, personal communication). From this report, their speed was estimated from the nearest original habitat to the new one (distance = 264 m) as 264 m/11 months = 24 m/month ≈ 0.8 m/day. In the present study, the stained galatheid crabs mostly stayed at the place of staining for 2 days, but their distribution was slightly widened (Fig. 5c and d). It is still not clear whether or not they have a loose territory in the habitat and if they compete for the methane and sulfide.

The present vital staining method provides a unique and practical technique to understand how the deep-sea galatheid crabs move within jostling crowds, and how they interact each other, without any lethal effects to the animal. However, the stained color was not contrasting enough with the non-stained ones for easier recognition through video observations in seawater. It is better to find a better dye or method to improve the intensity and longevity of the staining. In the present study, the setae were stained darker in the laboratory than in case of the *in situ*-stained wild galatheid crabs. If the ectosymbionts could be stained without damaging them, it would be possible to monitor the growth of these bacteria in this galatheid crab *in situ*, in which the newly grown parts of the fibrous bacterial cells would be unstained, but the older stained parts should remain colored.

5. Conclusion and perspectives

The present study showed that: 1) the deep-sea crustacean *Shinkaia crosnieri* could be marked by a vital staining without apparent damage to its survival or activity. Neither an avoiding nor an escaping reaction was observed. They were recognizable at least for a few days in the deep-sea habitat. 2) the marked individuals seemed to stay at the place where they were stained, for two days. This may indicate that *S. crosnieri* does not move around much and remains localized.

Vital staining is a mild marking method for invertebrates in various habitats. However, the blue color of the stain did not give a very high contrast for recognizing the stained galatheid crab in the deep-sea habitat. It is desirable to search for more suitable dyes, including fluorescent dyes, which may have high stainability for various organisms, a more easily recognizable color, and more stable and long-lasting color in the dark deep-sea environments, than the dyes being used currently, though we may need a strong excitation light source on the ROV. The present vital staining approach may be applicable to not only crustaceans with chitinous exoskeletons, but also to other various invertebrates that have proteinaceous outer coats.

In the present study, staining and tracing *S. crosnieri* were performed by using an ROV. Since the monitoring of the dyed galatheid crabs was performed visually, unmanned platforms such as autonomous underwater vehicles (AUV) equipped with visual mapping instruments to efficiently analyze the distribution of dyed individuals would be used. Such new technologies using unmanned robotics are becoming more and more important for future studies about marine ecology.

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References

Baba, K. and A.B. Williams (1998), New Galatheoidea (Crustacea, Decapoda, Anomura) from hydrothermal systems in the West Pacific Ocean: Bismarck Archipelago and Okinawa Trough, *Zoosystema*, *20*, 143–156.

Bergquist, D.C., F.M. Williams, and C.R. Fisher (2000), Longevity record for deep-sea invertebrate, *Nature*, *403*, 499–500.

Bergquist, D.C., I.A. Urcuyo, and C.R. Fisher (2002), Establishment and persistence of seep vestimentiferan aggregations on the upper Louisiana slope of the Gult of Mexico, *Mar. Ecol. Prog. Ser.*, *241*, 89–98.

Bernhard, J.M., D.R. Ostermann, D.S. Williams, and J.K. Blanks (2006), Comparison of two methods to identify live benthic foraminifera: A test between Rose Bengal and CellTracker Green with implications for stable isotope paleoreconstructions, *Pleoceanography*, *21*, PA4210, doi:10.1029/2006PA001290.

Chan, T., D. Lee, and C. Lee (2000), The first deep-sea hydrothermal animal reported from Taiwan: *Shinkaia crosnieri* Baba and Williams, 1998 (Crustacea: Decapoda: Galatheidae), *Bull. Mar. Sci.*, *67*, 799–804.

Haddaway, N.R., R.J.G. Mortimer, M. Christmas, and A.M. Dunn (2011), A review of marking techniques for Crustacea and experimental appraisal of electric cauterisation and visible implant elastomer tagging for *Austropotamobius pallipes* and *Pacifastacus leniusculus*, *Freshwater Crayfish*, *18*, 55–67.

Kitajima, M., M. Sugimura, S. Nemoto, and S. Nakagawa (2012), Feeding behavior of the captured deep-sea galatheid crab, *Shinkaia crosnieri*, observed during cultivation, Proceedings of Blue Earth 2012, (in Japanese), <https://www.jamstec.go.jp/maritec/j/blueearth/2012/pdf/poster/BE12-P20.pdf>.

Nagasawa, H. (2012), The crustacean cuticle: structure, composition and mineralization, *Frontiers in Bioscience*, E4, 711–720.

Nakajima, R., H. Yamamoto, S. Kawagucci, Y. Takaya, T. Nozaki, C. Chen, K. Fujikura, T. Miwa, and K. Takai (2015), Post-drilling changes in seabed landscape and megabenthos in a deep-sea hydrothermal system, the Iheya North Field, Okinawa Trough, *PLoS ONE*, *10*, 0123095.

Tokeshi, M. (2011), Spatial structures of hydrothermal vents and vent-associated megafauna in the back-arc basin system of the Okinawa Trough, western Pacific, *J. Oceanogr.*, *67*, 651–665.

Tsuchida, S., Y. Fujiwara, and K. Fujikura (2003), Distribution and population structure of the Galatheid crab *Shinkaia crosnieri* (Decapoda: Anomura: Galatheidae) in the southern Okinawa Trough, *Jpn. J. Benthol.*, *58*, 84–88.

Tsuchida, S., Y. Suzuki, Y. Fujiwara, M. Kawato, K. Uematsu, T. Yamanaka, C. Mizota, and H. Yamamoto (2011), Epibiotic association between filamentous bacteria and the vent-associated galatheid crab, Shinkaia crosnieri (Decapoda: Anomura), *J. Mar. Bio. Ass. UK*, *91*, 23–32.

Van Dover, C.L. (2000), The Ecology of Deep-Sea Hydrothermal Vents, Princeton University Press., pp. 424.

Watsuji, T., S. Nakagawa, S. Tsuchida, T. Toki, A. Hirota, U. Tsunogai, and K. Takai (2010), Diversity and function of epibiotic microbial communities on the galatheid crab, *Shinkaia crosnieri*, *Microb. Environ.*, *25*, 288–294.

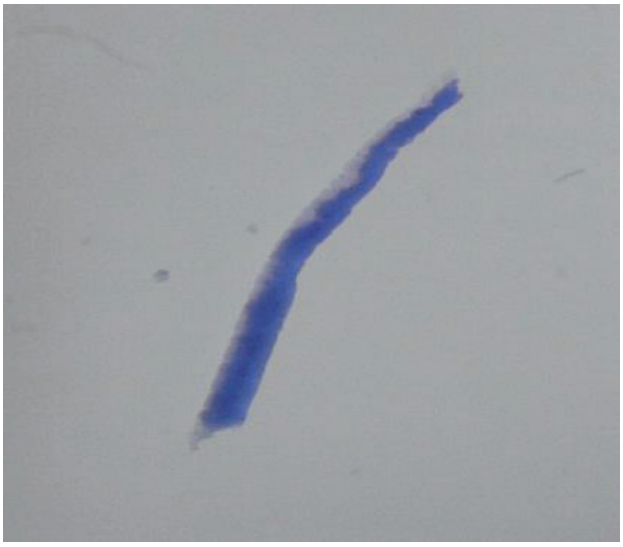
Watsuji, T., M. Nishizawa, Y. Morono, H. Hirayama, S. Kawagucci, N. Takahata, Y. Sano, and K. Takai (2012), Cell-specific thioautotrophic productivity of epsilon-proteobacterial epibionts associated with *Shinkaia crosnieri*, *Plos One*, e46282.

Watsuji, T., A. Yamamoto, K. Motoki, K. Ueda, E. Hada, Y. Takaki, S. Kawagucci, and K. Takai (2015), Molecular evidence of digestion and absorption of epibiotic bacterial community by deep-sea crab *Shinkaia crosnieri*, *ISME J.*, *9*, 821–831.

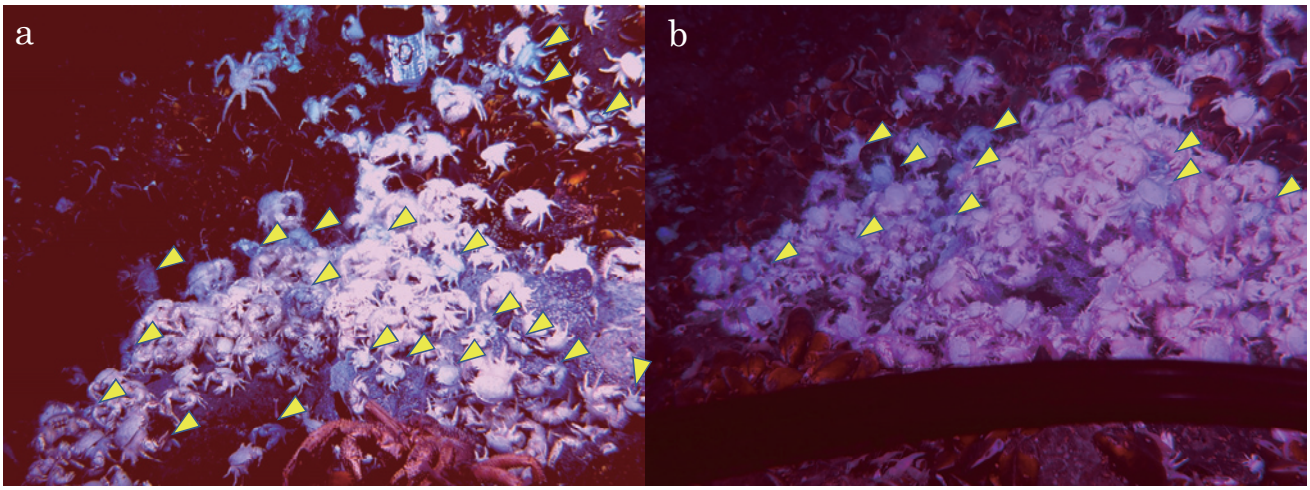
Watsuji, T., R. Tsubaki, C. Chen, Y. Nagai, S. Nakagawa, M. Yamamoto, D. Nishiura, T. Toyofuku, and K. Takai (2017), Cultivation mutualism between a deep-sea vent galatheid crab and its chemosynthetic epibionts, *Deep-sea Res. Part I*, *127*, 13–20.

Yang, C-H., S. Tsuchida, K. Fujikura, Y. Fujiwara, M. Kawato,

and T-Y. Chan (2016), Connectivity of the squat lobsters *Shinkaia crosnieri* (Crustacea: Decapoda: Galatheidae) between cold seep and hydrothermal vent habitats, *Bul. Mar. Sci.*, *92*, 17–31.



Appendix 1. Blue feces of the stained *Shinkaia crosnieri* voided 37 days post the CBB staining. Length of the feces was 5.2 mm.



Appendix 2. Color enhancement of the stained galatheid crabs in the field. Color balances of Figures 5c (**a**) and 5d (**b**) were changed to improve the recognizability of the stained galatheid crabs. Yellow arrows in **a** and **b** indicate the dyed galatheid crab individuals, which correspond to the circular marks in Figures 5c and 5d, respectively. Some apparently blue dyed individuals in the distant are excluded because the color might be shifted to blue by the longer light pass effect.