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Settlement behaviour of the early megalopae of the land hermit crab *Coenobita violascens* (Decapoda: Coenobitidae) under laboratory conditions: Effects of inshore odours and salinity

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Abstract: The distribution of the land hermit crab *Coenobita violascens* is restricted to the vicinity of rivers, particularly mangrove estuaries. To infer the recruitment mechanisms of *C. violascens*, we examined settlement behaviour, such as swimming, walking, shell-inspection, and shell-wearing activities, in 0-day-old to 6-day-old megalopae under different seawater conditions: 1) offshore salinity (34 ppt, control), 2) offshore salinity (34 ppt) with inshore odours (riverine water), and 3) inshore salinity (24 ppt). Salinity was regulated using artificial seawater salts. *Coenobita violascens* megalopae exhibited nocturnal swimming activity, whereas other activities significantly increased during the daytime period. Inshore odours did not affect the swimming and walking activities, whereas the inshore salinity conditions decreased the swimming activity and enhanced the walking activity, i.e., stimulated the settlement behaviour of megalopae. Shell-related activities were not influenced by any of the seawater types. Our results suggest that megalopae might migrate to the coast using nocturnal flood-tide transport and settle on or near inshore habitats under the reduced salinity conditions that occur as a result of the inflow of groundwater and river water. Further studies are required to elucidate the role of inshore odours in the recruitment of *C. violascens* megalopae in mangrove estuaries.

Key words: early life history; endangered species; mangrove estuary; recruitment

Introduction

Terrestrial hermit crabs of the family Coenobitidae Dana, 1851 comprise approximately 17 land hermit crab species in the genus *Coenobita* Latreille, 1829 and the coconut crab *Birgus latro* (Linnaeus, 1767) (Hartnoll, 1988; Poupin, 1996; McLaughlin *et al.*, 2010; Rahayu *et al.*, 2016). They are mainly distributed in subtropical and tropical coastal regions (Hartnoll, 1988). Terrestrial hermit crabs have a planktonic larval phase for dispersal in the sea. Female crabs hatch their eggs in the sea, and the larvae develop in the ocean through several zoeal stages before they metamorphose into the megalopal stage

(Hartnoll, 1988; Nakasone, 2001; Hamasaki *et al.*, 2014, 2015a). The megalopae migrate to the inshore region by swimming; after settlement, they acquire empty gastropod shells and then migrate onto land (Reese, 1968; Harvey, 1992; Brodie, 1999; Hamasaki *et al.*, 2011, 2014, 2015b, 2015c).

In Japan, six coenobitid species, including *B. latro*, *Coenobita brevipennis* Dana, 1852, *C. cavipes* Stimpson, 1858, *C. purpureus* Stimpson, 1858, *C. rugosus* H. Milne-Edwards, 1837, and *C. violascens* Heller, 1862, commonly occur on southern islands of the Ryukyu Archipelago (Nakasone, 1988, 2001; Asakura, 2004; Fujikawa *et al.*, 2017). We previously investigated the distributional characteristics of juvenile and adult terrestrial hermit crabs along the coasts of Ishigakijima Island and Iriomotejima

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Island in the Ryukyu Archipelago and revealed that the distribution of *C. violascens* was restricted to the vicinity of the river, mainly in the mangrove estuaries (Fujikawa *et al.*, 2017; Hamasaki *et al.*, 2017, 2018). *Coenobita violascens* has been listed as a 'near-threatened species' on the Red Lists by the Ministry of the Environment of Japan. Additionally, we genetically identified the species of recently landed juveniles of coenobitid crabs collected in the beach and river mouth areas of Ishigakijima Island (Hamasaki *et al.*, 2018). In this survey, two species, *C. rugosus* and *C. violascens*, were dominant. The early juveniles of *C. rugosus* occurred in both beach and river mouth areas, and they were abundant in beach areas, whereas the early juveniles of *C. violascens* were only found in river mouth areas associated with mangroves or rich in nutrients. Thus, our previous findings indicate that *C. violascens* complete their life cycles in eutrophic river mouth areas in subtropical and tropical coastal regions.

Environmental stimuli, such as salinity changes and chemical cues, i.e., odours derived from conspecific adults and/or nursery areas, such as aquatic vegetation and biofilms, have been known to affect settlement behaviour and accelerate metamorphosis in the megalopal stage of many decapod crustacean species (Tankersley *et al.*, 1995; Anger, 2001, 2006; Forward *et al.*, 2001, 2003; Welch and Forward, 2001; Gebauer *et al.*, 2003; Epifanio and Cohen, 2016). It has been suggested that organic materials and/or nutrients from mangrove areas on Ishigakijima Island are exported to the coastal lagoon, which has high salinity conditions (Akamatsu *et al.*, 2002a, 2002b; Takada *et al.*, 2005; Terada *et al.*, 2007, 2009, 2012), and that reductions in salinity widely occur in inshore areas due to the inflow of groundwater and river water (Tottori *et al.*, 2004). It is therefore hypothesized that *C. violascens* megalopae swim towards suitable settlement habitats with the aid of odours exported from the eutrophic river and settle based on salinity changes in inshore areas. To infer the recruitment mechanisms of *C. violascens*, the

present study aimed to examine the settlement behaviour of early megalopae and tested the following hypotheses: 1) seawater (offshore salinity) containing chemical cues from the eutrophic river stimulates the swimming behaviour of megalopae towards settlement habitats and 2) reduced salinity in inshore areas decreases the swimming behaviour of megalopae, leading to settlement. These hypotheses were tested by examining larval behaviour in relation to settlement, such as swimming, walking, shell-inspection and shell-wearing activities, in laboratory-raised *C. violascens* megalopae under different seawater conditions.

Materials and Methods

Experimental animals

Ovigerous females of *C. violascens* were captured during late June 2013 on Ishigakijima Island (24°23'N, 124°08'E). They were transferred to the laboratory at Tokyo University of Marine Science and Technology, Tokyo, where the air temperature was controlled at approximately 28°C, and maintained in tanks equipped with simulated land and sea areas (artificial seawater, 34 ppt salinity; Sealife, Marineteck Co. Ltd., Tokyo, Japan) until hatching occurred according to the methods of Hamasaki *et al.* (2009) and Hamasaki (2011). After the larvae had hatched, all the female crabs were released back into the habitats from which they were captured.

Larvae that hatched from two females on July 10 (brood 1) and July 16 (brood 2), 2013, were stocked in ten 1-l beakers at a density of 50 individuals beaker⁻¹ (approximately 28°C and 34 ppt salinity) and cultured until metamorphosis to the megalopal stage according to the methods of Hamasaki *et al.* (2013). Larvae from broods 1 and 2 metamorphosed into megalopae on July 27 and August 3, 2013, respectively. A total of 120 megalopae from each brood were placed individually in six-well cell culture plates on the day of metamorphosis (0 days old). Each well contained 10 ml artificial seawater (ap-

proximately 28°C and 34 ppt salinity) without substrate materials or gastropod shells. The megalopae were transferred daily to clean six-well cell culture plates with newly prepared seawater and fed commercially available frozen mysid shrimps (Seahorse Ways Co. Ltd., Minamikyushu, Kagoshima, Japan) before use in the experiments.

Experimental seawater treatments

To examine the effects of seawater (offshore salinity) containing inshore chemical cues and inshore salinity conditions on the settlement behaviour of *C. violascens* megalopae, we prepared three types of seawater: 1) artificial seawater with offshore salinity (control), 2) seawater with offshore salinity and inshore odours, and 3) artificial seawater with inshore salinity. The offshore salinity was adjusted to 34 ppt because a salinity of approximately 34–35 ppt was recorded in the open ocean around the Ryukyu Archipelago (<http://www1.kaiho.mlit.go.jp/KAN11/atlas/sal/>). Seawater (34 ppt) containing inshore odours was prepared by adding artificial seawater salts to riverine water (16 ppt). This riverine water was collected during an ebb tide on June 24, 2013, from a small river mouth located in the southern part of a brackish estuary system with a tidal flat and mangrove tree area (50 ha) called ‘Nagura Amparu’ on Ishigakijima Island. *Coenobita violascens* is abundant along the shoreline of the tidal flats in Nagura Amparu (Fujikawa *et al.*, 2017; Hamasaki *et al.*, 2017, 2018). Organic materials and nutrients from the mangrove swamps move to the coastal area through river basins in Nagura Amparu (Akamatsu *et al.*, 2002a, 2002b). The riverine water collected from Nagura Amparu was stored at –60°C until use in the experiments. It has been reported that the chemical cues that induce the megalopal metamorphosis of *Uca pugnax* (S. I. Smith, 1870) and *Panopeus herbstii* H. Milne-Edwards, 1834 maintain their activity even after freezing (O’Connor and Gregg, 1998; Andrews *et al.*, 2001). The inshore salinity was adjusted to 24 ppt based on the fluctuation in salinity in

accordance with the tidal cycle (approximately 0–34 psu) in the major river mouth of Nagura Amparu (Kawachi and Ishikawa, 2008; Kawachi *et al.*, 2009) and the incidence of a few mortalities after 24 h in *C. violascens* megalopae that were abruptly transferred from 34 ppt to 20 ppt salinity but no mortalities at 24 ppt (Hamasaki *et al.*, unpublished data).

Settlement behaviour experiments

Coenobita violascens megalopae spend about three weeks before moulting to the first crab stage (Hamasaki *et al.*, 2015c). Settlement behaviour experiments were conducted using 0-day-old to 6-day-old megalopae. Small transparent plastic containers (0.6 cm wide, 4.6 cm long, and 5.8 cm high) with a hard 2.8 cm² substrate consisting of black sand (1.7 mm mean diameter, approximately 5 mm thickness) were used for observing megalopal behaviour. A cleaned gastropod shell of *Littoraria undulata* (Gray, 1839) (shell length 4.6–4.8 mm) was placed at the bottom of each container. The containers were placed in a dark room (approximately 28°C) equipped with a fluorescent lighting system. The photoperiod was regulated based on a 13L:11D cycle, and the light intensity was 4 $\mu\text{mol s}^{-1} \text{m}^{-2}$ near the observation containers during the light period (5:30–18:30). A total of ten 0-day-old megalopae, five individuals from each brood, were individually housed in containers with the designated test seawater (15 ml) in the evening, and their behaviour was continuously recorded from 18:00 using a video recording system with CCD cameras (WM-N041DNR, $f = 3.6$ mm, Sony Co., Ltd., Tokyo, Japan) and an infrared light source (850 nm). Coenobitid crabs have no spectral sensitivity to this wavelength (Cronin and Forward, 1988). To avoid the possible effects of prey organisms on their behaviour, the megalopae were not fed during the observation period. To avoid the influence of starvation on megalopae, individual test megalopae were exchanged daily with new ones from the same cohort between 13:00 and 14:00. Thus, we assigned a change in the age of the megalopae at

13:00–14:00 each day. No megalopa was used more than once. The water temperature and salinity in the test containers were measured before exchanging the test megalopae; the water temperature was $29.4 \pm 0.3^\circ\text{C}$ (mean \pm standard deviation), and the salinity slightly increased to 34.6 ± 0.5 ppt and 25.5 ± 0.9 ppt in the offshore salinity (34 ppt) and inshore salinity (24 ppt) conditions, respectively, because the test containers were not covered with a lid.

The behaviour of individual megalopae was quantified using the video recordings. The individuals were treated as replicates. We determined the total number of the following behavioural events from counts every 5 s for 10 min (120 counts) every two hours from 18:00 at 0 days old to 12:10 at 6 days old: swimming using the pleopods in the water column (swimming activity), walking using the thoracic legs on the substrate before acquiring a shell (walking activity), inspecting a shell using the chelipeds (shell-inspection activity), and wearing a shell (shell-wearing activity). No deaths occurred, but behavioural data could not be obtained from two 5-day-old megalopae stocked in inshore salinity containers that had accidentally been placed down sideways after the initiation of the recordings.

Data analysis

Statistical analyses were performed using the R statistical software (R3.3.2; R Core Team, 2016) with a 5% significance level. Megalopal behavioural data were treated as proportional data because they had an upper limit (120 counts). A generalized linear mixed-effects model (GLMM) of the binomial family (logit link; Zuur *et al.*, 2009) was used to evaluate the effects of the experimental seawater treatments, observation time, and megalopal age on the settlement behaviour of the test individuals. The counts for a designated behaviour and those not showing the behaviour were the two-vector response variable, the different seawater treatments (A, offshore salinity; B, offshore salinity with inshore odours; or C, inshore salinity) and observation time (day or night) were

categorical explanatory variables, and megalopal age was a continuous explanatory variable. The data were collected longitudinally for individual megalopae 10–12 times during one day (i.e., repeated-measures data). Additionally, the megalopae originated from two broods. Therefore, to account for potential autocorrelation within the data for individual megalopae and megalopal origin (brood), the identification numbers for each megalopa and each brood were included in the GLMM as random intercept effects (Zuur *et al.*, 2009). The GLMM parameters (with standard errors; z-values with probabilities) were estimated using the *glmer* function implemented in the *lme4* package (Bates *et al.*, 2015) in R. In the GLMM analyses, the coefficient estimates of the categorical explanatory variables were provided for the respective treatments of offshore salinity with inshore odours, inshore salinity and night-time observation, which represent the change in the response variable relative to the baseline category (control treatment of offshore salinity or daytime observation).

Results

The proportions of behavioural events, such as swimming activity, walking activity, shell-inspection activity and shell-wearing activity, in 0-day-old to 6-day-old megalopae are shown in Figs. 1–4, respectively. The coefficient estimates from the GLMM evaluating the effects of the seawater treatments (offshore salinity, offshore salinity with inshore odours or inshore salinity), observation time (day or night) and age on individual behaviour are summarized in Table 1. The plus (or minus) signs of the coefficient estimates for the explanatory variables indicate positive (or negative) effects on the response variables.

Significant ontogenetic changes were observed in all megalopal behaviours except for shell-inspection activity; swimming and walking activities tended to decrease, and shell-wearing activity clearly increased in accordance with age (Figs. 1–4; Table 1).

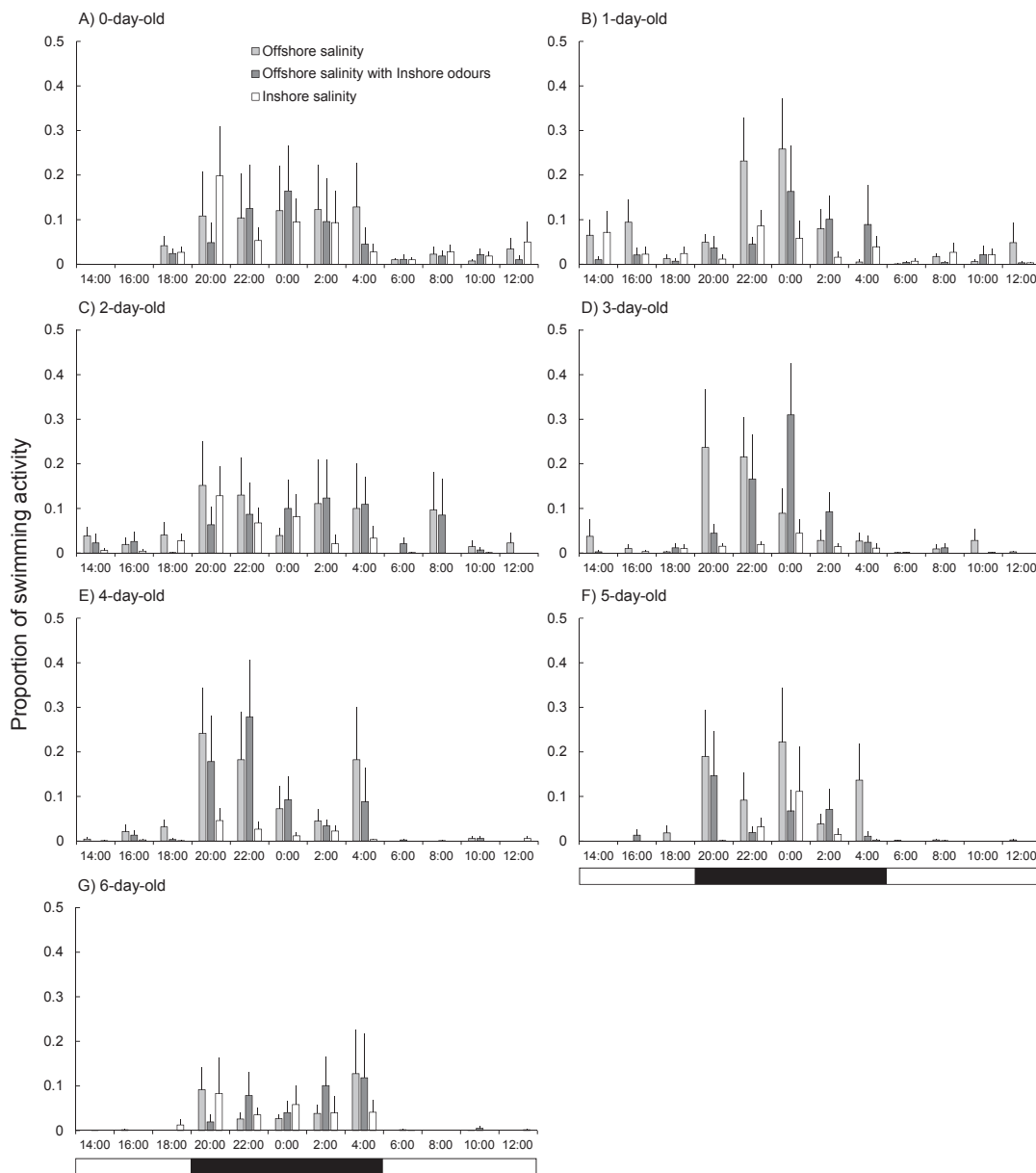


Fig. 1. Changes in the swimming activity of 0-day-old to 6-day-old *Coenobita violascens* megalopae (A–G) individually housed in small containers under different seawater conditions (offshore salinity, offshore salinity with inshore odours or inshore salinity). Number of replicate individuals was 10. Vertical bars represent the standard errors. The white and black areas of the horizontal rectangular bars under the graphs show daytime and night-time observation periods, respectively.

The observation period also significantly affected megalopal behaviour (Table 1); a nocturnal rhythm was evident in swimming activity (Fig. 1), whereas the other activities significantly decreased during the night-time period (Figs. 2–4).

The swimming activity of megalopae was not stimulated in the seawater of offshore salinity with inshore odours compared to the control group (offshore salinity), whereas the inshore salinity conditions significantly decreased the swimming activity

of the megalopae (Fig. 1; Table 1). The inshore odours also did not affect the walking activity of the megalopae, but their walking activity was significantly stimulated by the inshore salinity conditions (Fig. 2; Table 1). Thus, the settlement behaviour of *C. violascens* megalopae was not influenced by offshore salinity with inshore odours, but it was induced by reduced salinity conditions.

Shell-inspection activity was observed in 0-day-old to 6-day-old megalopae, but it was not significantly influenced by the seawater conditions (Fig. 3; Table 1). Shell-wearing activity mainly appeared in 4-day-old megalopae, and it tended to be stimulated by the seawater conditions of offshore salinity with inshore odours and inshore salinity in 5-day-old megalopae, but this trend was not observed in 6-day-old megalopae (Fig. 4). Overall, the seawater conditions did not significantly affect the shell-wearing activity (Table 1).

Discussion

The present study demonstrated that the early megalopae of *C. violascens* changed their settlement behaviour; the swimming activity in the water column tended to decrease with megalopal age, while the shell-wearing activity clearly increased. A diel rhythm in activity was also evident in the settlement behaviour of *C. violascens* megalopae; swimming activity had a clear nocturnal rhythm, and the walking, shell-inspection, and shell-wearing activities increased during the daytime, when swimming activity largely decreased. Similar phenomena have been observed in the settlement behaviour of coconut crab megalopae in the laboratory (Hamasaki *et al.*, 2015b).

Females of *C. violascens* hatch their eggs in mangrove areas (Doi *et al.*, 2016). In *C. violascens*, larval tolerance to low salinity conditions decreased after the second zoeal stage and became the lowest in

Table 1. Evaluation of the effects of seawater treatment (categorical explanatory variable; A, offshore salinity; B, offshore salinity with inshore odours; C, inshore salinity), observation time (categorical explanatory variable; daytime or night-time), and age on individual behavioural traits (proportional data) of *Coenobita violascens* megalopae.

Response variables	Coefficients	Estimates	SE	z values	P (> z)
Swimming activity	Intercept	-4.9042	0.4046	-12.12	< 0.0001
	Treatment (B)	-0.2746	0.3374	-0.81	0.4158
	Treatment (C)	-1.0505	0.3410	-3.08	0.0021
	Time (night-time)	2.4579	0.0277	88.90	< 0.0001
	Age	-0.2411	0.0698	-3.45	0.0006
Walking activity	Intercept	-2.0480	0.1704	-12.017	< 0.0001
	Treatment (B)	-0.1355	0.0956	-1.417	0.1564
	Treatment (C)	0.3179	0.0955	3.328	0.0009
	Time (night-time)	-0.3934	0.0139	-28.265	< 0.0001
	Age	-0.1239	0.0196	-6.332	< 0.0001
Shell-inspection activity	Intercept	-3.6994	0.2093	-17.678	< 0.0001
	Treatment (B)	-0.0980	0.2237	-0.438	0.661
	Treatment (C)	0.2266	0.2232	1.015	0.310
	Time (night-time)	-0.0903	0.0191	-4.729	< 0.0001
	Age	-0.0131	0.0456	-0.286	0.775
Shell-wearing activity	Intercept	-12.8160	0.9226	-13.89	< 0.0001
	Treatment (B)	0.5414	0.7185	0.75	0.451
	Treatment (C)	0.6046	0.7195	0.84	0.401
	Time (night-time)	-0.7697	0.0182	-42.32	< 0.0001
	Age	1.8437	0.1610	11.45	< 0.0001

Note: Data were analysed using a generalized linear mixed-effects model of the binomial family. Coefficient estimates of the categorical explanatory variables are provided for treatment B, treatment C and night-time observation, which represent the change in the response variable relative to the baseline category (treatment A or daytime observation). Number of observations for each response variable was 2436. Bold values are significant.

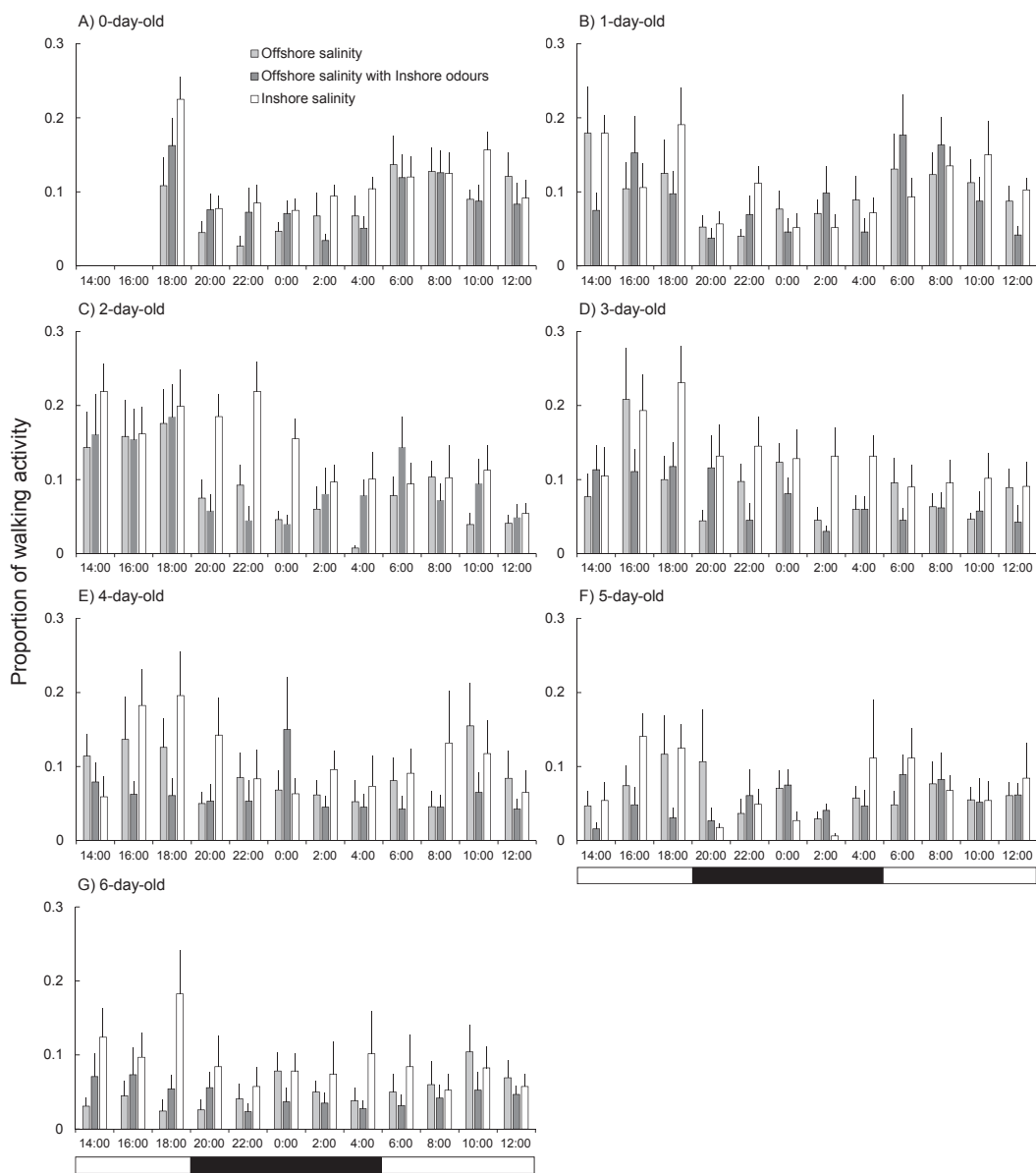


Fig. 2. Changes in walking activity before acquiring a shell by 0-day-old to 6-day-old *Coenobita violascens* megalopae (A–G) individually housed in small containers under different seawater conditions (offshore salinity, offshore salinity with inshore odours or inshore salinity). Number of replicate individuals was 10. Vertical bars represent the standard errors. The white and black areas of the horizontal rectangular bars under the graphs show daytime and night-time observation periods, respectively.

the fourth (last) zoeal stage; then, it increased in the megalopal stage (Hamasaki *et al.*, unpublished data). Therefore, the larvae of *C. violascens* might migrate offshore during the early zoeal stage and then immigrate to the inshore region in the megalopal stage. Thus, swimming activity in early megalopae is likely

linked to their immigration behaviour in the inshore habitats where they settle.

The horizontal movement of the megalopae of decapod crustaceans can be facilitated by their vertical migratory behaviour associated with the tidal cycle (Forward and Tankersley, 2001); they ascend

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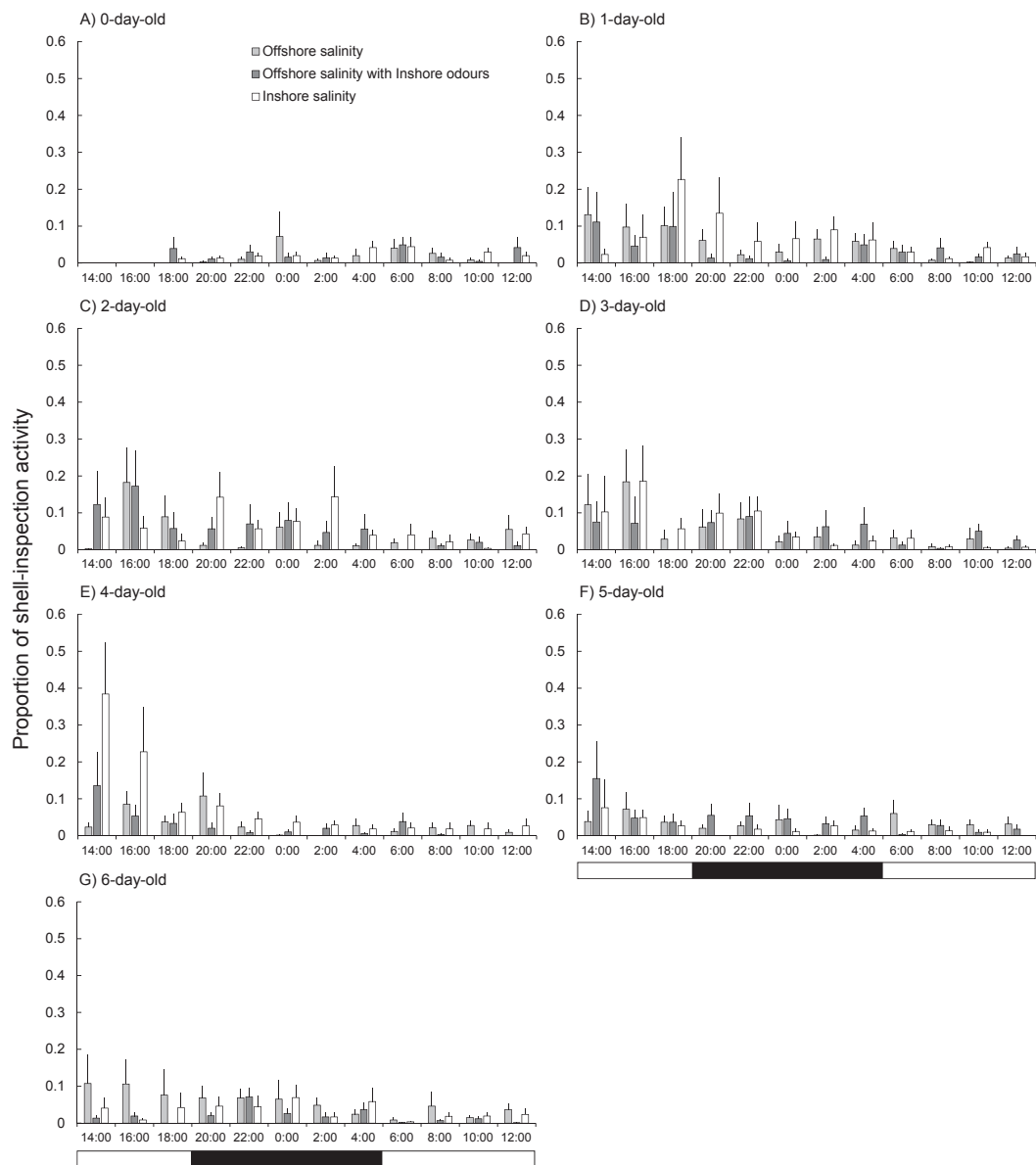


Fig. 3. Changes in shell-inspection activity by 0-day-old to 6-day-old *Coenobita violascens* megalopae (A–G) individually housed in small containers under different seawater conditions (offshore salinity, offshore salinity with inshore odours or inshore salinity). Number of replicate individuals was 10. Vertical bars represent the standard errors. The white and black areas of the horizontal rectangular bars under the graphs show daytime and night-time observation periods, respectively.

in the water column during a flood tide and descend to or near the bottom during an ebb tide, resulting in up-estuary movement (flood-tide transport). Subtropical Japanese waters have a mixed semidiurnal tidal cycle (http://www.data.jma.go.jp/kaiyou/db/tide/suisan/s_nansei.php). Therefore, *C. violascens* meg-

alopae might exhibit ascending (i.e., swimming) behaviour during the nocturnal flood tide and descending (i.e., walking, shell-inspection and shell-wearing) behaviour during the daytime, thereby enhancing the likelihood of their inshore transport while avoiding predation by pelagic visual feeders during the day

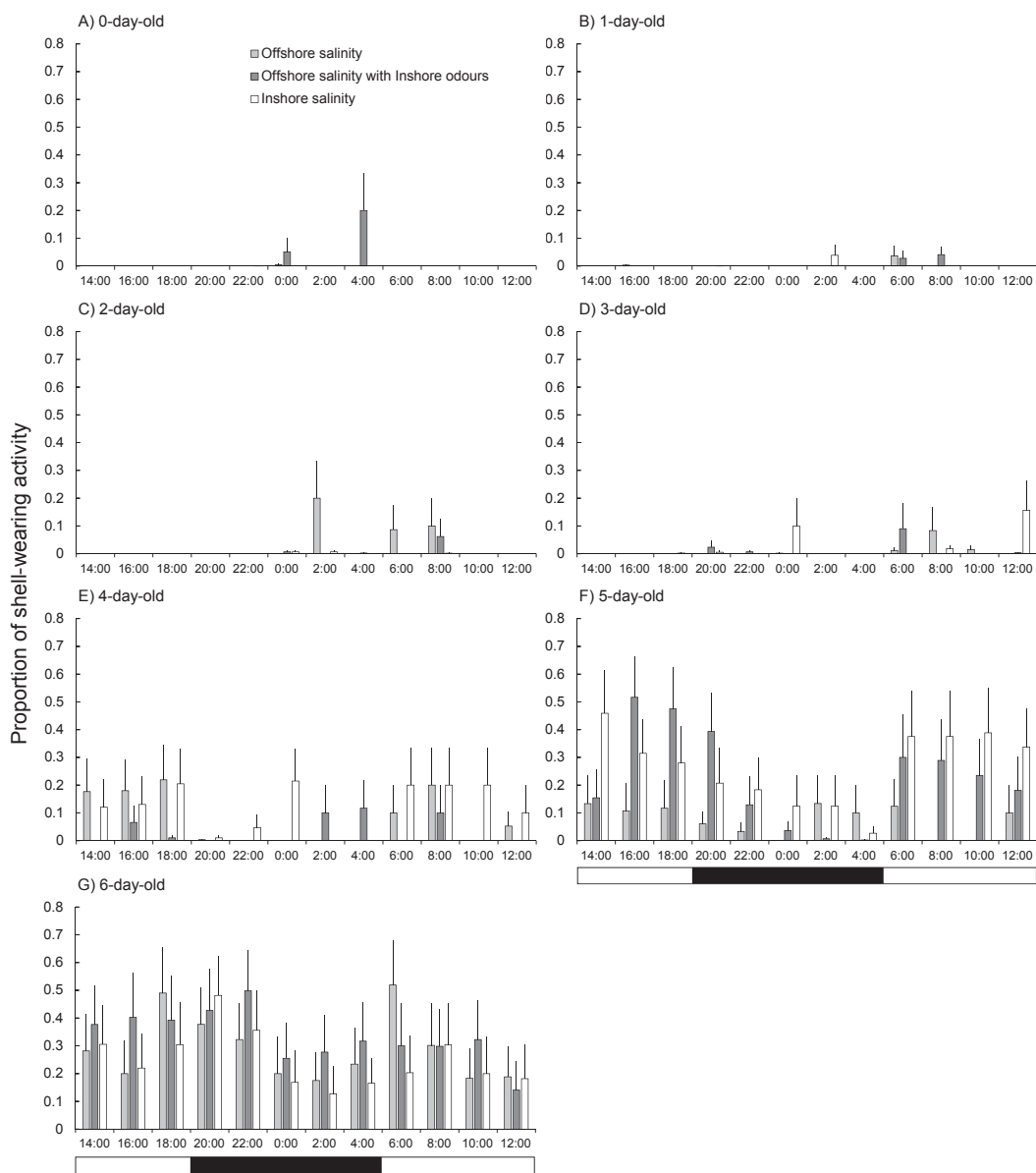


Fig. 4. Changes in shell-wearing activity by 0-day-old to 6-day-old *Coenobita violascens* megalopae (A–G) individually housed in small containers under different seawater conditions (offshore salinity, offshore salinity with inshore odours or inshore salinity). Number of replicate individuals was 10. Vertical bars represent the standard errors. The white and black areas of the horizontal rectangular bars under the graphs show daytime and night-time observation periods, respectively.

(Zaret and Suffern, 1976; Stich and Lampert, 1981).

Coenobita violascens inhabits the vicinity of eutrophic rivers, particularly mangrove estuaries. Therefore, we hypothesized that seawater (offshore salinity) containing chemical cues from eutrophic river basins would stimulate the swimming behaviour

of megalopae towards settlement habitats and that the reduced salinity in inshore areas decreases the swimming behaviour of megalopae, leading to settlement. Our hypothesis regarding inshore chemical cues, however, was not supported, as the offshore salinity conditions with inshore odours derived from the

riverine water of a mangrove estuary did not affect megalopal behaviour, including swimming activity. In contrast, our experiment demonstrated that the inshore salinity conditions decreased the swimming activity and increased the walking activity of megalopae, thereby enhancing their settlement behaviour. The megalopae of the blue crab *Callinectes sapidus* Rathbun, 1896 migrate from offshore to the estuary for settlement, and salinity has been known to be an important environmental factor affecting larval behaviour, i.e., swimming activity was found to decrease in association with decreasing salinity (Tankersley *et al.*, 1995; Welch and Forward, 2001), as demonstrated for *C. violascens* in the present study.

Lower salinity conditions might widely occur along shorelines due to the inflow of groundwater (Tottori *et al.*, 2004). Therefore, *C. violascens* megalopae must find suitable habitats in which they can settle, e.g., mangrove estuaries. It has been demonstrated that chemical cues from inshore habitats, such as seagrass odours, affect the orientation of blue crab megalopae (Diaz *et al.*, 1999; Forward *et al.*, 2003; Epifanio and Cohen, 2016). The megalopae of *C. violascens* might utilize the concentration cline of the eutrophic river odours diffusing into the coastal waters as an orientation cue to find settlement habitats. In Nagura Amparu, a brackish estuary system with a tidal flat and mangrove tree area where *C. violascens* is most abundant on Ishigakijima Island (Fujikawa *et al.*, 2017; Hamasaki *et al.*, 2017, 2018), it has been reported that brachyuran megalopae recruit to the tidal lagoon for a short time during flood tides with high salinity conditions (approximately 34 psu) before dawn around new moon periods (Kawachi *et al.*, 2009). This suggests that the megalopae of *C. violascens* might immigrate to settlement habitats through a flood-tide transport mechanism with the aid of eutrophic riverine odours, their settlement behaviour might be stimulated by the low salinity conditions that occur during the ebb tide near the river mouth area and that they might then enter the tidal lagoon by swimming during the nocturnal flood tide,

when the salinity increases.

Carrying a shell plays an important role in reducing the risk of predation in megalopae, including cannibalism by other hermit crabs (Thompson, 1903; Roberts, 1971; Bookhout, 1964; Hamasaki *et al.*, 2015b), and avoiding desiccation in terrestrial environments (Brodie, 2005; Hamasaki *et al.*, 2011, 2013). Therefore, shell-carrying behaviour is indispensable for the survival of megalopae. Consequently, it is inferred that shell-inspection activity appeared in newly metamorphosed *C. violascens* megalopae, and the different seawater treatments did not affect their shell-inspection activity in our experiments. On the other hand, older megalopae appeared to exhibit more shell-wearing activity, and the inshore odours and inshore salinity conditions tended to stimulate these activities in 5-day-old megalopae, although the overall shell-wearing activity was not significantly influenced by the seawater types. Hamasaki *et al.* (2015c) reported that laboratory-raised megalopae of *C. violascens* could walk steadily while wearing a shell at approximately two weeks after metamorphosis; then, they began to land on a sandy substrate in the culture containers. Therefore, to evaluate the effects of environmental cues on shell-wearing activity, megalopal behaviour should be monitored for longer periods than those in the present study. Additionally, to better understand the recruitment mechanisms of *C. violascens* megalopae, further studies will be required to investigate the effects of chemical cues from eutrophic river basins and salinity conditions on megalopal orientation in the sea and emigration behaviour during the sea-to-land transition.

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