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Thermal adaptations of embryos of six terrestrial hermit crab species

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ABSTRACT: We evaluated the thermal adaptations of embryos of 6 terrestrial hermit crab species in the family Coenobitidae (genera *Birgus* and *Coenobita*): *B. latro*, *C. brevimanus*, *C. cavipes*, *C. purpureus*, *C. rugosus*, and *C. violascens*. Embryos of each species were cultured *in vitro* at 6 different temperatures (18 to 34°C) in artificial seawater to avoid air desiccation; the lower threshold temperatures for embryonic development were estimated using heat summation theory equations. Additionally, partial effective cumulative temperatures (> lower threshold temperature) until hatching were determined for ovigerous females of each species cultured in containers. The relationships between the embryonic growth index values (relative area of the embryonic body vs. total embryo surface) and effective cumulative temperatures were expressed using cubic equations. Lower threshold temperature was estimated to be 12.7 to 14.5°C. The effective cumulative temperature and egg incubation period estimates from the appearance of the embryonic body to hatching were higher in *B. latro* and *C. brevimanus*, followed by *C. rugosus*, *C. cavipes*, and *C. violascens*, and lower in *C. purpureus*, suggesting that *C. brevimanus* may retain an ancestral thermal adaptation trait for embryos, as in *B. latro*, which is considered the most ancestral species in the coenobitid phylogeny. Egg size varied among species but did not affect the thermal adaptations of embryos. The lower effective cumulative temperature and shorter egg incubation period may be advantageous to producing broods during the shorter summer breeding season in *C. purpureus*, which has the northern-most geographical distribution.

KEY WORDS: Coconut crab · Land hermit crab · Embryonic development · Lower threshold temperature · Effective cumulative temperature

INTRODUCTION

Terrestrial hermit crabs in the family Coenobitidae diverged from a marine ancestor between 84 and 39 million years ago (Bracken-Grissom et al. 2013). They comprise land hermit crabs of the genus *Coenobita*, with 16 species, as well as the coconut crab *Birgus latro* (Linnaeus, 1767), which is the only species in the genus *Birgus* (McLaughlin et al. 2010). Land hermit crabs carry gastropod shells; however, the shell-carrying behavior of *B. latro* appears only during the juvenile stage (Harms 1938, Reese 1968, Kadiri-Jan & Chauvet 1998, Hamasaki et al. 2014b).

Terrestrial hermit crabs live in a potentially desiccating environment except during the larval phase. Females extrude their eggs terrestrially, which are subsequently attached to the setae of the pleopods on the left ventral side of the abdomen (de Wilde 1973, Greenaway 2003, Sato & Yoseda 2008, 2009, Drew et al. 2010). The *B. latro* egg mass is afforded little physical protection from the environment by the mother's abdomen and is therefore susceptible to

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desiccating conditions. To minimize dehydration of the egg mass, ovigerous *B. latro* females require a high-humidity shelter (Schiller et al. 1991). Additionally, *B. latro* females maintain hydration of their eggs by using branchial water reserves and body fluids while grooming the egg mass with their fifth pair of pereiopods (Schiller et al. 1991). In contrast, the egg mass of *Coenobita* spp. land hermit crabs is well protected against physical damage and desiccation by the female's shell (de Wilde 1973). Land hermit crabs carry water within their shells (de Wilde 1973, Greenaway 2003), and ovigerous females often moisten their eggs using shell water but never permanently bathe them (de Wilde 1973). Coenobitid females return to the sea to hatch the embryos (Hartnoll 1988, Schiller et al. 1991, Nakasone 2001). Newly hatched larvae develop through planktonic zoeal stages to megalopae in the sea (Hamasaki et al. 2015b), similar to marine hermit crabs. After settlement, coenobitid megalopae recognize and acquire gastropod shells and then migrate onto land (Reese 1968, Reese & Kinzie 1968, Harvey 1992, Brodie 1999, Hamasaki et al. 2011, 2015a).

Coenobitid crabs mainly occur in subtropical and tropical coastal regions and have been divided into 2 groups based on their geographical occurrence patterns: (1) widely distributed species in the Indo-West Pacific, and (2) relatively narrower distributed species in particular regions, such as the western Atlantic, West Africa, west coast of North America, Northern Australia, and the northwestern Pacific (Hartnoll 1988, Nakasone 1988, Harvey 1992, McLaughlin et al. 2007, Wang et al. 2007, Hamasaki et al. 2015b).

Coenobita purpureus Stimpson, 1858 has a limited distribution and mainly occurs in oceanic islands north of 24° N in the Ryukyu Archipelago, Izu Islands, and the Ogasawara (Bonin) Islands, Japan. This crab is also found on the Pacific coasts of mainland Japan (<35° N) (Hamasaki et al. 2016). Additionally, several widely distributed coenobitids, including *Coenobita brevimanus* Dana, 1852, *C. cavipes* Stimpson, 1858, *C. rugosus* H. Milne-Edwards, 1837, *C. violascens* Heller, 1862, and *B. latro*, commonly occur in the southern oceanic islands, Japan (Nakasone 1988, 2001, Hamasaki et al. 2016). Hamasaki et al. (2016) examined the phylogenetic relationships between *C. purpureus* and its widely distributed congeners based on partial 16S mitochondrial rDNA sequences. Their phylogenetic tree demonstrated that *C. purpureus* clustered with *C. rugosus*. They also hypothesized that ancestral *Coenobita* species may have expanded their distribution into the northwestern Pacific region (>24° N) and evolved into *C. purpureus* in the Ryukyu region land masses during the Pliocene.

Temperature is the most important environmental factor affecting biological processes of ectothermic organisms, including behavior, physiology, growth, and survival of all life history stages. Therefore, ectothermic organisms must adapt to the thermal conditions of their habitats (Stillman & Somero 2000, Hall & Thatje 2009), and thus temperature may have acted as a selective force when *C. purpureus* diverged in the Ryukyu Archipelago region. We hypothesize that the thermal adaptations of *C. purpureus* may differ from those of other widely distributed coenobitid species. Our objective in this study was to test this hypothesis and discuss thermal adaptations of coenobitid crabs in the context of evolutionary and ecological traits. We investigated thermal adaptations of embryos in 6 coenobitid crabs (*B. latro*, *C. brevimanus*, *C. cavipes*, *C. purpureus*, *C. rugosus*, and *C. violascens*) by estimating the lower threshold temperatures (LTT, °C) at which embryonic development ceases and the sum of daily temperatures above LTT, i.e. effective cumulative temperatures (ECT, degree-days [°D]), required for embryonic development to hatching. LTT and ECT have been estimated in order to evaluate thermal adaptations of ectothermic organisms including insects (Honěk 1996, Kiritani 2012) and decapod crustaceans (Hamasaki 1996, 2002, 2003, Hamasaki et al. 2009). To obtain the biological data for estimating these 2 life history parameters, we conducted 2 laboratory experiments. In Expt 1, we incubated embryos of each species at different temperatures to estimate the LTT values. In Expt 2, we cultured ovigerous females to hatch their embryos and examined the relationships between growth of the embryos and ECT values. Additionally, we tested whether or not egg size affected the thermal adaptation traits of the embryos of these coenobitids, because it has been suggested that larger eggs show slower developmental rates in closely related decapod crustaceans (Wear 1974), and interspecific variation in egg size is known for coenobitid species (Nakasone 2001).

MATERIALS AND METHODS

Culture of test animals

We captured ovigerous females of *Birgus latro*, *Coenobita brevimanus*, *C. cavipes*, *C. purpureus*, *C. rugosus*, and *C. violascens* during late June to early July of 2005 to 2015 on Hatomajima Island (24° 28′ N, 123° 49′ E), Ishigakijima Island (24° 23–31′ N, 124° 07–18′ E), and/or Miyakojima Island (24° 43–50′ N,

125° 15–21′ E), Okinawa Prefecture, Japan, and cultured them until their embryos hatched into larvae that were used in experiments. In 2005, ovigerous females of *B. latro* were cultured in a laboratory (29 to 30°C) at Yaeyama Station, Seikai National Fisheries Research Institute, Fisheries Research Agency on Ishigakijima Island, and from 2006 to 2015, ovigerous females of all species were cultured in a laboratory (27 to 28°C) at the Tokyo University of Marine Science and Technology (TUMSAT), Tokyo, Japan. Ovigerous females were maintained in tanks equipped with simulated land and sea areas (34 ppt salinity), according to the methods of Hamasaki et al. (2009) and Hamasaki (2011). The photoperiod of the culture room was 13–14 h light:11–10 h dark. Air temperatures were recorded by data loggers every 20 to 60 min during the culture period.

All *Coenobita* spp. in Japan are recognized as a Natural Monument Animal to promote their conservation. Therefore, *Coenobita* spp. were collected and cultured with permission of the Agency for Cultural Affairs, Ministry of Education, Culture, Sports, Science, and Technology of Japan. In addition, *B. latro* is listed as 'vulnerable' in the Red Data Book by the Ministry of the Environment of Japan. Therefore, these crabs were returned to their natural habitat after the culture experiments were completed.

Expt 1: LTT for embryonic development

In general, to investigate the effects of temperature on the embryonic development of decapod crustacean species, individual females are cultured in containers at different temperatures from spawning to hatching (e.g. Wear 1974, Hamasaki et al. 2003, Stevens et al. 2008). However, this methodology requires a large number of test animals. For our experiments, the number of females of each coenobitid species available was limited because they are a protected and/or endangered species, and we have not yet developed the culture methodology to spawn coenobitid females in the laboratory; therefore, we adopted an *in vitro* incubation methodology for embryos separated from ovigerous females. In decapod crustacean aquaculture, artificial incubation of embryos separated from the mother is considered beneficial because the potential negative effects of the mother (e.g. egg loss and death of the mother) can be alleviated (Balasundaram & Pandian 1981, Nakata et al. 2004, Zeng 2007). Several methodologies for *in vitro* incubation of embryos have been employed (e.g. Balasundaram & Pandian 1981, Zeng 2007). We used the simple method developed by Nakata et al. (2004) for artificial incubation of embryos of Japanese crayfish *Cambaroides japonicus* De Haan, 1841. Crayfish embryos were successfully reared individually in wells of cell-culture microplates with sterile water (0.125 to 10 ml) without aeration or water exchange. Nakata et al. (2004) stated that an advantage of this method is that it avoids negative effects caused by the presence of other eggs, such as a decline in water quality caused by dead eggs.

Two different ovigerous females (Females 1 and 2) of each species were used for the embryo incubation experiments conducted during 2012 to 2014 at TUMSAT. In 2012, 2 females each of *B. latro* and *C. rugosus*; in 2013, 1 female of *C. cavipes* and 2 females of *C. purpureus*; and in 2014, 1 female of *C. cavipes* and 2 females each of *C. brevimanus* and *C. violascens* were used. To avoid desiccation of the embryos and to maintain the same humid conditions for the *in vitro* incubation, we cultured the embryos by immersing them in artificial seawater. The concentration of the artificial seawater was regulated at 2 salinity levels (27 and 34 ppt), considering the osmolality of early stage *B. latro* embryos (807 mOsm kg^{-1} H_2O ≈ 27 ppt salinity) (Schiller et al. 1991). Early stage embryos were carefully removed from ovigerous females of each species using small forceps before eye pigmentation had appeared on the eye placodes, and were placed in a plastic dish with seawater (27 ppt). Individual embryos were separated carefully with small dissecting needles (see Fig. S1 in the Supplement at www.int-res.com/articles/suppl/b025p083_supp.pdf for photographs of embryos from females of each species) and rinsed with the designated salinity seawater in another dish. One embryo was stocked in each of 10 plastic tubes (volume: 2.8 ml) containing 2 ml of the designated salinity seawater. A total of 20 tubes for the 2 salinity levels were set in each of 6 incubation chambers (14 h light:10 h dark cycle) (MT1-201, Tokyo Rikakikai) controlled at 6 different temperatures between 18 and 34°C (120 embryos for each female). This temperature range was adopted considering the LTT estimate for *B. latro* zoeal development (Hamasaki et al. 2009) and the high temperature that coenobitids can experience in their natural habitats in the Ryukyu Archipelago. The seawater used for culture was not renewed during the incubation period, and evaporation was prevented by placing caps on the tubes. Incubation temperatures were recorded every 30 min with data loggers placed in the incubation chambers; the mean (±SD) incubation temperatures during the culture period are shown in Table S1 in the Supplement for each female.

To estimate the LTT for embryonic development, the number of days required to reach the designated developmental stage should be determined for embryos incubated at different temperatures. In the present study, we counted the number of days from the onset of incubation until eye pigmentation appeared on the eye placodes because of its easy detection by daily observation of embryos under a stereomicroscope. Embryos that appeared cytologically normal were considered survivors, and dead embryos became cloudy. Additionally, to compare the development of *in vitro* and *in situ* embryos, we attempted to determine the number of days required until eye pigmentation appeared on embryos of *C. brevimanus*, *C. cavipes*, and *C. violascens* ovigerous females cultured in 2014.

The relationship between mean temperature (T) and the number of days (D) required until eye pigmentation appeared on embryos incubated at the respective salinity levels was expressed by the following equation for each female: $D = a/(T - b)$. In this equation, the parameter a varies within species; it is larger when younger embryos are used for incubation. The parameter b is the so-called 'LTT' for embryonic development which is to be estimated in the present study. The parameters were estimated using a non-linear ordinary least-squares method and evaluated with t-tests.

Our experimental design—incubating embryos from 2 different females at different salinity levels—allows us to statistically test the interspecific variation of the LTT estimates. We applied a linear mixed-effects model (Everitt & Hothorn 2009, Zuur et al. 2009) to examine differences in the LTT among the 6 coenobitids using estimates for embryos incubated at 2 different salinities. In this analysis, species was the categorical explanatory variable, and female identity was included as a random effect to account for a potential correlation of repeated measurements (2 salinity levels) within females. Statistical significance of the explanatory variable was evaluated using the 'lme' and 'anova' functions in the 'nlme' package (https://cran.r-project.org/web/packages/nlme/nlme.pdf) in R v.3.1.0 (R Core Team 2014).

Expt 2: ECT for embryonic development

If coenobitid females had been available for controlled spawning in the laboratory, we could have evaluated the ECT for the entire embryonic period. However, we used wild ovigerous females which were cultured to obtain newly hatched larvae in the laboratory. Therefore, we developed an alternative methodology to estimate the ECT for partial embryonic development using ovigerous female coenobitids based on the method of Hamasaki (1996). In his study, the embryonic growth of laboratory-spawned ovigerous females of the swimming crab *Portunus trituberculatus* (Miers, 1876) was examined daily from the onset of appearance of embryonic body to hatching, and development was quantified by an embryonic growth index (EGI) measured as the relative area of the embryonic body versus the total embryo surface in a lateral view of the egg. ECT values were calculated as the sum of the daily effective temperature above LTT (°D) for partial embryonic development from the day of appearance of embryonic body to the day when hatching occurred in the designated females. Hamasaki (1996) showed that the ECT required for partial embryonic development to hatching decreased with increasing EGI values. When the relationship between the EGI and ECT values can be formulated, the intercept of the equation is interpreted as the mean ECT required for partial embryonic development, starting when the embryonic body appeared until hatching. Additionally, the equation between the EGI and ECT values allows us to predict the hatching day of embryos.

B. latro females are highly fecund and, depending on body size, produce ~50 000 to 250 000 eggs brood^{-1} (Sato & Yoseda 2008). Moreover, it is easy to sample eggs from ovigerous females because the egg mass is not protected by a gastropod shell. Therefore, we measured the EGI daily and calculated the partial ECT from the time of appearance of embryonic body to hatching in 4 different ovigerous females of *B. latro* in 2005; the number of individual estimates of partial ECT based on 1 observation of EGI and known hatch time for each female ranged from 21 to 24 (total: 91) (see Table S2 in the Supplement). An additional 1 to 16 estimates (total: 89) of the partial ECT were obtained for 20 different *B. latro* females during 2006 to 2014. In contrast to *B. latro*, females of *Coenobita* spp. land hermit crabs are less fecund, and it is difficult to sample eggs from the egg mass because females must be partially pulled out from their shell. To reduce stress to the *Coenobita* ovigerous females from egg sampling, we collected only a small number of estimates of the partial ECT (1 to 8) for each female. The dates of measurements of EGI were arbitrarily determined for each female so as to cover the entire period from the appearance of embryonic body to hatching in the species. The total number of partial ECT estimates ranged from 33 to 82 and were based on 18 to 36 different females of each *Coenobita* species (Table S2).

When we observed embryos, a small cluster of ~10 eggs was removed from the outer margin of the egg mass, placed on a glass slide in seawater (34 ppt) under a stereomicroscope, and lateral views of 5 eggs were photographed with a digital camera (Nikon Digital Sight). Lengths of the major axis (L) and minor axis (S) at the outer egg membrane, and the areas of the embryonic body (EA) and yolk mass (YA) were determined from the digital photographs using an image analyzing system (NIS-Elements software; Nikon). Egg volume was calculated as $\pi LS^2/6$. The EGI was calculated as: EA/(EA + YA) × 10^2. In the present study, the date of egg extrusion was not known but the date of hatching was determined for each female in Expt 2; therefore, the EGI values and egg sizes can be presented in relation to the number of days before embryo hatching.

Hatching occurred at night in all species. The partial ECT from the day when the EGI value was measured to the day when hatching occurred in each female was calculated as the sum of the daily effective temperature above the LTT for embryonic development (°D). The relationships between EGI and ECT were empirically fitted with the following cubic equation: ECT = aEGI^3 + bEGI^2 + cEGI + d; where a, b, c, and d are parameters, determined by a linear mixed-effects model using the 'lme' function and evaluated with t-tests in R. In this analysis, female identity was included as a random effect to account for a potential correlation of repeated measurements within females.

Effect of egg size on thermal adaptations of embryos

Intraspecific variation of offspring size occurs in many decapod crustacean species (e.g. Gardner 1997, Moland et al. 2010, Guay et al. 2011), including *B. latro* (Sato & Suzuki 2010) in which larger females produce larger offspring. Although intraspecific variation of offspring size dependent on maternal size has not been reported for other coenobitid species, possible maternal size effects should be considered as a confounding factor in interspecific comparisons of egg size. However, we were not able to conduct such a statistical test because we did not measure female body size in the present study. Alternatively, to examine interspecific variation in egg size while accounting the potential effects of female identity on egg size, we applied a nested ANOVA, in which species was the independent factor and female identity was nested within each of the 6 species.

To evaluate differences in egg diameters and volumes at the same embryo stage among the 6 coenobitids, measurements of eggs within 1 d of hatching, determined for 4 to 8 different females of each species were summarized. Interspecific variation in egg size (volume) was statistically compared using the 'aov' function. Finally, we used the Pearson's product-moment correlation (r) analysis, whose significance was determined by t-test, to evaluate the relationship between mean egg volume of each species and 2 measures of thermal adaptation of embryos, i.e. LTT from Expt 1 and ECT as represented by parameter d in the cubic equation from Expt 2.

RESULTS

Expt 1: LTT for embryonic development

Embryos of all of the coenobitid crab species developed in artificial seawater, although embryo survival tended to be low at lower and higher temperatures in *Coenobita cavipes* Female 1 (see Fig. S2 in the Supplement at www.int-res.com/articles/suppl/b025p083_supp.pdf). Temperature strongly affected the rate of embryonic development; the number of days from the onset of incubation until eye pigmentation appeared decreased exponentially with increasing incubation temperature (Fig. 1). Among the female broods, the number of days until eye pigmentation appeared on embryos attached to the female pleopods was determined for *C. brevimanus* Female 2 (3 d), *C. cavipes* Female 2 (8 d), and *C. violascens* Females 1 (5 d) and 2 (6 d). These developmental times coincided with those of embryos incubated *in vitro* at the same temperature (~28°C) (*C. brevimanus* Female 2: 3–4 d; *C. cavipes* Female 2: 7–8 d; *C. violascens* Females 1 and 2: 5–6 d and 6–7 d, respectively).

The heat summation theory equation seemed to be a good fit to the relationship between mean temperature and the number of days until eye pigmentation appeared, as shown by the regression curves in Fig. 1 which were drawn using the parameter estimates of equations in Table S3 in the Supplement. The LTT for embryonic development (i.e. parameter b in the equation) was estimated to vary from 12.4 to 15.0°C (Table S3 in the Supplement). LTT values are summarized by box plots in Fig. 2 using estimates for the 27 and 34 ppt seawater incubations from 2 females of each species. The LTT was significantly different among species (F = 6.018, df = 5, 6, p = 0.0247). The means ± SD of the LTT estimates for 2 females were: 13.5 ± 0.3, 13.6 ± 0.5, 12.7 ± 0.3, 14.5 ± 0.6, 13.5 ± 0.6,

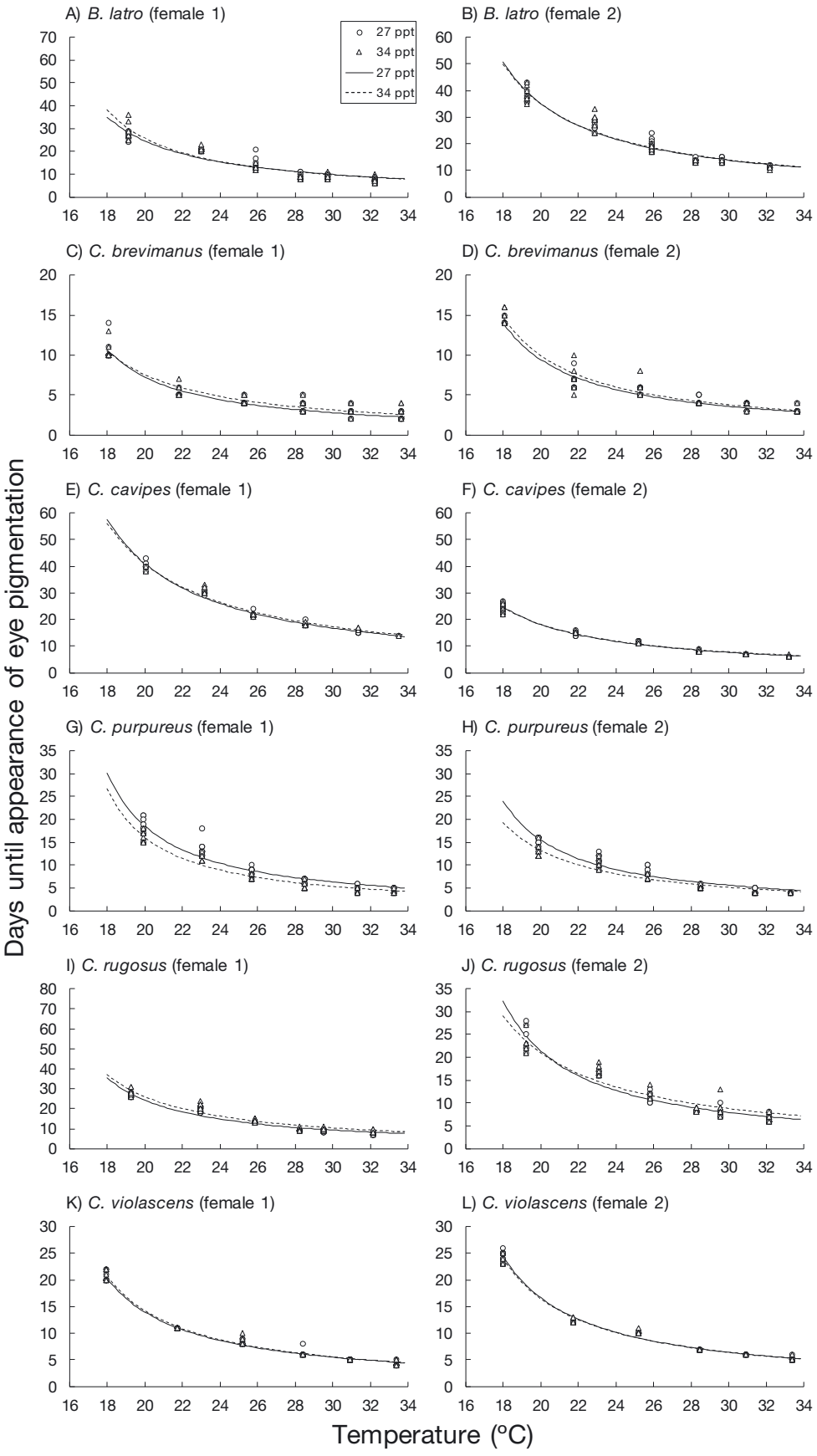

Fig. 1. Relationships between mean incubation temperature and the number of days until eye pigmentation appeared on embryos of the 6 coenobitids incubated *in vitro* in seawater at 2 salinity levels and 6 different temperatures. Experiments were conducted with 2 different females of each species: (A,B) *Birgus latro*, (C,D) *Coenobita brevimanus*, (E,F) *C. cavipes*, (G,H) *C. purpureus*, (I,J) *C. rugosus*, and (K,L) *C. violascens*. Regression curves were drawn for each salinity based on the heat summation theory equations applied to the relationship between temperature and incubation period shown in Table S3 in the Supplement at www.int-res.com/articles/suppl/b025p083_supp.pdf

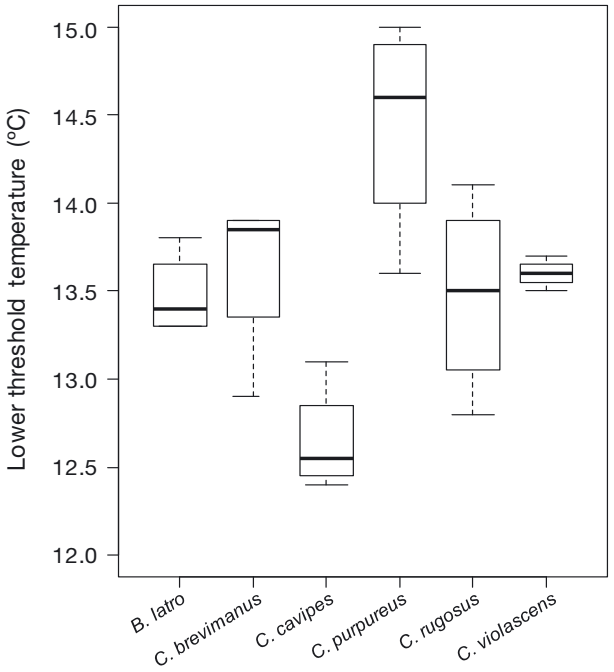

Fig. 2. Lower threshold temperatures for embryonic development in the coenobitids *Birgus latro*, *Coenobita brevimanus*, *C. cavipes*, *C. purpureus*, *C. rugosus*, and *C. violascens* using estimates for 2 different females from each species incubated in 27 and 34 ppt seawater (see Fig. 1)

and 13.6 ± 0.1°C for *Birgus latro*, *Coenobita brevimanus*, *C. cavipes*, *C. purpureus*, *C. rugosus*, and *C. violascens*, respectively, and they were slightly lower in *C. cavipes* and higher in *C. purpureus*.

Expt 2: ECT for embryonic development

Changes in the external morphology of the embryos in lateral view from the day when the embryonic body appeared to the day of hatching are represented in photographs of a *B. latro* female cultured in 2005 shown in Fig. 3. Additionally, changes in the EGI values and egg diameters of 4 *B. latro* females observed daily during incubation in 2005 are shown in Fig. 4, and those for all females are shown in Figs. S3 & S4 in the Supplement. The germinal disk formed as a yolk-free portion (Fig. 3A); that yolk-free portion (i.e. the embryonic body) then increased, while the rudimentary appendages, including antennules, antennae, mandibles, maxillules, maxillae, and mandibles as well as the eye placodes and pleon, developed (Fig. 3B–H). Pigmentation first appeared on the eye placodes (Fig. 3I) when the EGI value was around 25%. The eye-pigmented area then increased with decreasing yolk mass while red/brownish pigmentation developed on the body (Fig. 3J–X). The rate of increase in the EGI value was slow until it reached about 15%, at which point EGI values increased fairly linearly in all species (Fig. 4 & Fig. S3 in the Supplement). The *S* length of the eggs changed little during embryonic development, whereas *L* length clearly increased in all species (Fig. 4 & Fig. S4).

The partial ECT values calculated from the day of measurement until the day of hatching are plotted against the EGI values of each species in Fig. 5. The cubic equations seemed to be a good fit to the relationship between the EGI and ECT values as shown by the regression curves in Fig. 5, which were drawn using the parameter estimates of equations in Table 1. The estimates of parameter *d* in these equations are the mean ECTs required for partial embryonic development starting when the embryonic body appeared until hatching. The egg incubation periods from the appearance of the embryonic body to hatching were calculated under air temperatures of 24 to 29°C during the main reproductive season of coenobitid species as: partial ECT from the appearance of the embryonic body to hatching / (mean T – LTT). As shown in Fig. S5 in the Supplement, *B. latro*: 23 to 35 d; *C. brevimanus*: 24 to 38 d; *C. cavipes*: 18 to 27 d; *C. purpureus*: 15 to 25 d; *C. rugosus*: 20 to 32 d; and *C. violascens*: 17 to 26 d. The partial ECT and egg incubation period estimates from the appearance of the embryonic body to hatching were higher in *B. latro* and *C. brevimanus*, followed by *C. rugosus*, *C. cavipes*, and *C. violascens*, and lower in *C. purpureus* (Fig. 6 & Fig. S5).

Effect of egg size on thermal adaptations of embryos

The dimensions (lengths of *L* and *S*) and volume of eggs within 1 d of hatching for each species are summarized in Table 2. The values of egg size (volume) are also summarized by box plots for each female in Fig. S6 in the Supplement. Egg volume varied significantly among species ($F = 313.177$, df = 5, $p < 0.001$) and among females (nested in each species) ($F = 26.934$, df = 27, $p < 0.001$), showing that the factor species accounted for 65% and females for 30% of the total variance (Table S4 in the Supplement). Egg size tended to be larger in *B. latro* and *C. brevimanus*, followed by *C. purpureus*, *C. rugosus*, and *C. violascens*, but was smaller in *C. cavipes*. Mean egg size did not significantly affect the LTT or ECT values for embryonic development in the 6 coenobitid species (LTT: r = 0.374, $t = 0.808$, df = 4, p = 0.465; ECT: r = 0.501, $t = 1.159$, df = 4, p = 0.311).

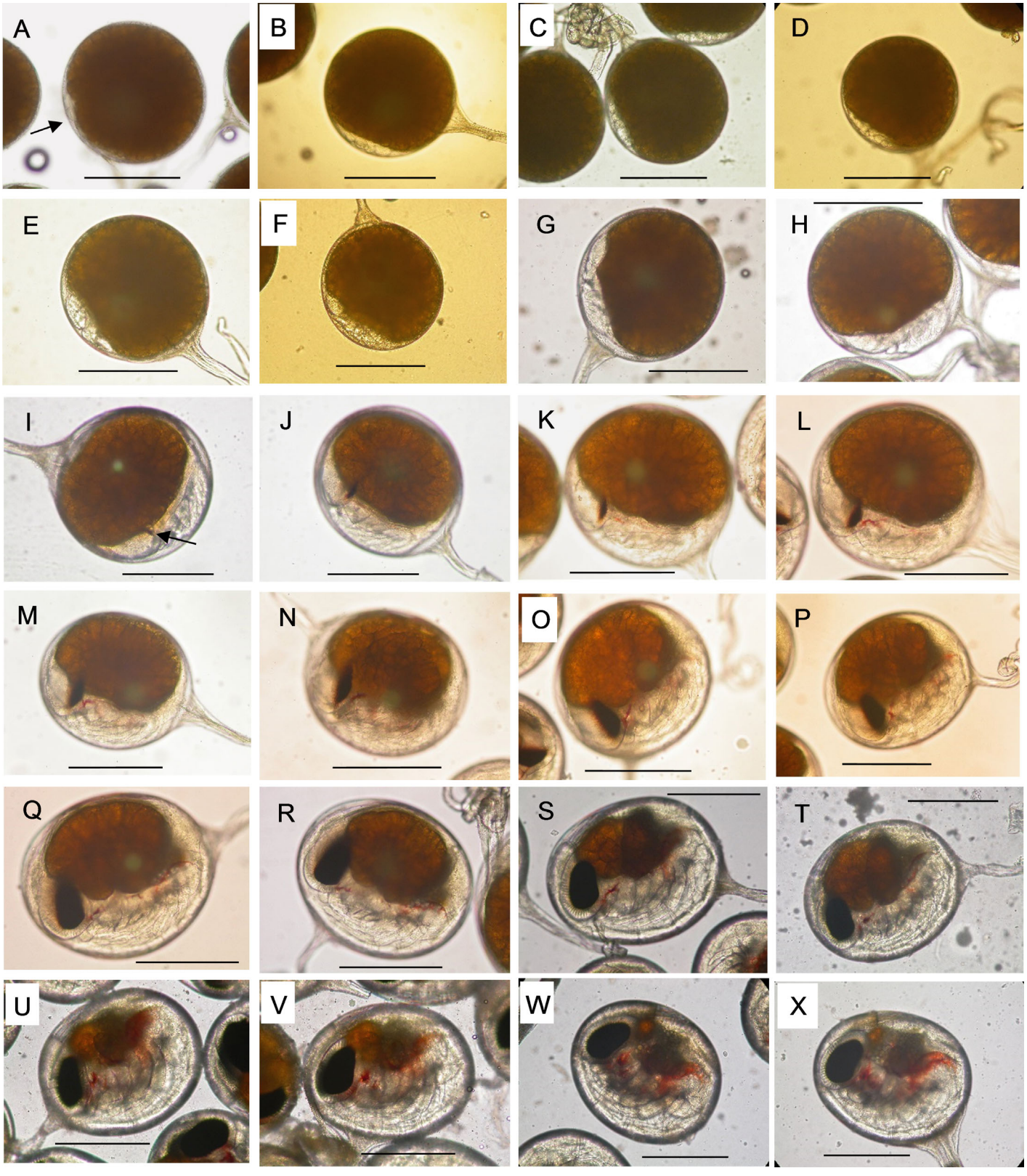

Fig. 3. (A–X) Embryonic development of *Birgus latro* observed daily for 24 d before hatching. Hatching occurred on the night of the last observation of embryos (panel X). Arrows in (A) and (I) show the embryonic body and pigmented eye placode, respectively. Scale bars = 0.5 mm

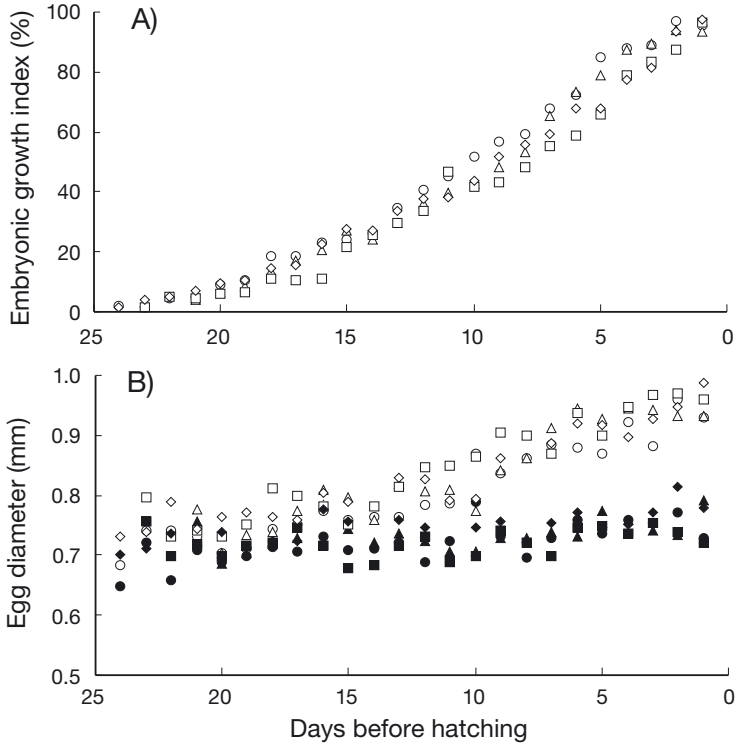

Fig. 4. Changes in (A) the embryonic growth index (EGI) and (B) egg diameter before hatching in 4 *Birgus latro* broods. The EGI represents the relative area of the embryonic body vs. the total embryo surface measured in lateral view of the egg. Egg diameter is shown as major axis (white symbols) and minor axis (black symbols) lengths. Different symbols indicate data from 4 different females

DISCUSSION

In the present study, the eggs of 6 terrestrial coenobitid species were incubated in seawater of different salinities (27 and 34 ppt) to avoid air desiccation during *in vitro* incubation, and they successfully developed. In a previous experiment, we successfully cultured *Birgus latro* embryos *in vitro* (34 ppt seawater) until hatching occurred, and the larvae were reared to metamorphosis into megalopae (Hamasaki et al. unpubl. data). These results indicate that coenobitid embryos can breathe in air and seawater, which has been demonstrated for some subtidal and semi-terrestrial brachyuran crabs by measuring embryo oxygen uptake in air and seawater (Cannicci et al. 2011, Simoni et al. 2011, 2013). We confirmed for *Coenobita brevimanus*, *C. cavipes*, and *C. violascens* that the developmental rates of the embryos were similar during the *in vitro* (with seawater) and *in situ* (with mothers) incubations under identical temperature conditions as in the present study, suggesting that coenobitid embryos may have similar physiological performance in air and seawater.

Changes in external morphology, including size increases in eggs during embryonic development of the 6 coenobitid species observed in the present study, were similar to those documented for many other decapod crustacean species (e.g. Nagao et al. 1999, Yamaguchi 2001, García-Guerrero & Hendrickx 2005, Stevens 2006), including *B. latro* in Vanuatu, South Pacific (Schiller et al. 1991). The increase in egg volume is attributed in part to slow but steady osmotic uptake of water (Davis 1968). Additionally, the egg volume increases sharply because of rapid embryonic growth after the metanaupliar stage in some decapod crustaceans (Furota 1996, Nagao et al. 1999, Hamasaki et al. 2003), and the EGI is about 15% when eggs of *Portunus trituberculatus* initiate a large increase in volume (Hamasaki et al. 2003), which is similar to the embryonic developmental profiles of the coenobitid crabs observed in the present study.

The present study demonstrated that temperature largely affected the rate of embryonic development in 6 coenobitid species, and the duration of embryo incubation decreased exponentially with increasing temperature. While this has been shown in many marine decapod crustaceans (e.g. Wear 1974, Hamasaki 2002, 2003, Hamasaki et al. 2003), this may be the first report demonstrating temperature-dependent embryonic development in terrestrial decapod crustaceans. In this study, we incubated embryos in seawater without aeration or water exchange. Oxygen is less soluble in warmer than in colder water and it could be depleted more rapidly in warmer water due to the higher metabolic rate of the embryo. This may affect embryonic development negatively at higher incubation temperatures. However, apparent negative effects, such as prolonged developmental periods of embryos, were not observed at higher temperatures in any species tested. Therefore, we believe that the culture methodology had little influence on interspecific comparisons of thermal adaptations of embryos in these 6 coenobitid species.

Nakasone (2001) documented that the breeding season based on the chronology of larval release and the occurrence of ovigerous females was similar in *C. cavipes* and *C. purpureus*, extending from late May to late August, but the season was somewhat longer in *C. rugosus* (late May to November) on Okinawajima Island and/or Kudakajima Island (26° 8–10′ N, 127° 45–54′ E), Okinawa Prefecture, Japan. Sato &

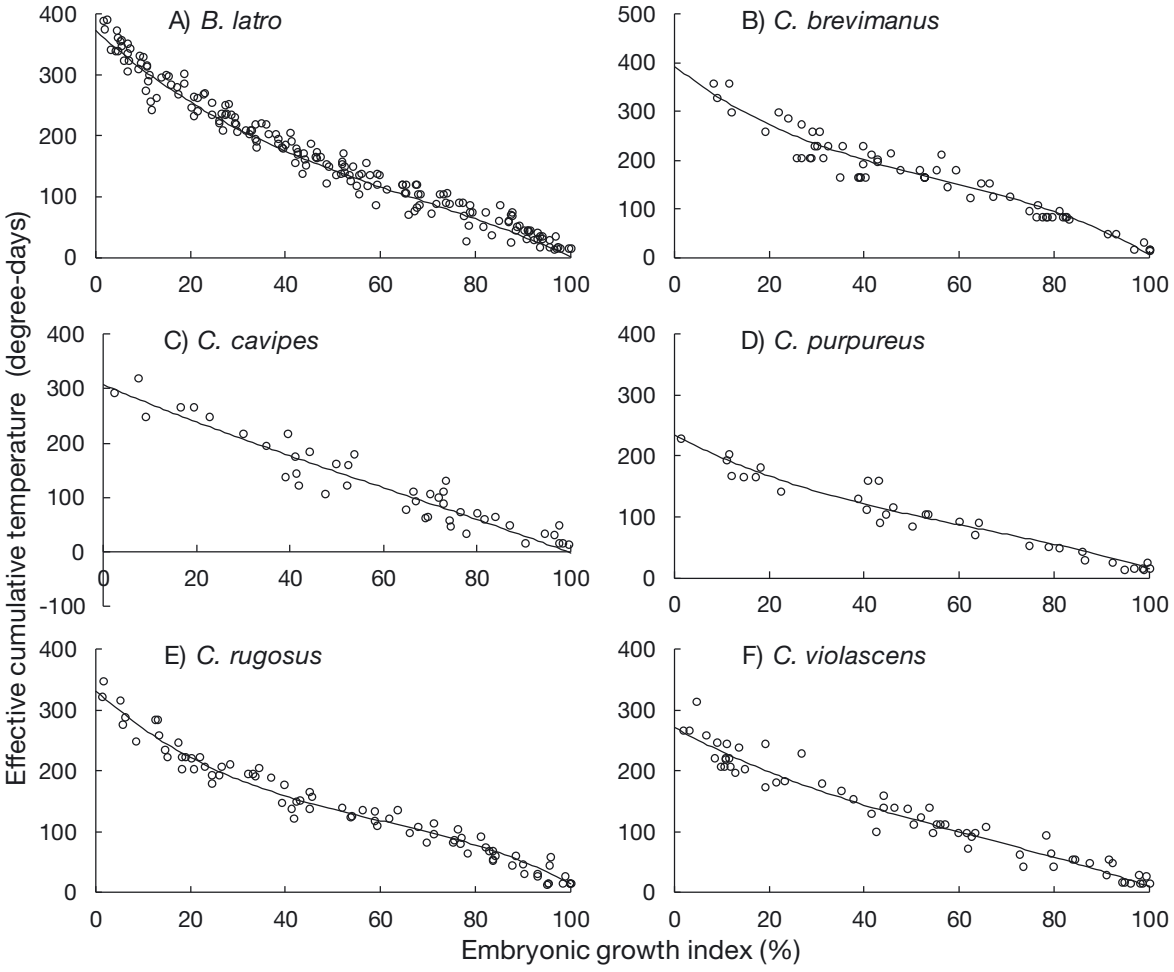

Fig. 5. Relationships between the embryonic growth index (EGI) and partial effective cumulative temperatures (ECT) from the day when EGI values were measured to the day of hatching in 6 coenobitids: (A) *Birgus latro*, (B) *Coenobita brevimanus*, (C) *C. cavipes*, (D) *C. purpureus*, (E) *C. rugosus*, and (F) *C. violascens*. The partial ECT values were calculated as the sum of the daily effective temperature for embryonic development (> lower threshold temperature). Regression curves were drawn from the cubic equations applied to the relationships between the EGI and ECT values as shown in Table 1

Yoseda (2008) reported that ovigerous *B. latro* females were found from early June to late August on Hatomajima Island (24° 28′ N, 123° 49′ E), Okinawa Prefecture. Monthly (10 d interval) mean temperatures at Naha (26° 12′ N, 127° 41′ E) on Okinawajima Island, the capital of Okinawa Prefecture, fluctuate from 24.5°C (late May) to 29.0°C (mid-July) and 28.6°C (late August) to 21.1°C (late November) and those on Ishigakijima Island (24° 20′ N, 124° 9′ E), near Hatomajima Island, range from 26.1°C (early June) to 29.4°C (late August) during the breeding seasons of coenobitids (www.data.jma.go.jp). Therefore, the incubation periods of coenobitid crab embryos vary with these temperature changes during the breeding season.

The heat summation theory equations fit the relationship between mean incubation temperature and the number of days the embryos were incubated until eye pigmentation appeared in the 6 coenobitid species. LTT estimates for embryonic development were slightly lower in *C. cavipes* (12.7°C) but higher in *C. purpureus* (14.5°C), compared with those of *B. latro*, *C. brevimanus*, *C. rugosus*, and *C. violascens* (13.5 to 13.6°C). Hamasaki et al. (2009) estimated a LTT of ~18°C for *B. latro* zoeal development. Thus, the LTT for development was lower for embryos than for zoeal larvae. This is a common finding because the embryonic developmental period precedes that of larvae during the reproductive season. It has been suggested that the LTT represents the degree of lower-temperature adaptation in ectothermic organisms. Honěk (1996) examined variation of the LTT estimates for 355 insect species in relation to their geographic origins, demonstrating that the LTT esti-

Table 1. Parameter estimates for the cubic equations describing the relationship between degree of embryonic development expressed by the embryonic growth index (EGI, %) and the effective cumulative temperature required until hatching (ECT, °D) in 6 coenobitid species in the genera *Birgus* and *Coenobita*: ECT = $aEGI^3 + bEGI^2 + cEGI + d$, where *a*, *b*, *c*, and *d* are parameters. N: number of observations; SE: standard error

Species	N	Parameter *a*			Parameter *b*			Parameter *c*			Parameter *d*		
		Estimate (SE)	*t*-value	p	Estimate (SE)	*t*-value	p	Estimate (SE)	*t*-value	p	Estimate (SE)	*t*-value	p
B. latro	180	-3.181×10^{-4} (4.517×10^{-5})	−7.042	<0.001	6.514×10^{-2} (6.847×10^{-3})	9.514	<0.001	−7.036 (0.292)	−24.111	<0.001	372.4 (4.6)	81.138	<0.001
C. brevimanus	62	-5.588×10^{-4} (1.112×10^{-4})	−5.025	<0.001	9.376×10^{-2} (1.902×10^{-2})	4.930	<0.001	−7.654 (0.998)	−7.672	<0.001	392.8 (15.9)	24.742	<0.001
C. cavipes	44	-6.266×10^{-5} (8.016×10^{-5})	−0.782	0.434	1.179×10^{-2} (1.349×10^{-2})	0.874	0.382	−3.638 (0.675)	−5.389	<0.001	307.1 (11.3)	27.266	<0.001
C. purpureus	33	-2.074×10^{-4} (9.994×10^{-5})	−2.076	0.038	3.988×10^{-2} (1.661×10^{-2})	2.401	0.016	−4.105 (0.766)	−5.361	<0.001	234.7 (9.8)	24.022	<0.001
C. rugosus	82	-4.822×10^{-4} (5.230×10^{-5})	−9.219	<0.001	8.693×10^{-2} (8.236×10^{-3})	10.555	<0.001	−7.032 (0.371)	−18.948	<0.001	331.5 (5.1)	64.516	<0.001
C. violascens	62	-1.723×10^{-4} (1.173×10^{-4})	−1.469	0.142	3.359×10^{-2} (1.849×10^{-3})	1.816	0.069	−4.242 (0.800)	−5.304	<0.001	270.8 (8.6)	31.568	<0.001

mates were high with little geographic variation in the tropics, and tended to decrease with increasing geographical latitude, although the scatter of data was large in subtropical to temperate regions. Though little is known about the LTT for embryonic development in decapod crustaceans, Hamasaki (2003) compared LTT estimates for embryos of 2 closely related mud crabs, *Scylla serrata* (Forsskål, 1775) (Hamasaki 2003) and *Scylla paramamosain* Estampador, 1949 (Hamasaki 2002). The results showed that LTT was higher in *S. serrata* (15.7°C), which mainly occurs in subtropical and tropical areas, than in *S. paramamosain* (14.0°C), which occupies the northern limit of the mud crab species distribution, extending into warm temperate areas. They suggested that *S. paramamosain* may be adapted to the lower-temperature region. However, the LTT for embryonic development in the present study did not appear to be related to the geographical distributions of the coenobitid species because the highest LTT estimate was determined for *C. purpureus*, which has the most northern geographical distribution, extending to the main island of Japan.

We also successfully estimated partial ECT from the appearance of the embryonic body to hatching in the 6 coenobitids; the estimates were higher in *B. latro* and *C. brevimanus*, followed by *C. rugosus*, *C. cavipes*, and *C. violascens*, and lower in *C. purpureus*. Wear (1974) suggested that interspecific variation in egg size affects embryonic development; larger egg size slows the developmental rate in closely related decapod species. Although significant interspecific differences were found in the egg sizes of the 6 coenobitids, as previously shown for *C. cavipes*, *C. purpureus* and *C. rugosus* by Nakasone (2001), egg size did not affect embryonic development, such as the LTT and ECT values. Hamasaki et al. (2016) examined the phylogenetic relationships among 7 coenobitid species based on 16S rDNA sequences. They found that *B. latro* branched off from the outer diogenids (sister marine hermit crabs) first, followed by *C. brevimanus*, and that the other species comprised 3 clusters: (1) *C. cavipes* and *C. perlatus* H. Milne-Edwards, 1837, (2) *C. violascens*, and (3) *C. rugosus* and *C. purpureus*. Bracken-Grissom et al. (2013) analyzed 2 mitochondrial and 3 nuclear markers as well as 156 morphological characters for 19 families, 77 genera, and 137 species, including *B. latro* and 3 *Coenobita* species, to reconstruct the evolutionary history of Anomura, and demonstrated that *B. latro* first evolved from sister diogenid species. Additionally, Hamasaki et al. (2014a) demonstrated that the larval morphology of *C. brevimanus* is more similar to that of *B. latro*

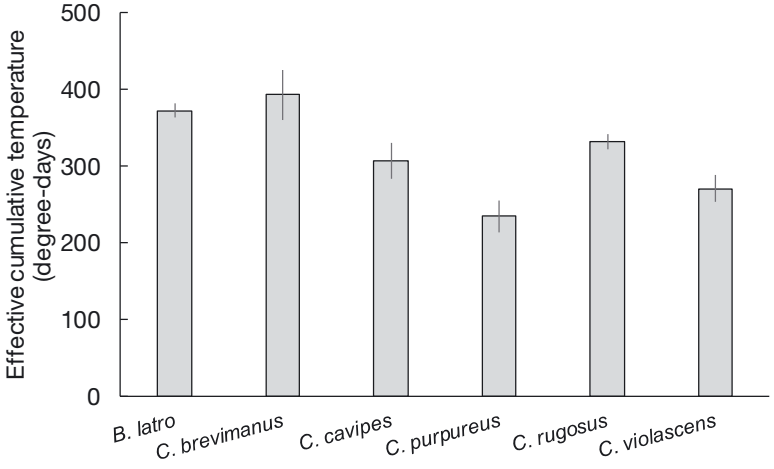

Fig. 6. Estimates of effective cumulative temperature (ECT) required from the appearance of the embryonic body to hatching in 6 coenobitids (*Birgus latro*, *Coenobita brevimanus*, *C. cavipes*, *C. purpureus*, *C. rugosus*, and *C. violascens*). Estimates are intercept values of the cubic equations applied to the relationship between embryonic growth index (EGI) and ECT shown in Table 1. Vertical bars: 95% CI of the estimates

Table 2. Mean dimensions of eggs within 1 d of hatching in 6 coenobitid species in the genera *Birgus* and *Coenobita*. N: number of different females examined; SD: standard deviation

Species	N	Major axis (mm)	SD	Minor axis (mm)	SD	Volume (mm^3)	SD
B. latro	8	0.991	0.050	0.788	0.047	0.320	0.058
C. brevimanus	4	0.919	0.028	0.743	0.017	0.266	0.018
C. cavipes	4	0.783	0.032	0.591	0.038	0.144	0.023
C. purpureus	4	0.890	0.038	0.687	0.028	0.221	0.028
C. rugosus	7	0.877	0.052	0.678	0.047	0.213	0.039
C. violascens	6	0.927	0.060	0.693	0.036	0.235	0.038

rather than other *Coenobita* spp. Thus, *C. brevimanus* is thought to have retained the ancestral traits of *B. latro*, which is considered the most ancestral species in the coenobitid phylogeny. Therefore, the ECT and egg incubation period estimates may be similar in *B. latro* and *C. brevimanus*. *C. purpureus* and other coenobitid crabs are abundant in the northern (>27° 19′–26′ N) and southern (<27° 01′–04′ N) Ryukyu Archipelago regions, respectively (Kagoshima Prefectural Board of Education 1987, Okinawa Prefectural Board of Education 1987, 2006). Hamasaki et al. (2016) suggested that an ancestral *C. purpureus* population may have been isolated in the Ryukyu region land masses during the Pliocene, considering the divergence time between *C. purpureus* and *C. rugosus* and the paleogeography of the Ryukyu Archipelago region. It has been inferred that *B. latro* females produce only one brood per reproductive season (Schiller et al. 1991, Sato & Yoseda 2008). Meanwhile, Nakasone (2001) reported that some *C. cavipes*, *C. purpureus*, and *C. rugosus* females produce at least 2 broods during the breeding season because ovigerous females with brooded embryos have mature oocytes simultaneously; moreover, some *C. rugosus* females, a species with a protracted breeding season, may produce a third brood. Therefore, it can be assumed that a reduction in the ECT values and incubation periods for *C. cavipes*, *C. purpureus*, and *C. rugosus* embryonic development may be an adaptive trait to produce more than one brood by accelerating the embryonic development rate during the reproductive season. A higher ECT value and longer egg incubation periods of *C. rugosus* among these 3 species may also relate to its protracted breeding season. In particular, a lower ECT values and shorter egg incubation periods in *C. purpureus* may be advantageous to produce broods during the shorter summer that occurs in the northern Ryukyu Archipelago region (see Fig. S7 in the Supplement for fluctuations in monthly [10 d interval] mean temperatures at 3 locations from the southern, middle, and northern Ryukyu Archipelago regions).

Little is known about the egg incubation periods of coenobitid crabs. Schiller et al. (1991) attempted to culture *B. latro* females with freshly extruded eggs in enclosures to monitor egg development, but all animals aborted their eggs. Alternatively, Schiller et al. (1991) estimated that the egg incubation period is 25 to 45 d (majority: 27 to 29 d) using radio-tracking and mark–recapture techniques. It has also been estimated that wild *Coenobita clypeatus* (Fabricius, 1787) incubate their eggs for 3 to 4 wk until hatching (de Wilde 1973). The cubic equations used to apply the relationships between the EGI and ECT values for the 6 coenobitid species provided the egg incubation periods from the appearance of the embryonic body to hatching as shown in Fig. S5 in the Supplement. These equations can also be used to estimate the hatching periods by measuring the EGI values and using the *in situ* temperature data from natural and laboratory settings. Further studies that examine the duration of the egg cleavage stage would be required to estimate the entire egg incubation period from egg laying to hatching in coenobitid crabs.

Our results suggest that coenobitid embryos have adapted to thermal conditions within an evolutionary constraint and/or in terms of an ecological trait. The reproductive biology and ecology of other species, such as *C. brevimanus* and *C. violascens*, should be investigated to explain the interspecific variations of the thermal adaptations in coenobitid crabs. Additionally, the methodological approach employed in the present study, i.e. estimates of LTT and ECT required for embryonic development based on *in vitro* and *in situ* culture experiments, would also be effective to examine thermal adaptations of the embryos of threatened crustacean species, of which available numbers of test animals are limited.

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