

Population Dynamics and the Effects of Temperature on the Eggs of the Seawater-dispersed Stick Insect *Megacrania tsudai* (Phasmida: Phasmatidae)

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Shun Kobayashi, Ryota Usui, Kouta Nomoto, Mineyuki Ushirokita, Tetsuo Denda, and Masako Izawa (2016) Although the stick insect *Megacrania tsudai* cannot fly, its eggs are dispersed by seawater, which allows the species to distribute itself widely via the ocean. The life history of this non-volant insect that establishes settlements by egg dispersal remains poorly understood. We aimed to clarify the population dynamics and effects of temperature on the eggs of *M. tsudai* through field observation in near the northern limits of the distribution and laboratory experiments. In the wild, all instars appeared over a period of several months, with a larger proportion of first instars emerging from December to March, and a greater number of adults being observed from June to August. Laboratory experiments showed that the hatching rate of *M. tsudai* was not different between conditions of 25 and 30°C, whereas it was significantly lower at 20°C. The egg-development period was shorter when eggs were maintained under high temperature conditions, and longer for eggs kept at 20°C. The effective cumulative temperature was estimated as 1561.5-2000.0°C, and the developmental zero was estimated as 12.2-13.9°C. The peak appearance of the first instar in the laboratory, which was used to obtain an estimate for the effective cumulative temperature and peak appearance of adults in the wild, was not entirely accurate because the peak appearance of the first instars in the wild was not consistently observed when the estimation indicated that they should appear. *Megacrania tsudai* is a univoltine near the northern limit of its distribution, and temperature has a strong effect on its egg development. Our estimation of population dynamics by laboratory experiments did not exactly predict what was observed in the field and it may be controlled by other factors. However, our findings indicate that the limiting factor of the distribution of this species is likely the effect of temperature on egg hatching.

Key words: Developmental zero, Hatching rate, *Megacrania tsudai*, Non-volant, Northern limit, Population dynamics, Seawater dispersal, Temperature.

BACKGROUND

Temperature affects the life history of many animals, and in particular, insects (Speight et al. 1999; Gullan and Craston 2010). Many current previous studies that focused on how temperature

affects insects have aimed to either predict the population dynamics of insects that are agricultural pests or helpers, or estimate the ability of an insect to adapt to climate change (e.g., Porter et al. 1991; Bale et al. 2002; Thomson et al. 2010). Predictions that combined data from both field observation

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and laboratory experiments are lacking, and have only been conducted in species of importance for agriculture, such as the diamondback moth or lady beetle (e.g., Yamada and Kawasaki 1983; Xia et al. 2003). Studies on the unique responses of other insects to temperature changes are needed, as this information is needed to understand the life history of each species.

The genus *Megacrania* (Phasmatidae) is primarily distributed in tropical regions (Hsiung 2007), and *Megacrania tsudai* occurs in the subtropical region, with its northernmost borders formed by the Hengchun Peninsula, Lyudao and Lanyu islands in Taiwan, and Iriomote-jima, Ishigaki-jima, and Miyako-jima islands in the Ryukyu Archipelago, Japan (Azuma et al. 2002; Liu 2002) (Fig. 1). The species feeds on the leaves of the screw pine *Pandanus odoratissimus* L. f., which grows in coastal forest. The populations of *M. tsudai* are small and currently protected in Taiwan (Council of Agriculture, Executive Yuan, R.O.C. 2015), as well as listed as Near Threatened in the Red List in Okinawa, Japan (Okinawa Prefectural Government 2005).

Megacrania tsudai has two pairs of wings but lacks the ability to fly, making dispersion across an ocean difficult. However, because of its parthenogenetic reproductive system (Yamasaki 1991), the special structure of the eggs that enables them to float on water (Wang and Chu

1982), and the tolerance of the eggs to seawater (Ushirokita 1998; Kobayashi et al. 2014), this species can disperse across oceans via its eggs. Wu et al. (2012) support these claims. In order to understand the life history of this insect with such a special dispersal pattern, its response to temperature, an important environmental factor, must be clarified.

The first objective of this study is to clarify the population dynamics on Iriomote-jima Island near the northern limits of the distribution of this species. The second aim is to identify the effect of temperature on egg hatching. Thirdly, the appearance pattern in relation to environmental temperature will be calculated. Finally, we discuss the landing and settlement process in relation to the limiting effects of temperature on the distribution of *M. tsudai*.

MATERIALS AND METHODS

Route census

A route census was conducted more than two times per month from January 2011 to February 2013 to determine the appearance pattern of *M. tsudai* on Iriomote-jima Island in the Southern Ryukyus (S1 in Fig. 1). The census was conducted along a 210-m route that extended from the seashore to a coastal forest. Approximately 43% of this route was covered by *P. odoratissimus*. The census started at 1-2 h after sunset. We counted the number of individuals and measured their body lengths during each census, after which the insect was released at its capture point. A data logger (CO-U23-001, Onset, MA, USA) recorded hourly measurements of temperature and humidity at the study site from February 2012 to January 2013.

Temperature control experiment during the egg-development period

An egg-hatching experiment was conducted from 2011 to 2013 at the University of the Ryukyus on Okinawa-jima Island. The temperature in the laboratory was 26-28°C, and the day length was 12 h (0600-1800). Seven adults collected on Iriomote-jima Island from 1998 to 2010 had been maintained at the University of the Ryukyus and were used as the source of eggs for this experiment. The individuals from these eggs were reared together until eclosion, and then they were maintained individually in plastic cages (410 mm ×

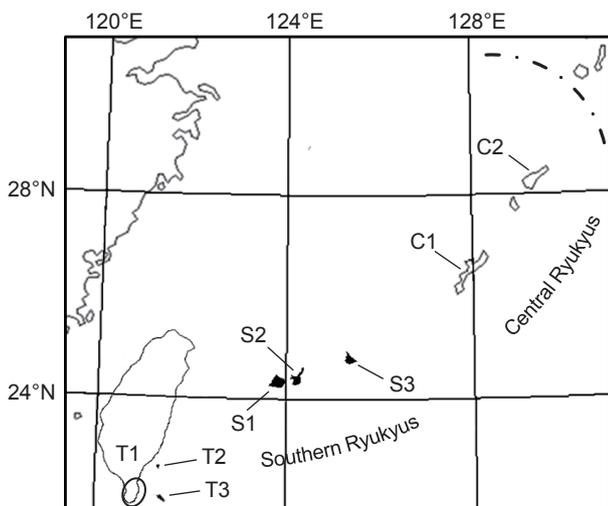


Fig. 1. Map of the distribution of *Megacrania tsudai* and its food plant *Pandanus odoratissimus*. *Megacrania tsudai* is distributed in T1 to T3 and S1 to S3. T1: Taiwan, T2: Lyudao Island, T3: Lanyu Island, S1: Iriomote-jima Island, S2: Ishigaki-jima Island, S3: Miyako-jima Island, C1: Okinawa-jima Island, and C2: Amami-Oshima Island. The dashed line shows the northern limit of *P. odoratissimus* (Nakanishi 1991).

310 mm × 550 mm) made of 3-mm mesh. Screw pine leaves were collected on Okinawa-jima Island, and the insects were fed fresh leaves every 4 days.

Eggs were collected from the adults every day. Individual eggs were placed on wet cotton swabs in a plastic case (30 mm × 30 mm × 15 mm) with holes for aeration. The cases were incubated (LP-30CCFL-8CTAR, Nippon Medical & Chemical Instruments Co., Ltd, Japan) at 20, 25, or 30°C for 12 h each day (0600-1800). Every day, the egg condition was checked and the eggs were sprayed with fresh water. Eggs incubated at 25 and 30°C were considered dead if they had not hatched at 365 days after being laid. Several of the eggs incubated at 20°C hatched after 365 days; therefore, eggs incubated at 20°C were considered dead if they had not hatched at 550 days after being laid. The 550-day limit was based on 100 days after the last egg hatched. The unhatched eggs were dissected to determine whether they were alive or dead. The number of days required for egg development (egg-development period), from oviposition to hatching, was used in subsequent analyses. All statistical analyses were performed using the R ver. 2.15.2 software package.

Egg-development period and estimation of the hatching season in nature

The results from the temperature experiment and route census enabled us to estimate the hatching season and appearance pattern in nature. To estimate the hatching season, we calculated the parameter for the rate of egg development using the law of total effective cumulative temperature. We used two equations: one is the traditional equation,

$$D(T - t) = k \quad (1),$$

and the other is an equation proposed by Ikemoto and Takai (2000),

$$DT = k + tD \quad (2),$$

where D is the egg-development period, T is temperature (°C), t is developmental zero temperature (°C), and k is the effective cumulative temperature (°C). The rate of egg development per day ($1/D$) at each temperature (20, 25, and 30°C) was estimated using both equations. Equation (2) is the deformation equation of equation

(1). However, equation (2) has remedied the problem that estimated error becomes high in low temperatures, which results in poor estimates of t and k in cold temperature experiments (Ikemoto and Takai 2000).

To estimate the appearance pattern of *M. tsudai* in nature, we used air temperatures recorded by the data logger. Each month was divided into three periods of approximately 10 days (early, middle, and late), and the mean 10-day temperature (T) of each period was calculated. The developmental rate for each 10-day period was calculated as $(1/D) \times 10$. The hatching time was estimated to be the point when the cumulative value of $[(1/D) \times 10]$ from the time of egg laying exceeded 1. We calculated the egg-development period and hatching season in nature using this equation.

In addition, the developmental zero temperature was calculated using both equations: t_1 was calculated from equation (1), and t_2 was calculated from equation (2) in order to determine the limiting factor on the distribution of this species. We calculated the number of days that the temperature was below the developmental zero temperature on three islands: Iriomote-jima Island (Fig. 1, S1), where *M. tsudai* lives; Okinawa-jima Island (Fig. 1, C1), in the Central Ryukyus; and Amami-Oshima Island (Fig. 1, C2), which is near the northern limit of *P. odoratissimus*. *Megacrania tsudai* does not occur in either of the latter two islands. For the analyses, we used the daily mean temperature from 2001 to 2010 from the recording stations of the Japan Meteorological Agency (Japan Meteorological Agency 2015) at Iriomote-jima Island, Naha (Okinawa-jima Island), and Naze (Amami-Oshima Island).

RESULTS

Pattern of instar appearance in nature

This species is reported to molt six times (Ushirokita 1998; Wu et al. 2011), and therefore we determined the range of body lengths for each instar. Seven clusters of body lengths were observed in the body-length data measured in the field (Table 1). Based on the data from Table 1, the monthly change in appearance of each instar was obtained (Fig. 2). First instars appeared from December to May, and were not observed from June to September. In contrast, adults (seventh instar) primarily appeared from June to August, but

were observed in all months except for January and April during the 2-year survey. In 2012, the appearance of a peak of the second- to seventh-

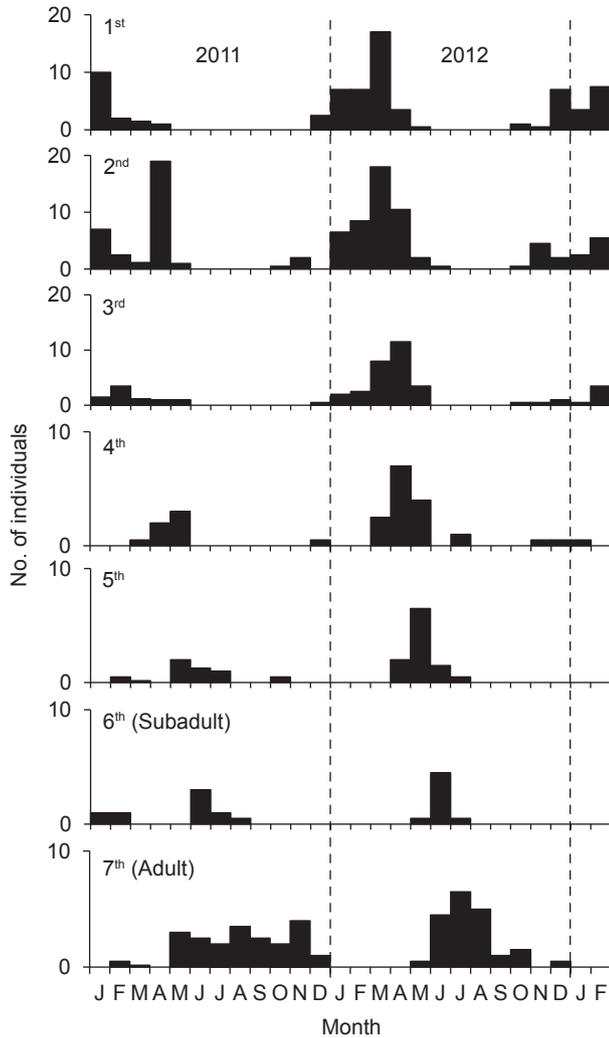


Fig. 2. Monthly appearance of each instar on Iriomote-jima Island.

instar individuals was observed one month after the appearance of the peak of the previous instars. In 2011, appearance patterns were similar to those in 2012, but the peaks for appearance were indistinct; a large number of the second instars suddenly appeared in April, and adults appeared consistently from May to November.

Hatching ratio and egg-development period

Seven adults laid 956 eggs during the egg hatching experiment, and we obtained egg-laying dates and hatching or death dates for 928 of those eggs. Each adult laid 1.0 ± 0.2 (Mean \pm SD) eggs per day. A total of 33-228 eggs were laid per adult, indicating a large variation in the number of days that each of the adults oviposited. One adult (N1 in Table 2) was omitted from the analyses because only two of her eggs hatched: one at 20°C and the other at 25°C.

The hatching rate was significantly different among adults (Fisher’s exact test, $p < 0.05$) (Table 2). Therefore, we compared the hatching rate among the three temperatures for each adult. The hatching rate of eggs at 20°C was significantly lower (Fisher’s exact test, $p < 0.05$) than at 25 and 30°C for four of the adults (indicated by * in Table 2), but was not significantly different among temperatures for the other three adults (Fisher’s exact test, $p > 0.05$). The combined hatching rate for eggs from all of the adults was significantly lower (Fisher’s exact test, $p < 0.05$) at 20°C than at 25 and 30°C.

No significant difference was observed in the egg-development period among eggs between the adults for any of the three temperature conditions (Kruskal-Wallis test, $p > 0.05$). However, for all adults combined, the egg-development period at 20°C was 260.0 ± 3.1 (183-449) days (Mean

Table 1. Body length of each instar

	1st	2nd	3rd	4th
n	153	153	76	47
Mean \pm SD	30.0 ± 1.8	39.7 ± 1.9	49.5 ± 2.1	60.6 ± 2.0
Range of body length (mm)	BL < 35	$35 \leq$ BL < 45	$45 \leq$ BL < 55	$55 \leq$ BL < 67
	5th	6th	7th (Adult)	
n	43	44	135	
Mean \pm SD	76.6 ± 3.9	94.5 ± 2.5	114.8 ± 5.3	
Range of body length (mm)	$67 \leq$ BL < 87	$87 \leq$ BL < 100	$100 \leq$ BL	

± SE (Min-Max)), which was significantly longer than at 25°C (121.2 ± 1.2 (84-213) days) and at 30°C (109.8 ± 1.1 (91-182) days) (Scheffe's test, $p < 0.001$) (Fig. 3). The longest egg-development period was observed at 20°C, followed by 25°C. Furthermore, a much longer egg-development period was observed at all temperatures when data for eggs from all adults were combined (Fig. 3). These eggs were observed without regard to individuals. The effective cumulative temperature and developmental zero temperature were 2000.0 and 12.2°C, respectively, based on (1), and 1561.5 and 13.9°C based on (2). The developmental zero temperature calculated by these two equations had a 1.7°C difference.

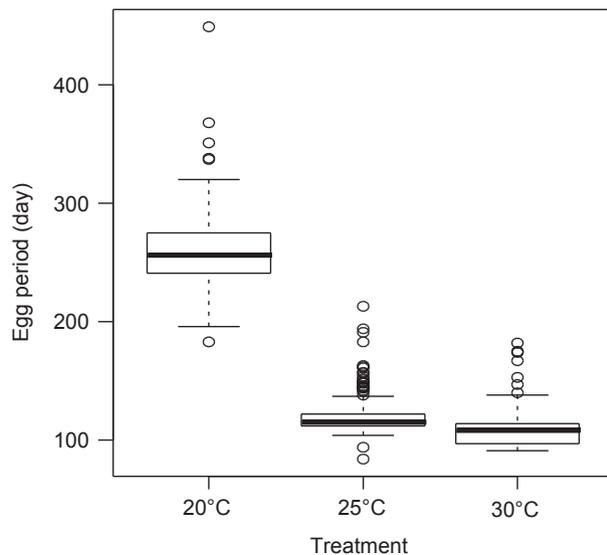


Fig. 3. The egg-development period according to temperature conditions. Boxes indicate the median with first and third quartiles. Whiskers indicate data points located within a range of 1.5 times the respective interquartiles. Open circles indicate outliers.

Egg-development rate

The annual mean temperature at the study site on Iriomote-jima Island was 23.5°C and the annual mean relative humidity was 87.5%. The lowest monthly mean temperature (17.8°C) was observed in January, and the highest was in July (Fig. 4). This pattern is the same as shown by the data collected from 2001 to 2010 and recorded by the Japan Meteorological Agency, except for 2008, when the lowest monthly mean temperature was observed in February. Relative humidity was constantly > 70%, except for 10 days (October-April).

After dividing the months into early, middle, and late periods, the lowest 10-day mean temperature (T) was recorded in the middle of February (17°C), and the highest was recorded in the middle of July (29°C). We calculated $(1/D) \times 10$ from these temperature records, and figure 5 shows the estimated egg-development period for each month. When estimated using equation (1),

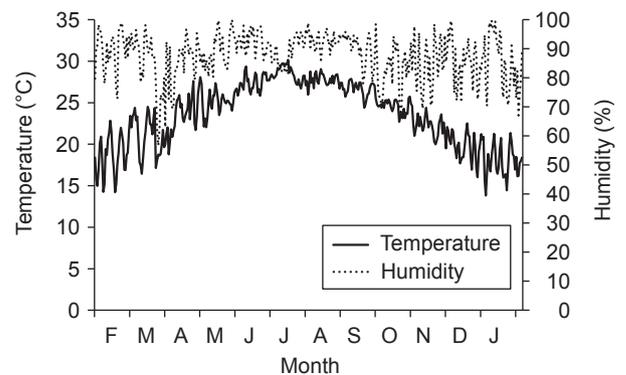


Fig. 4. Daily mean temperature and humidity in the *Pandanus odoratissimus* dominated coastal forest on Iriomote-jima Island.

Table 2. Hatching rate (%) for each treatment. Number in parentheses is the sample size

Female ID	20°C	25°C	30°C
D1*	48.0 (50)	83.3 (48)	80.4 (51)
N1	9.1 (11)	9.1 (11)	0.0 (11)
N6*	35.0 (40)	67.5 (40)	67.5 (40)
N8	21.3 (47)	34.0 (47)	37.8 (45)
N11	22.7 (22)	31.8 (22)	47.6 (21)
N17*	60.6 (66)	73.9 (69)	61.8 (68)
N19*	54.8 (73)	78.4 (74)	76.4 (72)
Total*	43.3 (309)	64.3 (311)	62.3 (308)

*Hatching rate was significantly lower in 20°C condition (Fisher's exact test; $p < 0.05$).

eggs deposited from September through October would have the longest egg-development period (approximately 230 days), and eggs deposited from April to June would have the shortest egg-development period (approximately 140 days) (Fig. 5a). When estimated using equation (2), the eggs laid from September to November would have the longest egg-development period (approximately 220 days), and eggs laid in May through June would have the shortest egg-development period (approximately 120 days) (Fig. 5b). The estimations differed by 10-20 days between equations (1) and (2), although the longest and shortest egg-development periods were observed in similar seasons. The fluctuation among the nearest plots in figure 5 was smaller in equation (2) than in equation (1).

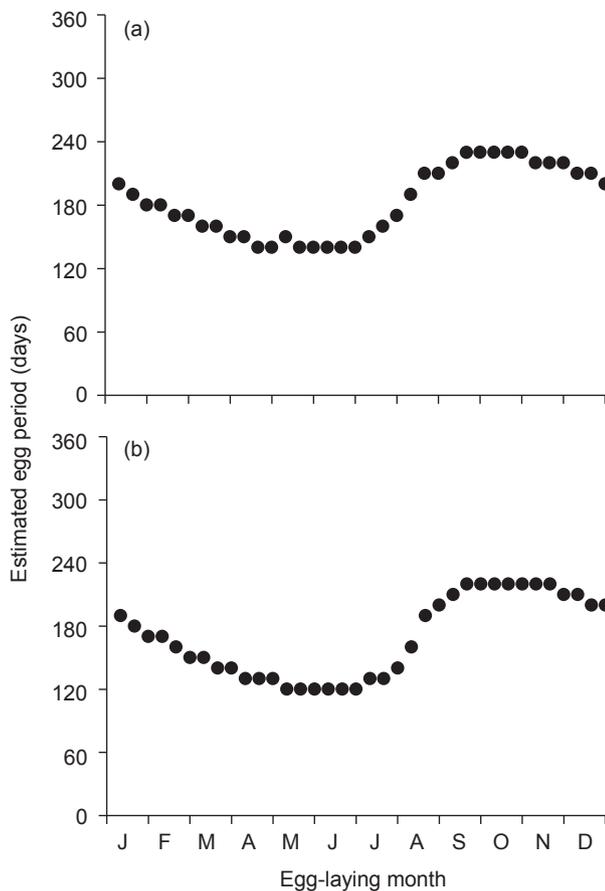


Fig. 5. Estimated egg-development period. (a) Estimated by equation 1: $D(T - t) = k$; (b) Estimated by equation 2 (Ikemoto and Takai 2000): $(DT) = k + tD$.

Temperature on the three islands

The number of days per year during the period from 2001 to 2010 when the daily mean temperature and daily lowest temperature were below t_1 (12.2°C) and t_2 (13.9°C) on Iriomote-jima, Okinawa-jima, and Amami-Oshima islands is shown in figure 6. On Iriomote-jima Island (where *M. tsudai* is found), the daily mean temperature was below t_1 for 0.4 days per year and below t_2 for 2.7 days per year. On the same island, the daily mean temperature did not decrease below t_1 for 6 years and did not decrease below t_2 for 1 year. However, the daily mean temperature decreased below the developmental zero temperature for some year on the Okinawa-jima and Amami-Oshima islands (areas where *M. tsudai* does not live). On Okinawa-jima Island, the daily mean temperature was below t_1 for 1.0 day per year and below t_2 for 6.1 days per year. Furthermore, the number of days when the daily mean temperature was below these developmental zero temperatures dramatically increased on Amami-Oshima Island and was 7.0 days per year for t_1 and 27.8 days per year for t_2 .

DISCUSSION

Life history of *Megacrania tsudai*

Field observations revealed that *M. tsudai* is univoltine and hatches in the winter, with adults appearing in the summer in near the northern limits of its distribution. The population dynamics of phasmid species in the Ryukyu Archipelago

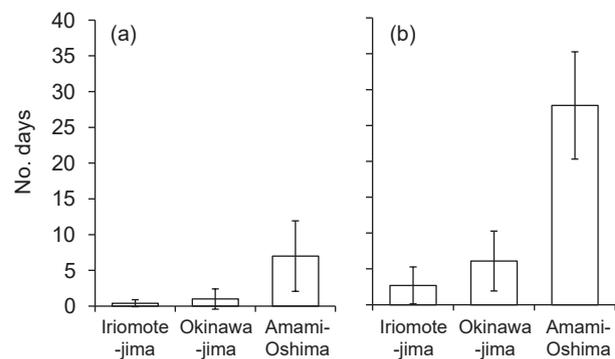


Fig. 6. Number of days below the developmental zero temperature per year on three islands. (a) Number of days when the daily mean temperature was below 12.2°C; (b) Number of days when the daily mean temperature was below 13.9°C. Whiskers indicate standard deviation.

are not well studied, but several species such as *Entoria okinawaensis* and *Sipyloidea sipylus*, which live in many types of the vegetation including the coastal forest, have been observed in all seasons (Fukuda et al. 2009). Therefore, even in a similar environment, there are both seasonal and non-seasonal phasmid species in the Ryukyu Archipelago. Adult *M. tsudai* appeared from winter to summer, which means that the egg stage is from summer to winter. An average of 7.4 typhoons passed near the Ryukyu Archipelago from April to December (the majority occurring in August and September) during the past 50 years (1965-2014) (Japan Meteorological Agency 2015). During a typhoon, the habitat of *M. tsudai* can be affected by strong winds and seawater, and therefore being in the egg state, which is tolerant of seawater (Ushirokita 1998; Kobayashi et al. 2014) during typhoon season (summer to winter) may be of benefit to this species.

The temperature control experiment showed that the hatching rate of *M. tsudai* decreased with low temperatures. Kiritani (1997) suggested that most insects originating in tropical regions do not have a mechanism for diapause, an adaptation that permits tolerance to low temperatures. As the genus *Megacrana* originated from a tropical region (Hsiung 2007), *M. tsudai* likely does not have a diapause mechanism.

The mean egg period was 121 days under 25°C conditions, whereas some individuals had a longer egg period and larger variation in length of egg period. Bedford (1976) studied the hatching rate and egg period of two non-diapause stick insects, *Eurycantha calcarata* and *Anchiale maculata*, in New Guinea. Their hatching rates were 50% and 77%, respectively, and mean egg periods were 101 days and 114 days, respectively, under room temperature conditions (21-35°C). In addition, Bedford (1976) showed that some eggs of both species had an egg period of over 180 days. Eggs of some phasmids (including diapause species) were reported to have the longest egg periods from several to 30 months (reviewed by Bedford 1978). Thus, the long egg period and large variation in egg period of *M. tsudai* is a common characteristic in Phasmida.

We calculated the population dynamics of *M. tsudai* using laboratory experiments. The estimated hatching season for eggs deposited in August coincided with the actual appearance of the first instar in the wild, whereas the hatching season for eggs deposited in June and November did not agree with the predicted season. Considering

that the egg-laying period of a female was long and the egg-development period showed wide variation (this study; Kobayashi et al. 2014), all instars in fact appeared throughout the year. This finding suggests that other factors, in addition to temperature, affect seasonal prevalence. The following four hypotheses are possible reasons for variations in seasonal prevalence in regions of recent settlement: (1) A lower hatching rate is present in overwintering eggs. When eggs are laid in the winter or autumn, the insect spends the season with the lowest temperatures as an egg, which leads to many eggs dying from exposure to cold temperatures. (2) Day length affects hatching. Some insects, and potentially *M. tsudai*, use day length to determine the timing of hatching (e.g., Danilevsky 1965; Masaki 1972; Speight et al. 1999). (3) Natural events may affect hatching. The coastal environment is strongly affected by typhoons and other natural disturbances (McLachlan and Brown 2006), which can wash away the eggs and cause mortality among the nymphs. (4) A change in predator may affect survival. The survival rate of eggs and nymphs is highly affected by predators (Hairston et al. 1960), and if the density and species of predators changes, then the total survival rate of the species is also affected, along with predation pressure on each life stage. Furthermore, changes in predation pressure can cause seasonal changes. Further research is needed to determine which, if any, of these hypotheses are plausible explanations for the population dynamics of *M. tsudai*.

Predicting the limiting factor of distribution

Ushirokita (1998) and Kobayashi et al. (2014) found that *M. tsudai* expands its distribution through the dispersal of eggs by seawater, which is a rare phenomenon among insects. Because Kobayashi et al. (2014) have already described the dispersal and drift processes, we attempted to determine the course of settlement for this species. In this study, we also examined the possibility of *M. tsudai* settling in the Central Ryukyus.

First, we examined the opportunities to drift ashore and the distribution range of the food resources of *M. tsudai*. One factor that may potentially affect the likelihood of landing on an island is either or both the size and coastal length effect (MacArthur and Wilson 1967). However, even though there are 16 islands larger than the smallest island on which *M. tsudai* is distributed in the Southern and Central Ryukyus (The

Geospatial Information Authority of Japan 2014), *M. tsudai* is absent from these bigger islands. Therefore, the difficulty of drifting ashore and size of the target location may not limit the distribution of this species. Furthermore, the food of *M. tsudai*, the screw pine, also disperses via seawater and is widely distributed in the tropical and subtropical zones, with a range that extends far north of the distribution of *M. tsudai* (Nakanishi 1988; 1991) (Fig. 1). Therefore, the distribution and availability of food does not appear to limit the distribution of *M. tsudai*.

Another factor affecting the settlement of this species is that even though eggs can be dispersed to the islands in the Central Ryukyus, the eggs or nymphs may not be able to survive in that specific region. To understand this, we must first examine the effect of temperature on egg hatching. As seen in many other insects (e.g., Howe 1967; Ratte 1985), the hatching rate decreases and the egg-development period lengthens in *M. tsudai* under low temperature conditions. The number of days per year in which the temperature fell below the developmental zero temperature was significantly less on Iriomote-jima Island (where this species is present) than on Okinawa-jima and Amami-Oshima islands (where *M. tsudai* is not found), and that number increased in the higher latitude regions. Although not all eggs stop developing at lower temperatures, even when temperatures fall below developmental zero (e.g., Wagner et al. 1984; Liu et al. 1995), the lack of adaptation to cold temperatures may limit the settlement of this species in higher latitude regions. In this regard, however, additional field survey is needed.

However, even if the egg can hatch in its new territory, the species will not successfully establish itself if the nymph is unable to survive. For example, the climate and predators are both factors that can inhibit the settlement of nymphs. Several reports show that the developmental period of the nymph is affected by climate, and especially temperature (e.g., Champlain and Butler 1967; Tu et al. 2012). The average nymph period (first to sixth instar) surveyed in laboratory was 173 days under L:D (12 h:12 h; 25°C:25°C) conditions (Wu et al. 2011), and 169 days under L:D (16 h:8 h; 25°C:20°C) conditions (Ushirokita 1998). Both studies showed that the total nymph period was 5–6 months, which is consistent with the appearance peaks of the first to sixth instars in the wild in this study. However, the average temperature at the peak of the first instar (March) to the sixth instar (June) did change, and ranged from 21 to 27°C

in 2012 in the field, and the range of temperature was not much difference in the experimental conditions in previous studies. In this way, different stages were influenced by varying temperature conditions in the wild. Thus, additional research is needed to clarify the effect of temperature on the development speed of each instar of nymph. The second factor potentially affecting the ability of a nymph to establish itself in a new territory is predators or parasites. *Megacrania tsudai* uses a defensive secretion to avoid predation and parasites (Chow and Lin 1986; Ho and Chow 1993). Although the target predator of this species is unknown, mammals, birds, insects, and spiders are plausible predators and insects such as wasps or the horsehair worms parasitize in other phasmid species (reviewed by Bedford 1978). These animals are found in the distributed islands of *M. tsudai*, but it does not appear that they have larger abundance on islands where the species is not distributed as compared to islands inhabited by *M. tsudai*. The density may be different among the islands, but it is not likely that predators or parasites are factors limiting the ability of nymphs to inhabit high latitude regions. However, further research is needed to determine whether overall nymph survival is a limiting factor in the distribution of this species.

CONCLUSIONS

This study clarified the parts of the life history of *Megacrania tsudai*, which is non-volant but disperses its eggs via seawater. The population of *M. tsudai* on Iriomote-jima Island showed a clear seasonality, and thus this species may be univoltine. In laboratory experiments, temperature strongly affected egg development; low temperatures caused decreased hatching rates and longer egg periods. These characteristics are considered to be common in Phasmids. The results suggest that *M. tsudai* cannot hatch in low temperature regions and that temperature can be one of the important limiting factors of the distribution of this species. We estimated the seasonal appearance pattern of this species from the results of temperature control experiments and temperatures in the wild. However, there was some difference between estimation and field observation, which suggests that the population dynamics of this species are controlled not only by temperature, but also by other factors.

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