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ORIGINAL RESEARCH PAPER

Geographical variation in diapause development in eggs of *Sympetrum frequens* (Odonata: Libellulidae)

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Abstract Geographical differences causing variations in the egg period and the effects of environmental factors on diapause development were examined in *Sympetum frequens* (Selys), a univoltine species with an obligatory egg diapause for overwintering. Eggs were obtained from females collected from 11 localities in Japan and incubated under six different combinations of photoperiod and temperature. No clear geographical trends were found in the average egg period under any experimental treatment. Average hatch period (i.e., period from the date when 10 % of the eggs were hatched to the date when 90 % of the eggs were hatched) did not display any geographical trend at 25 and 20 °C. However, at 15 °C, a significant negative correlation was observed between the hatch period and the latitude of the collection site. Similarly, a significant correlation was also detected between the coefficient of variation in the egg period and the average annual temperature near the collection site, but only at 15 °C. Because each egg batch was divided into six groups which were then incubated under different experimental conditions, it was possible to discern that the rate of diapause development at 15 °C varies among eggs from southern populations. The large variations in the egg period in the southern populations at 15 °C were considered to be a risk-spreading strategy: a certain proportion of the eggs were able to maintain diapause until winter, even if the adults laid the eggs early in the season. These differences in the rate of diapause development within a population may be an adaptation to the unpredictable length of the summer–autumn period.

Keywords *Sympetrum frequens* · Egg diapause · Diapause development · Temperature · Geographical variation

Introduction

Diapause induction and intensification are followed by a period of diapause maintenance (Tauber et al. 1986). During the diapause maintenance period, diapause development—a physiological process—progresses toward the end of diapause; the intensity of the diapause gradually decreases (Andrewartha 1952; Danks 1987; Hodek 1996, 2002; Košťál 2006; Lees 1955; Tauber et al. 1986). Upon completion of diapause development, insects resume morphogenesis or reproduction. In nature, diapause for overwintering is usually terminated before the arrival of spring. This is because diapause development can progress at temperatures lower than those of the developmental threshold for non-diapause development (Hodek 1996, 2002; Košťál 2006). Therefore, insects usually resume morphogenesis or reproduction as the temperature increases in spring, enabling them to synchronize the life cycles within a population (Danks 1987; Hodek 2002).

The environmental regulation of diapause development has been investigated in several members of Odonata that have univoltine life cycles with an obligatory egg diapause (see Corbet 2004). Low temperatures facilitate diapause completion (Corbet 1956; Sawchyn and Church 1973; Schaller 1968). Temperature and photoperiod have both been shown to affect the maintenance and termination of diapause in some species (Corbet 2004). For example, the egg periods in *Lestes disjunctus* Selys and *L. unguiculatus* Hagen are prolonged by photoperiods shorter than 12–14 h (Sawchyn and Church 1973).

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Table 1 Collection site of *Sympetrum frequens*

Collection site	Latitude/longitude	Approximate altitude (m)	Date	Number of egg batches	Average annual temperature (°C)
Engaru	44.0°N, 143.3°E	140	August 30, 2010	6	5.8
Sapporo	43.0°N, 141.2°E	210	August 31, 2010	9	8.9
Ohnuma	42.0°N, 140.6°E	180	August 22, 2012	5	9.1
Aomori	40.8°N, 140.5°E	70	October 11, 2008	8	10.3
Iwate	39.7°N, 140.9°E	300	September 16, 2009	5	9.4
Niigata	38.0°N, 139.1°E	60	September 27, 2012	4	12.2
Nagano	36.1°N, 137.9°E	680	September 18, 2011	8	11.1
Shiga	35.3°N, 135.9°E	450	October 19, 2012	6	14.4
Hiroshima	34.4°N, 132.9°E	330	October 12, 2009	7	14.3
Kochi	33.5°N, 133.5°E	10	November 4, 2010	8	16.7
Kumamoto	32.7°N, 131.0°E	440	October 17, 2009	12	16.1

Temperature data were obtained from the Japan Meteorological Association (http://www.data.jma.go.jp/obd/stats/etrn/index.php)

Sympetrum frequens (Selys) is widely distributed in the main Japanese islands, and is known to overwinter as eggs (Sugimura et al. 1999). The southern lowland *S. frequens* adult populations do not begin reproduction. Alternately, they enter summer diapause and migrate to higher altitudes (Asahina 1984); following estivation in the mountains, they return to the lowlands in order to reproduce during autumn. Therefore, reproduction occurs in the southern populations of *S. frequens* at the same time or even later than it does in the northern populations (Uéda 1988, 1993).

Takashima and Nakamura (2014) compared the egg period in *S. frequens* among three populations under L16:D8 or L12:D12 at 25, 20, or 15 °C. However, a clear geographic trend was not observed at 25 and 20 °C, suggesting little geographical variation in the diapause intensity with a change in latitude. In contrast, the mean length of the embryonic period tended to be negatively correlated with the latitude of the collection site at 15 °C; at this temperature, the embryonic period in the Hiroshima population displayed a bimodal distribution (Takashima and Nakamura 2014). These results indicated that the rate of diapause development at low temperatures was slower in a certain number of eggs (Takashima and Nakamura 2014).

Under exceptionally cool summer temperatures, nondiapause adults of *S. frequens* were observed in the lowlands of central Japan (Arai 2007). This observation appeared to suggest that summer diapause in adult *S. frequens* is not obligatory but facultative, and is regulated by ambient temperatures. In this case, the eggs laid by nondiapause females early on in the season would undergo a long summer in low-latitude regions; consequently, the egg diapause is also maintained for a relatively long period until winter. We assumed that the geographical variation in egg period and the hatching bimodality in the low-temperature, low-latitude populations discovered by Takashima and Nakamura (2014) were adaptations to early oviposition in the southern *S. frequens* population. The risk of hatching and dying before the onset of winter would be decreased by including eggs that have a slow rate of diapause development within the population. In order to test this hypothesis, the variability of the egg period was compared among various populations in this study. *S. frequens* eggs were collected from 11 localities in Japan, and hatch times were recorded under different photoperiod and temperature combinations in the laboratory. The average egg period and duration of hatching were compared among the different populations. Geographic and seasonal adaptations in *S. frequens* were inferred from the results.

Materials and methods

Adult *S. frequens* females were collected from 11 localities in Japan from August to November during 2008–2012 (Table 1). The eggs were obtained from the females by dipping their abdomens in water immediately after collection. The eggs were subjected to the experimental conditions within a few days of being oviposited. They were maintained at room temperature in a cooler bag during transfer from the collection site to the laboratory. Each batch of eggs was approximately divided into six groups and placed in plastic cups (80 ml). These were subjected to a long-day (L16:D8) or a short-day (L12:D12) photoperiod at 25 ± 1, 20 ± 1, or 15 ± 1 °C in order to examine the combined effects of photoperiod and temperature. The water was replaced with tap water once every week. Moldy eggs were removed periodically, and the hatchings were recorded twice a week. The experiments were continued until all the eggs were hatched or dead. These experiments were not replicated. The annual average temperature data recorded at the meteorological station closest to each *S. frequens* collection site were obtained from the Japan Meteorological Association (http://www.data.jma.go.jp/obd/stats/etrn/index.php).

In the data analysis, average egg periods for each experimental treatment were calculated and compared among geographical populations. In order to detect the differences among experimental treatments, the mean values at each temperature were elaborated by analysis of variance (ANOVA), using the embryonic period as the dependent variable and the photoperiod and collection site as the independent variables. The Tukey test at $p = 0.05$ (Zar 2010) was used to perform multiple comparisons among the groups. As an index of variation in the duration of hatching within the treatments, we measured the period from the date when 10 % of the eggs were hatched to the date when 90 % of the eggs were hatched, hereafter designated the "10–90 % hatch period." The average (±standard deviation; SD) hatch period was therefore calculated for each experimental treatment. In order to detect any geographical trends in the egg period, the correlation coefficient between the 10–90 % hatch period and the latitude of the collection site was calculated for each experimental condition. The coefficient of variation in egg period was also calculated by dividing the standard deviation by the mean egg period. This was also used to estimate the variation in hatching pattern.

Results

The average egg period was observed to range from 71 to 111, from 90 to 144, and from 82 to 159 days when incubated at 25, 20, and 15 °C, respectively (Table 2). Significant differences were detected among populations subjected to all experimental treatments, regardless of the photoperiod or temperature ($p < 0.05$, ANOVA). No clear trend in the egg period with latitude was discerned. For example, the average egg period at 25 °C was shortest in the Aomori population (located at 40.8°N) and longest in the Iwate population (located at 39.7°N). We detected no clear increase or decrease in the egg period in populations obtained from latitudes either lower or higher than the Aomori and Iwate regions. The correlation coefficients (r) between the average egg period and the latitude of the collection site ranged from −0.410 to 0.364, meaning that they were not significant under any combination of temperature and photoperiod ($p > 0.05$). No significant correlation was observed between the average egg period and the collection date ($r = -0.421$ to 0.457, $p > 0.05$), or between the average egg period and the altitude of the collection site ($r = -0.249$ to 0.288, $p > 0.05$), under any combination of temperature and photoperiod.

A comparison of the average egg periods for the two photoperiods only revealed statistically significant differences for the populations obtained from the Kochi and Kumamoto regions at 25 °C. The average egg period for the Kochi population subjected to the L12:D12 photoperiod was significantly shorter than that of the Kochi population subjected to the L16:D8 photoperiod. On the other hand, the average egg period was longer in the Kumamoto population subjected to the L12:D12 photoperiod than in the Kumamoto population exposed to the L16:D8 photoperiod. At 20 °C, seven populations displayed significant differences in average egg period between the populations exposed to different photoperiods. The eggs from the Sapporo, Aomori, Iwate, Niigata, and Nagano populations incubated under the short-day photoperiod hatched later than those subjected to the long-day photoperiod. The opposite results were observed for the Hiroshima and Kumamoto populations. Significant differences were detected at 15 °C between the photoperiod treatments in eight populations, excluding the Engaru, Hiroshima, and Kumamoto populations. The egg period under the short-day photoperiod was longer than that under the long-day photoperiod in all eight populations ($p < 0.05$).

Next, in order to detect the variability in the egg period, the average 10–90 % hatch period was plotted against the latitude of the collection site (Fig. 1). At 25 and 20 °C, there was no significant correlation between the hatch period and the latitude ($p > 0.05$). However, at 15 °C, significant negative correlations were detected ($r = -0.707$ and −0.802 when eggs were exposed to the L12:D12 and L16:D8 photoperiods; $p < 0.05$ and $p < 0.01$, respectively). Similar negative correlations were also detected between the total (0–100 %) hatch period and the latitude of the collection site ($r = -0.651$ and −0.678 when eggs were subjected to the L12:D12 and L16:D8 photoperiods, respectively; $p < 0.05$), although these results are not shown in the figure. Furthermore, the standard deviation of the egg period when the eggs were incubated at 15 °C was observed to be larger in the populations from the lower latitudes (Table 2). No significant correlation was detected between the coefficient of variation in the egg period and the latitude of the collection site at 25 °C ($p > 0.05$, Fig. 2). In addition, no significant correlation was observed at 20 °C, although the coefficient of variation was observed to be negatively related to the latitude ($r = -0.546$ and −0.448 when eggs were exposed to the L12:D12 and L16:D8 photoperiods, respectively; $p > 0.05$). However, a significant correlation was detected at 15 °C regardless of the photoperiod ($r = -0.645$ and −0.648 when subjected to the L12:D12 and L16:D8 photoperiods, respectively, $p < 0.05$).

The average annual temperature recorded at the meteorological observatory closest to the *S. frequens* collection site was observed to increase with decreasing latitude of the collection site (Table 1). For example, the

Table 2 Average (±standard deviation) egg period of *Sympetrum frequens*

Temperature (°C)	Collection site	Number of hatched eggs		Average egg period (days)	
		L12:D12	L16:D8	L12:D12	L16:D8
25	Engaru	585	606	97.9 ± 25.2^{cde}	100.2 ± 25.6^{bcd}
	Sapporo	993	911	83.7 ± 19.2^{i}	85.5 ± 21.5^{hi}
	Ohnuma	348	283	92.1 ± 18.3^{fg}	94.5 ± 19.7^{ef}
	Aomori	832	876	71.1 ± 17.6^{m}	73.8 ± 18.4^{m}
	Iwate	284	258	104.6 ± 26.5^{ab}	110.5 ± 29.3^{a}
	Niigata	200	132	84.2 ± 16.6^{hijk}	86.2 ± 18.3^{ghij}
	Nagano	897	855	87.8 ± 20.7^{gh}	89.8 ± 22.1^{fg}
	Shiga	830	677	77.7 ± 21.3^{l}	79.5 ± 23.6^{jkl}
	Hiroshima	769	807	91.1 ± 19.9^{fg}	92.0 ± 21.2^{fg}
	Kochi	1070	1103	78.4 ± 20.1^{kl}	85.6 ± 21.0^{hi}
	Kumamoto	1545	1465	99.7 ± 21.8^{bc}	96.9 ± 22.9^{de}
20	Engaru	662	703	113.7 ± 25.8^{cd}	112.4 ± 23.1^{d}
	Sapporo	848	1015	101.3 ± 23.4^{ef}	96.8 ± 21.8^{g}
	Ohnuma	345	411	100.8 ± 27.0^{efg}	98.2 ± 21.8^{fg}
	Aomori	588	413	122.6 ± 23.9^{b}	118.4 ± 28.4^{cd}
	Iwate	252	278	143.6 ± 26.6^{a}	131.3 ± 30.0^{b}
	Niigata	155	185	114.7 ± 28.1^{cd}	93.5 ± 24.5^{gh}
	Nagano	923	755	100.9 ± 26.7^{efg}	104.9 ± 30.8^{e}
	Shiga	687	701	110.7 ± 32.6^{d}	100.3 ± 26.7^{efg}
	Hiroshima	760	767	103.9 ± 26.7^{ef}	120.0 ± 21.9^{c}
	Kochi	1033	1014	103.1 ± 30.6^{ef}	103.4 ± 31.8^{ef}
	Kumamoto	1809	1545	89.9 ± 22.2^{h}	104.7 ± 25.7^{e}
15	Engaru	623	705	113.2 ± 26.9^{g}	111.1 ± 20.4^{g}
	Sapporo	749	1069	103.5 ± 29.5^{h}	94.6 ± 20.4^{i}
	Ohnuma	411	418	102.5 ± 19.9^{h}	81.7 ± 24.8^{j}
	Aomori	767	863	130.9 ± 22.6^{d}	109.9 ± 23.5^{g}
	Iwate	181	268	159.4 ± 37.6^{a}	133.0 ± 28.4^{cde}
	Niigata	251	197	123.3 ± 46.9^{ef}	106.1 ± 33.0^{gh}
	Nagano	898	1071	119.5 ± 38.1^{f}	109.2 ± 26.0^{g}
	Shiga	686	745	140.4 ± 34.9^{bc}	121.3 ± 32.3^{f}
	Hiroshima	630	322	140.5 ± 41.3^{bc}	145.2 ± 42.1^{b}
	Kochi	829	1185	135.2 ± 49.1^{cd}	111.5 ± 36.9^{g}
	Kumamoto	1476	1294	99.6 ± 35.4^{h}	104.5 ± 30.1^{hi}

Values with same letter at each temperature do not differ significantly by Tukey test with $p = 0.05$ after ANVA (Zar 2010)

temperatures at low-latitude regions, such as the Kochi and Kumamoto regions, were at least 10 °C higher than that for the Engaru region. When the correlation between the average 10–90 % hatch period and the average annual temperature near the collection site was calculated, significant positive correlations were only detected at 15 °C ($r = 0.611$ and 0.834 when subjected to the L12:D12 and L16:D8 photoperiods; $p < 0.05$ and $p < 0.01$, respectively). Similarly, significant correlations were detected between the coefficient of variation in the egg period and the average annual temperature near the collection site only at 15 °C ($r = 0.610$ and 0.834 under L12:D12 and L16:D8 photoperiods; $p < 0.05$ and $p < 0.01$, respectively).

Discussion

Effect of temperature on diapause development

Diapause development progresses over a wide range of temperature. Within that range, there are temperatures that optimize its rate, and these temperatures are often lower than those for non-diapause development (Danks 1987). For example, in the false melon beetle, *Atrachya menetriesi* Faldermann, the optimal temperatures are between 5 and 10 °C for the termination of egg diapause, although diapause development proceeds at low temperatures ranging from 1 to 16 °C (Ando 1983). Further, the rate of diapause development is rapid at low temperatures of around

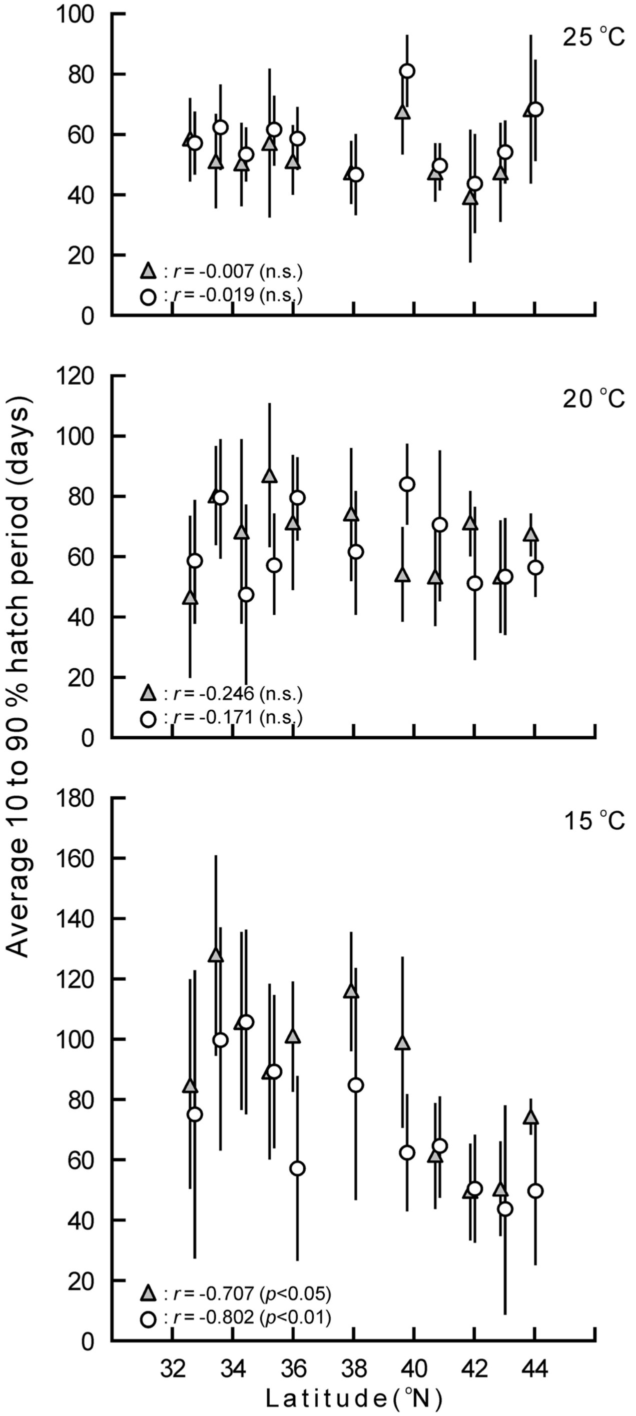

Fig. 1 Scattergrams of the average 10–90 % hatch period of *Sympetrum frequens* versus the latitude of the collection site. *Shaded triangles* L12:D12 photoperiod, *open circles* L16:D8 photoperiod, *error bars* standard deviations. Number of egg batches = 4–12

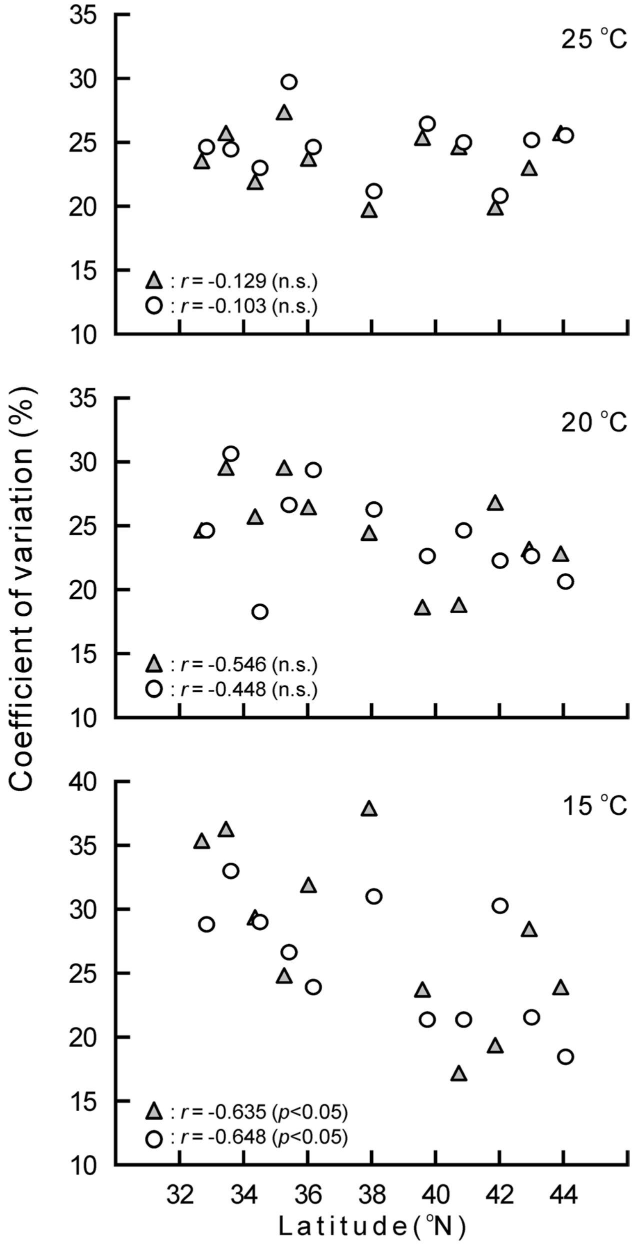

Fig. 2 Scattergrams representing the correlation between the coefficient of variation in the egg period of *Sympetrum frequens* and the latitude of the collection site. *Shaded triangles* L12:D12 photoperiod, *open circles* L16:D8 photoperiod

10 °C among Odonata (Corbet 2004). Takashima and Nakamura (2014) showed that variations in the egg period with changes in temperature were small. Indeed, variations in the average egg period with changes in temperature were small in the present study, regardless of the collection site. However, the average egg period tended to increase at lower temperatures. These results appear to confirm that the rate of diapause development in *S. frequens* eggs increases with decreasing temperature, whereas non-diapause development progresses faster at higher temperatures.

Diapause for overwintering is usually completed as early as around the winter solstice, long before the arrival of spring (Hodek 1996). Insects can resume development or reproduction with a rise in temperature. The physiological nature of diapause development is completely different from that of post-diapause development; diapause development can proceed at temperatures lower than the developmental threshold for non-diapause development, whereas post-diapause development proceeds faster at higher temperatures. Using the experimental design of this study, we were not able to distinguish the period of diapause development from that of post-diapause development. Jinguji et al. (2010) obtained *S. frequens* eggs (after completion of diapause) from the field and compared the egg periods that occurred under different incubating temperature conditions. They revealed that the average egg period was 9.7, 17.0, 21.3, and 47.0 days when the eggs were incubated at 23 18, 13, and 8 °C, respectively (Jinguji et al. 2010). They also demonstrated that the light conditions do not affect the hatching of *S. frequens* eggs by comparing the eggs exposed to an L14:D10 photoperiod to those exposed to constant darkness at 23 °C (Jinguji et al. 2010). From these results, it was suggested that the post-diapause development period was relatively short at ≥15 °C. Therefore, differences in the egg period observed among the experimental treatments in the present study were mainly due to differences in the diapause development at each temperature.

Effect of photoperiod on diapause development

Photoperiod is the most important environmental factor that regulates diapause (Danilevsky 1961; Danks 1987; Košťál 2006; Tauber et al. 1986). The photoperiod plays a significant role in the development of dragonflies, which undergo embryonic diapause for overwintering (Sawchyn and Church 1973). Takashima and Nakamura (2014) observed differences in the average egg period between short- and long-day photoperiods at 20 and 15 °C, although the photoperiodic response was not clear. In this study, the photoperiodic response during the egg period of *S. frequens* was dependent on the incubation temperature. At 25 °C, a statistically significant difference in the egg period between the photoperiod treatments was detected only in two populations, whereas such differences were detected in seven and eight populations at 20 and 15 °C, respectively (Table 2). Furthermore, at 15 °C, the egg period observed under the short-day photoperiod was statistically significantly longer than that observed under the long-day photoperiod in all eight populations. These results may suggest that the photoperiod plays a role in the regulation of egg diapause at low temperatures. When the eggs of *L. disjunctus* and *L. unguiculatus* were incubated at a photoperiod shorter than 12–14 h, the second phase of the egg diapause was observed to persist, demonstrating that a short day length helps maintain diapause during the winter (Sawchyn and Church 1973). According to the results of this study, it is possible that the egg diapause is prolonged in *S. frequens* as a result of the short day length in late autumn to prevent early hatching.

Variation in diapause development among populations and temperatures

Hatch periods in *S. frequens* were 40–90 days at 25 and 20 °C; no clear trend in hatch period with latitude was observed (Fig. 1). These results suggest that both diapause intensity and rate of diapause development did not vary significantly among and within populations. However, the hatch periods at 15 °C were longer than those seen at high temperatures in eggs laid by females collected from lower latitudes. If the long hatch periods in the low-latitude populations are due to physiological constraints (e.g., a slow non-diapause development resulting from low temperatures), the hatch periods at 15 °C should also be longer for eggs obtained from the high-latitude populations. However, the hatch periods at 15 °C of the eggs obtained from northern populations, such as Engaru, Sapporo, Ohnuma, and Aomori, were similar to those of the eggs incubated at higher temperatures. In addition, each egg batch was divided into six groups that were subjected to different experimental conditions in the present study. Therefore, the genetic compositions of the eggs included in the various experimental treatments for a particular geographical population are considered to be identical. It is unlikely that genetic differences among egg batches led to the large variations in hatch periods that were observed at 15 °C. It is, however, possible that there are genetic differences among populations: females of the southern populations lay eggs displaying different rates of diapause development within a batch.

The annual average temperature was significantly correlated with the average hatch period and the coefficient of variation in the egg period at 15 °C (Figs. 1, 2). These results suggest that the insects show different responses with regard to egg period as a result of climatic adaptation. Uéda (1988) assumed that *S. frequens* populations express three types of life-history patterns: type 1, Hokkaido populations, highland-type individuals, and autumn-emerging individuals that display neither a reproductive diapause nor migration to the highland regions; type 2, individuals of intermediate latitudes or altitudes that normally migrate to the highland area but do not enter a reproductive diapause; and type 3, early summer-emerging individuals of lowland populations in low latitudes that migrate to highland regions as diapausing adults. However, type 3 adults were observed during extremely cool summers even in the lowlands of central Japan (Arai 2007). This observation indicates that the onset of reproduction in *S. frequens* is dependent on the environmental temperature.

Consequently, the egg period between oviposition and the arrival of winter in the southern populations may vary widely within a population. It may be assumed that the large intrapopulation variations in the egg period observed in the southern populations is a risk-spreading strategy; at least some eggs will maintain diapause until winter, even if they are laid early in the season and subjected to a long summer. The termination of diapause is divided into two or more periods in several univoltine insects, suggesting that the bimodality of emergence is an adaptation to unpredictable spring conditions (Danks 1987). It is unlikely that the timing of diapause termination differs among eggs of *S. frequens* because the low temperatures during winter may enhance diapause development. However, it is possible that the variation in diapause development within a population is adaptive to the unpredictable length of the summer–autumnal period.

Geographical variation in egg period

Although no significant correlation was observed between the average egg period and the latitude of the collection site at any of the tested temperatures, the egg period in *S. frequens* was observed to differ significantly among the geographical populations (Table 2). Takashima and Nakamura (2014) suggested that, given a lack of geographical trends in the egg period, differences in the local temperature (see Table 1) may not be associated with the egg period. Therefore, it is difficult to identify the factors that lead to differences in the egg period among populations. In this study, the eggs of *S. frequens* were collected at different altitudes on different days. In several insects, the diapause induced early in the season is more intense (Masaki 2002). The altitude of the habitat also affects the intensity of diapause. For example, egg diapause in the grasshopper *Melanoplus sanguinipes* (F.) was observed to be more intense at high altitudes (Dingle et al. 1990). However, neither the altitude nor the day of egg collection was significantly correlated with the average *S. frequens* egg period under any of the tested experimental conditions. It is unclear whether the differences in egg period among populations are of any ecological significance.

Although the physiological mechanism of diapause development has not yet been elucidated, the responses of several insects alter upon the exogenous addition of hormones. For example, the application of juvenile hormone (JH) may facilitate diapause completion in *Leptinotarsa decemlineata* (say) during the beginning of diapause rather than during the later stages of diapause development (Schooneveld et al. 1977). It might be assumed that *S. frequens* females present in the lower-latitude regions lay eggs that vary in the rates of physiological processes, such receptivity to hormones, within egg batches. It is also possible that *S. frequens* undergoes more than one diapause during its embryonic development. In the eggs of *L. disjunctus* and *L. unguiculatus*, the first phase of diapause development is completed by late October, but it has been revealed that the second phase of diapause persists if the eggs are incubated at a photoperiod shorter than 12–14 h (Sawchyn and Church 1973). If *S. frequens* is assumed to undergo two diapauses, the wide variations in the egg period and the bimodality of hatching (Takashima and Nakamura 2014) may be explained as follows: some eggs undergo two diapauses during their embryonic development, whereas the others undergo only one diapause.

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