Relationship between cold hardiness and northward invasion in the great mormon butterfly, *Papilio memnon* L. (Lepidoptera: Papilionidae) in Japan

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Abstract

To investigate factors involved in the recent northward invasion of the great mormon butterfly, *Papilio memnon* L. (Lepidoptera: Papilionidae), cold hardiness of pupae was compared among 4 populations including those in a subtropical and a northernmost area of Japan. The mean supercooling points (SCP) of diapausing and non-diapausing pupae were lower than −20°C in all populations. The SCP was not affected by an acclimation period nor by pupal weight, although it became higher with inoculation. Diapausing pupae were placed in 4 sites at different altitudes in southern Osaka Prefecture during the winter, then survival rates were compared in the spring. All individuals died at altitudes of 1,100 and 800 m, while more than half of the individuals survived the winter at 400 and 30 m. There was no difference in the survival rate at each site among populations. Even the subtropical population showed cold hardiness to survive winter in Osaka, which is located near the northern edge of the distribution of this species. Based on the results, we inferred that the climatic lethal limits during the winter lie between −3.8 and −7.3°C for the minimum temperature, and between 52 and 68 frost days. We discuss the effect of climatic warming on the northward invasion of this species.

Key words: Cold hardiness, winter survival, distribution, climatic warming, *Papilio memnon*

INTRODUCTION

The great mormon butterfly, *Papilio memnon* L. (Lepidoptera: Papilionidae), has been expanding its distribution into northeastern areas in western Japan to the present northern limit of its distribution (Fukuda et al., 1982; Shirôzu, 1985; Yoshio, 1994, 1995; Yoshio and Ishii, 1998). In a previous study (Yoshio and Ishii, 1998), we investigated the characteristics of pupal diapause in 4 populations including a subtropical and a northernmost one, and concluded that the recent northward invasion of this species has occurred without any substantial changes in diapause.

Besides diapause, insects inhabiting the temperate and cold regions employ cold hardiness for enhancing their chances of winter survival (Danks, 1987; Denlinger, 1991; Pullin et al., 1991). There are many insects that increase cold hardiness in response to cool temperatures in overwintering and/or diapausing stage(s) (Baust and Rojas, 1985; Asahina, 1991). As for butterflies, diapausing pupae of the large white, *Pieris brassicae* L., were more cold hardy than non-diapausing ones (Pullin and Bale, 1989a). Troyer et al. (1996) reported in the monarch butterfly, *Danaus plexippus* L., the mean supercooling point (SCP) of migrants was significantly lower than that of non-migrants.

Low temperatures in mid-winter may threaten overwintering insects with death, and the mortality of insects in the temperate regions is influenced by the winter cold (Beirne, 1955; Bale, 1991). The winter survival for insects, especially at the northern limit of distributions, may be largely influenced by an interaction between the degree of insect cold hardiness at the overwintering stage and the magnitude of winter severeness. It is suggested that the winter temperature trend affects territorial fluctuations in population sizes of two British butterflies, *Pararge aegeria* L. and *Polygonia c-album* L. (Lees, 1962; Pratt, 1987).

In this study, we investigated the cold hardiness of *P. memnon* to elucidate the factors involved in the recent northward invasion of this species.

MATERIALS AND METHODS

Animals and rearing. Four Japanese popula-
tions of *P. memnon*, the Naze, Kagoshima, Wakayama and Mino populations, were used for this study. Female butterflies and/or larvae were collected in Naze (28°23′H11032′N) and Kagoshima Cities (31°36′H11032′N) in Kagoshima Prefecture, Wakayama City (34°11′H11032′N) in Wakayama Prefecture, and Mino City (34°51′H11032′N) in Osaka Prefecture from 1994 to 1997. Newly hatched larvae from eggs obtained from these females and/or the female progeny were reared according to Yoshio and Ishii (1996) on an artificial diet (Yoshio and Ishii, 1996) at 20°C ± 1°C under either a short day (12L–12D) or a long day (16L–8D) photoperiod to produce diapausing and non-diapausing pupae, respectively. All the pupae obtained under 12L–12D were kept at the same conditions for 30 days and individuals emerging within 25 days after pupation were regarded as non-diapausing (see Yoshio and Ishii, 1998).

**Supercooling points.** The pupae to be examined were put into a space (2.5 cm × 3.5 cm × 2 cm) produced by a pair of Styrofoam planks (6 cm × 7 cm × 3 cm in thickness) (Fig. 1), and cooled at a rate of about 1°C/min by a medical freezer (SANYO, MDF-330). A sensor was attached to the forewing bud of the pupae by adhesive tape and the body temperature was recorded every second by a data logger (RT-10, TABAI ESPEC Corp.). The SCP was regarded as the lowest temperature prior to a transient rise in temperature, indicating a release of latent heat concomitant with freezing.

Diapausing pupae were divided into 3 groups, and kept at 10°C ± 0.5°C under 12L–12D for 0, 40 and 90 days to elucidate the effect of an acclimation period on the SCP. After the acclimation, the pupae were weighed and put into the medical freezer to estimate the SCP. We also measured the SCP of non-diapausing pupae kept under the same conditions as their larval stage for 5 days after pupation. They were kept at room conditions after the measurement of SCP to observe adult emergence.

The effect of inoculation on SCP was investigated in the diapausing pupae of the Kagoshima population which was acclimated at 10°C for 40 days. The head or abdomen of the pupae was tied with a piece of tissue paper (1 cm × 8 cm, Hoxy Corp.) wetted in tap water and the inoculated SCP was measured.

**Winter survival in the field.** We conducted field experiments to compare the winter survival of the 4 populations at 4 sites in southern Osaka Prefecture. Site 1 (alt., 30 m) was in a shrubbery on the campus of Osaka Prefecture University, and Sites 2 (400 m), 3 (800 m) and 4 (1,100 m) inside forests in Mt. Kongo.

Diapausing pupae of the 4 populations acclimated at 10°C for about 10 days were placed at 2 sites (Sites 1 and 2) from December 14, 1994 to April 24, 1995, and those of the Wakayama and Mino populations at Sites 3 and 4 from January 20, 1996 to April 15, 1996. The pupae were placed about 1 m above the ground in all sites, and on the soil surface in Sites 2 and 3 to assess the influence of a microclimate on the winter survival rate. The ambient temperature was recorded every day during the experimental period by a maximum and minimum thermometer at Site 1, and every hour by the data logger at the other sites. In the spring, all pupae were collected and examined for survival. Individuals that survived were kept in a room without direct sunlight, enclosed partly with window screens, and the number of adults emerging was recorded. We used the hand-pairing method (Clarke and Sheppard, 1956) to examine the reproductive capacity of adults.

All data were analyzed and statistical comparisons were made using SPSS 6.1 J. for the Power Macintosh.

**RESULTS**

**Supercooling points**

*SCPs of diapausing and non-diapausing pupae*

All pupae were frozen to death in measuring the SCP. The mean SCPs of diapausing pupae accl-
mated for 40 days were −20.2, −21.9, −20.8 and −21.3°C in the Naze, Kagoshima, Wakayama and Mino populations, respectively, and those of non-diapausing were −21.0, −20.5 and −21.1°C in the latter 3 populations (Table 1). The mean SCP of diapausing pupae of the Naze population was significantly higher than that of the other 3 populations with no significant difference being found among the latter three. There was no significant correlation between SCP and body weight in all 4 populations.

**Effect of acclimation period on SCP**

Mean SCPs of diapausing pupae of the 4 populations acclimated for different periods ranged between −19.8 and −21.8°C (Fig. 2). There was no significant difference in the mean SCPs of each population for different acclimation periods and among the 4 populations for the same acclimation period (one-way ANOVA, d.f. = 11, p > 0.05), although responses to acclimation periods seemed to differ among populations.

**Inoculative SCP**

Table 2 shows the effect of inoculation on the SCP of diapausing pupae in the Kagoshima population acclimated at 10°C for 40 days. Mean SCPs of pupae inoculated at the head and abdomen were −17.2 and −16.2°C, respectively, and the latter was significantly higher than that without inoculation.

**Winter survival in the field**

The mean and minimum air temperatures during the experimental period were lower with increasing altitude (Table 3). Although the difference in minimum temperature between Sites 1 and 2 was small, there was a marked difference in the number of frost days, when the minimum temperature fell below 0°C. The minimum temperature ranged between −3.4 and −10.1°C, which was much higher than the SCP of *P. memnon* (Table 1).

More than half of the individuals survived the
winter at Sites 1 and 2 (Table 4), whereas all individuals died at Sites 3 and 4 (n: 43–55). The mortality at Site 1 was small and no significant difference was found in winter survival among populations (c²-test, d.f./H11005 3, p/H11022 0.05). Fertile eggs were obtained from parents that had overwintered at Site 2 in all populations.

At Site 2, the survival rate of pupae was lower on the soil surface than above the ground in all populations (c²-test, d.f./H11005 1, p/H11021 0.05), although the ambient temperature on the soil surface was higher than that above the ground (Table 4). As for the winter survival above the ground, the survival rate of pupae and the ratio of successful emergence ranged between 60 and 75%, and between 49 and 67% at Site 2. There were no significant differences in the two rates among the populations (c²-test, d.f./H11005 3, p/H11022 0.05).

Table 4. Number of individuals which emerged successfully and unsuccessfully, and which were dead in the pupal stage in diapausing pupae of P. memnon placed on and above the ground at 2 sites of different altitudes in Osaka from December, 1994 to April, 1995 after exposure at 10°C for 10 days

<table>
<thead>
<tr>
<th>Population</th>
<th>Site 1 (alt. 30 m)</th>
<th></th>
<th>Site 2 (alt. 400 m)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Above ground</td>
<td></td>
<td>Above ground</td>
<td>On ground</td>
</tr>
<tr>
<td></td>
<td>Emergence</td>
<td>Dead</td>
<td>Emergence</td>
<td>Dead</td>
</tr>
<tr>
<td></td>
<td>Success</td>
<td>Unsuccess</td>
<td></td>
<td>Success</td>
</tr>
<tr>
<td>Naze</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>Kagoshima</td>
<td>31</td>
<td>1</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>Wakayama</td>
<td>21</td>
<td>0</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>Mino</td>
<td>8</td>
<td>0</td>
<td>1</td>
<td>8</td>
</tr>
</tbody>
</table>

DISCUSSION

Supercooling point

It is clear from the results of this study that P. memnon is a freeze-intolerant species, and that all 4 Japanese populations examined including the subtropical one have the ability to supercool below −20°C in the pupal stage (Table 1 and Fig. 2). The SCP of diapausing pupae reported in other freeze-intolerant Papilio species ranged from −20.2 to −25.3°C in northern Japanese populations of P. xuthus (Shimada, 1988), and less than −20°C in P. bianor and P. maackii (Asahina, 1991). The mean SCP of P. memnon was within these ranges.

Sømme (1982) suggested that intraspecific variations of SCP would occur in terrestrial arthropods. Tanaka (1996) reported that the SCP of overwintering individuals decreased with increasing latitude of original habitats in the house spider, Achaearanea tepidariorum. By contrast, no remarkable clinal variation in the SCP was observed in P. mem-
non. However, the SCP of the subtropical population was as low as that of the northern population for other *Papilio* species (Shimada, 1988; Asahina, 1991).

A seasonal change in SCP has been reported in many insects (Sømme, 1982; Block, 1990; Bale, 1991), and a decrease in SCP in mid-winter has been described as one adaptation for winter survival. Some insects including butterflies change SCP in response to the period of acclimation in the laboratory (e.g. Pullin and Bale, 1989b). However, the SCP was not affected by acclimation in *P. memnon*. Pullin et al. (1991) pointed out that the SCP of diapausing *Pieris brassicae* pupae, which shows little change during winter, allow them to sufficiently avoid freezing in usual winters in England. Since the minimum temperature of the year never falls below the SCP of *P. memnon* in the southwest lowlands of Japan, a seasonal change in SCP may not be essential for hibernation of this species in the winter.

Inoculation increases the SCP or lower limit of temperature for survival in some insects including butterflies (Shimada, 1988; Larsen and Lee, 1994; Hoshikawa, 1996). Inoculative SCPs were higher than the uninoculative ones in *P. memnon*, which suggests that overwintering individuals will die at higher subzero-temperatures than the uninoculative SCP in the field. In addition, it was shown that non-diapausing pupae have a similar capacity to supercool to that of diapausing ones in this species (Table 2). As is pointed out by Bale (1987), it is doubtful whether the SCP reflects the cold hardiness of *P. memnon* in the field.

**Winter survival in the field**

The results of this study demonstrated that the winter survival rate of *P. memnon* pupae differed with elevations; all pupae died at higher altitudes (800 and 1,100 m), while more than half of the individuals survived at lower sites (30 and 400 m). Since the minimum temperature lowered with increasing altitudes (Table 3), subzero temperatures would be a possible factor causing the winter mortality at higher altitudes. It is noted that the minimum temperatures at Sites 3 and 4 were significantly higher than the inoculative SCP of the pupae (Tables 2 and 3). This suggests that the cause of winter mortality of *P. memnon* pupae is non-freezing cold injury rather than freezing injury. It has been reported that most freeze-intolerant insects are fatally damaged without freezing at a low temperature above their SCPs (Bale, 1987, 1991).

All populations of *P. memnon* examined in this study have the cold hardness to survive winter at an altitude of 400 m in the site located near the northern edge of their distribution. This means that there is no remarkable difference in the cold hardiness between the 4 populations.

This study also demonstrated that, even at the same elevations, the mortality rate differs with microsites where the pupae hibernated. At Site 2, the winter mortality was significantly higher in the pupae on the soil surface than those above the ground (Table 4), although the ambient temperatures were nearly the same between the two sites (Table 3). This suggests that the mortality during winter depends not only on low temperature but also on other physical and/or biological factors. Shimada (1988) reported in *P. xuthus* that all pupae placed on the soil surface died from infectious germs in the humid condition. Further studies are needed to elucidate the mortality factors of overwintering pupae placed on the soil surface in *P. memnon*.

**Relationship between northward invasion and climatic warming**

It was clear from the results of Yoshio and Ishii (1998) and this study that the recent northward invasion of *P. memnon* has occurred without substantial changes in the physiological traits such as diapause intensity and cold hardiness. These facts may suggest that some environmental factors are involved in this phenomenon. In recent years, climatic warming has become a global environmental problem. The influence of climatic warming on plants and animals including insects have been studied extensively (e.g. Houghton, 1997; Kiritani, 1997; Woiwod, 1997). Parmesan et al. (1999) reported that the distribution of 22 non-migratory butterfly species have shifted northward due to climatic warming during the 20th century in Europe. Yoshio and Ishii (1998) suggested that rising winter temperatures may be responsible for the northward invasion of *P. memnon*.

The lethal limit of *P. memnon* in the field is inferred to lie between −3.8 and −7.3°C for the minimum temperature of the year, and between 52 and 68 frost days (Table 3). *P. memnon* is consid-
ered to have established a pioneer population in Mino City (alt. ca. 230 m) in the late 1980’s (Yoshio, 1994). According to Osaka District Meteorological Observatory (1950–1959), the winter cold would have had severe effects on the winter survival of *P. memnon* around Mino City in the 1950’s. The mean minimum temperature of the year and the average number of frost days were \(-7.3^\circ C\) (\(-6.4 \text{ to } -9.0^\circ C\) in range) and 78 days (63 to 87 days) in Nose Town (34°57’N, alt. ca. 240 m) located north of Mino, and \(-5.4^\circ C\) (\(-4.3 \text{ to } -6.0^\circ C\)) and 54 days (43 to 71 days) in Ikeda City (34°48’N, ca. 60 m) located west of Mino in the 1950’s. In Toyonaka City (34°46’N, ca. 10 m) located south of Mino, the mean minimum temperature \(-3.3^\circ C\) \((-2.2 \text{ to } -4.6^\circ C\) in range) and the average number of frost days (26 days; 15 to 49 days) (Osaka District Meteorological Observatory, 1990–1999) were below the lethal level of *P. memnon* in the 1990’s, when this species had already reached this city but not Nose Town yet. Nose Town had severe winters even in the 1990’s, with the mean minimum temperature being \(-6^\circ C\); \(-5.0 \text{ to } -7.4^\circ C\) in range and the average number of frost days being 74 days; 59 to 91 days (Osaka District Meteorological Observatory, 1990–1999). Thus it is possible to conclude that the recent northward invasion of *P. memnon* is brought on by the climatic warming.

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