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Some Physiological Properties Associated with Freeze-tolerance in Diapausing Pupae of *Papilio machaon*[1]

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Abstract Physiological properties associated with freeze-tolerance of *P. machaon* diapausing pupae were studied under experimental conditions. Freeze-tolerance markedly increased after cold acclimation at 5°C, but decreased by prolonged chilling. A significant amount of glycerol was accumulated by diapausing pupae after the cold acclimation. The degree of freeze-tolerance seemed to be closely related with hemolymph glycerol content.

Introduction

Freeze-tolerance and glycerol accumulation in overwintering pupae of *P. machaon* were already reported by Asahina (1, 2), and Takehara and Asahina (7). According to them, the pupae survive after freezing at temperatures lower than −30°C and accumulate more than 3% of glycerol at the maximum. Overwintering *P. machaon* pupae have been known as one of the most freeze-tolerant insects. However, little is known about the physiological properties which contribute to the freeze-tolerance. The present report deals with additional and detailed study on some physiological properties of diapausing pupae under experimental conditions.

Materials and Methods

Animals

Diapausing pupae of *P. machaon* were obtained from labocultured colonies. Larvae were reared under a short-day photoperiodic condition (8 L-16 D per day at 22°C) with leaves of the *Aegopodium podagraria* throughout the larval stage. These larvae were destined to enter the pupal diapause. Obtained pupae were left at 22°C for one month, then they were transferred into a refrigerator kept at about 5°C.

Freezing procedure, detection of supercooling points and survival assay

Diapausing pupae were weighed just before use for freezing experiments. The pupae were cooled to −30°C at rates between 0.6 and 0.8°C/min. Each

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pupa was covered with a piece of cotton wool and hanged in a double-walled test tube. A tip of thermocouple was inserted into a space between pupa and cotton wool. The thermocouple was connected with a recorder (Rika-denki KB681H) to measure the temperature change. Supercooling points were determined from the cooling curves recorded. When the pupa freezes during a course of cooling, the body temperature abruptly raises to high at supercooling point, because of the heat of fusion.

The frozen pupae were placed in a room air (about 22°C) soon after the temperatures reached −30°C. Rewarmed pupae were stored at 22°C until they developed into adults. Dates of emergence and completion of adult development were recorded. The pupae unchanged after prolonged storage up to 6 months were dissected to confirm the deaths.

Estimation of hemolymph osmolality and glycerol content

Hemolymph was drawn from diapausing pupa by making a small hole in the pupal antennal cuticle. A certain amount (0.15 ml) of hemolymph was taken in a measuring vessel. Osmolality was measured by the Automatic Semi-Micro Osmometer (Knauer), which analyzes hemolymph osmolality automatically on the basis of freezing point of hemolymph.

The hemolymph sample used for the measurement of osmolality was also used for the estimation of glycerol content. The 0.1 ml out of 0.15 ml hemolymph sample was mixed with 1 ml of 99% ethanol and stored at about −20°C until the glycerol estimation. A definite amount of 1, 4-butanediol (Nakarai Chemicals) was added to the mixture as internal standard (9). The sample was evaporated to dryness under reduced pressure at 50°C. The residue was suspended in one ml of distilled water. The suspension was centrifuged at 3,000 rpm for 5 min. A small amount (2∼10 μl) of the supernatant was used for the estimation of hemolymph glycerol content. Glycerol content was determined by gas-chromatography (9), using a Hitachi model K-53 instrument with a 1 m by 3 mm glass column containing a Chromosorb 101 support. Samples were injected into the column kept at 200°C. Elution profiles were determined with a flame ionization detector.

Results

Changes of supercooling points and freeze-tolerance under artificial conditions

The degree of supercooling markedly increased after pupation. During one-month storage at 22°C, the mean value of supercooling points rapidly dropped from −11.2°C to −22.7°C (Fig. 1). The supercooling points did not change so much after cold acclimation. During the cold storage at about 5°C, the supercooling points slightly decreased and reached the maximum three months after the cold exposure. The cold storage prolonged more than three months, however, brought about a slight increase in the supercooling points (Fig. 1).

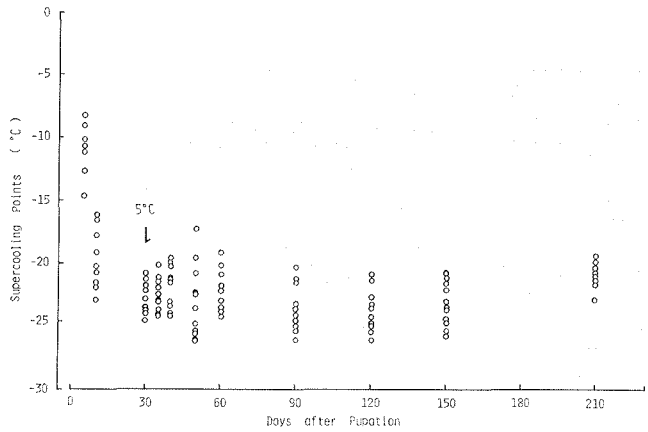

Fig. 1. Supercooling points of individual pupae before and during cold storage at 5°C

Throughout the experimental period, individual variation in the degree of supercooling was observed (Fig. 1). The supercooling points were plotted against the weights of diapausing pupae. There was no significant realtionship between the two variables (Fig. 2).

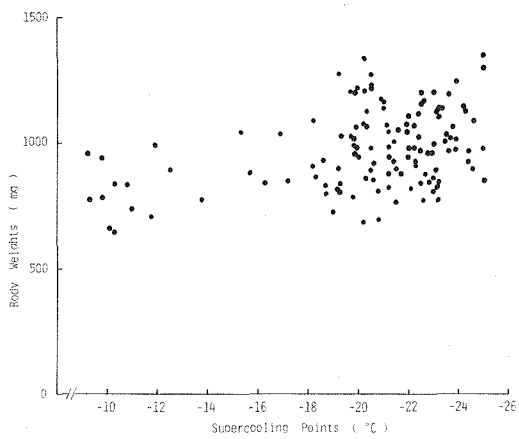

Fig. 2. Supercooling points in relation to individual body weights

Change of freeze-tolerance of diapausing pupae under artificial conditions is shown in Table 1. Before cold acclimation at 5°C, no pupae could survive after freezing to −30°C. Survival rates after freezing were considerably increased after the cold acclimation. Two months after the cold exposure, all pupae examined were tolerant to freezing at −30°C. Long-term cold storage more than two months resulted in a decrease of freeze-tolerance.

Many surviving pupae developed into adults, but the days required for emergence widely fluctuated from 97 to 230 days (Fig. 3). This suggests that the termination of diapause did not occur coincidentally. Individual

Table 1. Survivals of diapausing *P. machaon* pupae after freezing to −30°C

stage of pupae	number of pupae	number of survivals			survival rate (%)
		partially developed*	completelly developed**	emerged	
unchilled pupae (time after pupation)					
5 days	12	0	0	0	0
10 days	12	0	0	0	0
1 month	12	0	0	0	0
chilled pupae (duration of chilling)					
5 days	12	0	1	2	25.0
10 days	12	3	3	2	66.7
20 days	12	0	1	6	58.3
1 month	12	1	0	8	75.0
2 months	12	0	0	12	100.0
3 months	12	1	2	7	83.3
4 months	12	1	0	8	75.0
6 months	12	0	1	1	16.7

* developed into half-imago
** failed to emerge after completion of adult development

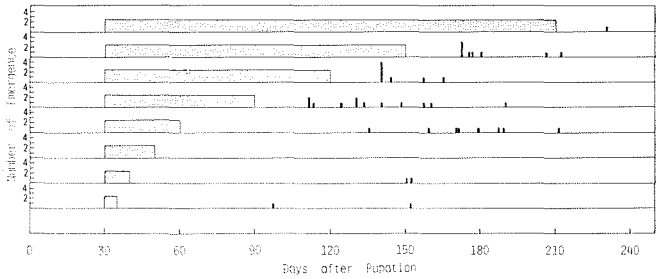

Fig. 3. The days requied for frozen-thawed pupae to reach adult emergence. ▨, duration of chilling at 5°C

variation in the termination of pupal diapause was observed previously in the same materials (6). The days required for emergence ranged from 120 to 266 days when the pupae were left at 20°C. Cold storage prolonged more than 4 months brought a coincidental emergence of adults, but did not shorten the pupal period of the diapausing insects. There is no substantial difference between pervious and present results. The present results therefore suggest that significant acceleration or delay in the termination of diapause did not occur by freezing. However, some surviving pupae could not complete the adult development (half-imgos) or failed to perform the pupal-adult ecdysis after the completion of adult development (Table 1).

Changes of hemolymph osmolality and glycerol content

The osmolalities gradually increased even if the pupae were left at 22°C. The mean value changed from 326.8 to 438 mOsm/kg during the one-month storage at 22°C (Fig. 4). Hemolymph osmolalities strongly raised after cold acclimation. After two months, mean value reached a maximum (875.2 mOsm/kg), then decreased gradually. Rewarming to 22°C after 6-month cold storage brought about an abrupt decrease in osmolality. Five days after the rewarming, the osmolalities fell down to the level of fresh pupae.

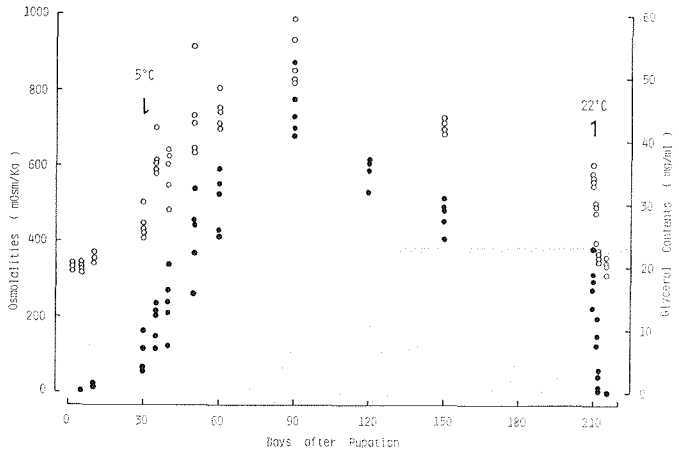

Fig. 4. Changes of hemolymph osmolality (○) and glycerol content (●) in diapausing pupae under artificial conditions

Glycerol contents also changed before, during and after the cold storage at 5°C. Soon after the pupation, hemolymph of diapausing pupae contained only a trace amount of glycerol. Glycerol contents slightly increased during

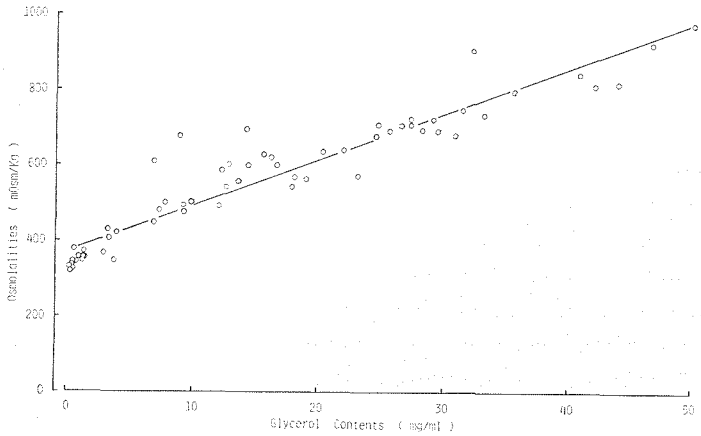

Fig. 5. Relation between hemolymph osmolalities and glycerol contents

the storage at 22°C. Cold exposure strongly stimulated the diapausing pupae to accumulate glycerol. During two-month cold storage, mean concentration of glycerol changed from 5.5 to 45.1 mg/ml. But prolonged cold storage resulted in a gradual decrease of glycerol content. Glycerol rapidly disappeared from hemolymph when the pupae were rewarmed to 22°C after 6-month cold storage.

The values of osmotic pressure were plotted against glycerol contents (Fig. 5). There was good correlation between hemolymph osmolalities and glycerol contents. The plotted points are distributed around a regression line,

$$Y = 1.216x + 368$$

where Y is osmolality (mOsm/kg) and x is glycerol content (mg/ml).

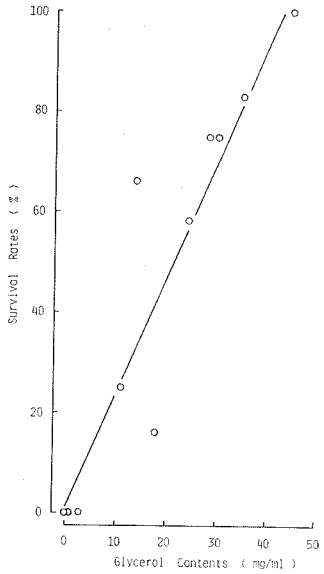

Fig. 6. Relation between survival rates and hemolymph glycerol contents

In addition, glycerol seemed to affect the survival of diapausing pupae after freezing at −30°C. Fig. 6 shows the relationship between glycerol contents and survival rates. It seemed that the increase of glycerol content brought about the increase of survival rate.

Discussion

Some physiological properties significantly change during diapause, although visible morphological changes are hardly observed in diapausing pupae. In the present work, some physiological changes associated with freeze-tolerance under artificial conditions were observed.

Supercooling points of *P. machaon* diapausing pupae rapidly decreased during storage at 22°C. Actual mechanism related with the rapid decrease remains uncertain. However, this rapid decrease seems to be useful to prevent freezing of the pupae. Since the pupae remained freeze-susceptible until they were exposed to cold temperature.

Salt (5) demonstrated a close relationship between supercooling points and glycerol contents in *Bracon cephi* larvae. In *P. machaon* diapausing pupae, a similar relation was observed during storage at 5°C. However, the rapid decrease of supercooling points brought about by the storage at 22°C was not related with glycerol contents. During this period the pupae accumulated only a small amount of glycerol.

Glycerol, on the other hand, was directly responsible for the change of hemolymph osmolality. The present results showed a close relation between hemolymph osmolality and glycerol content. The change of os-

molality was mainly caused by glycerol itself rather than the other substances.

Freeze-tolerance significantly increased after cold acclimation at 5°C. During the early period of cold acclimation hemolymph glycerol contents also increased. And survival rates after freezing at −30°C were closely related with the glycerol contents. Protective role of glycerol against freezing damages has been reviewed by several authers. Lovelock (4) concluded that the cryoprotective effect of glycerol is due to its colligative properties, which lower the injurious salt concentrations by the reduction of frozen water. The cryoprotective effect of glycerol in freeze-tolerant insects was explained by Zachariassen on the basis of the colligative properties of the substance (10). For cryopreservation of free living cells, 10 to 20% of glycerol solution is generally used. On this account, it is noteworthy that only about 4.5% of glycerol is sufficient to protect *P. machaon* diapausing pupae against freezing damages. Asahina (2) postulated a protoplasmic factor to explain the increase of freeze-tolerance in overwintering insects with only a small amount or without glycerol accumulation. No evidence to support this postulation has so far been obtained.

The other interest is the effect of temperature on glycerol accumulation in diapausing pupae. Changes of glycerol concentration under natural and artificial conditions were investigated by Takehara (8) in *Monema flavescens*, in overwintering prepupae of which temperature was very effective to regulate the concentration of glycerol. Ziegler and Wyatt (11) demonstrated the activation of glycogen phosphorylase by cold temperatures in *Hyalophora cecropia* diapausing pupae. It strongly supports the accumulation of glycerol under cold conditions. Similar results were obtained in *P. machaon* diapausing pupae by Hayakawa and Chino (3). Enzymatic regulations of glycerol formation under cold conditions are very attractive things to consider the chemical and physical basis of cold adaptation in diapausing and overwintering insects.

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