Low Temp. Sci., Ser. B 39: 31-39, 1981

Larval Diapause in *Chymomyza costata* (Diptera : Drosophilidae) II. Frost Avoidance[1]

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Abstract *Chymomyza costata* is frost susceptible. The diapausing larvae do not exhibit any stage-specific low supercooling point, but they posess the ability to lower the supercooling point by rapid defecation at low temperature and by dehydration both being effective to maintain a high viability under low temperature.

Introduction

The present knowledge on the overwintering strategies of insects provides us the two alternative ways of cold resistance, frost avoidance and frost resistance (1, 9, 10). The numerous contributions have been published to find out the mechanisms that enhance these two ways (1, 4, 7, 8, 11). As the study of diapause in Drosophilidae is a relatively new field, their overwintering strategies have been poorly known. *Chymomyza costata* Zetterstedt is one of few drosophilid species so far known to pass winter by the final (3rd) larval instar (5), in Hokkaido under bark of trees and stumps. Although its detailed habits during winter is still unknown, the experimental study on its cold tolerance may be not vain as only the diapausing individuals confront fairly low temperatures under natural conditions (3). The purpose of the present article is to report some aspects of frost avoidance using the artificially induced diapausing larvae.

Materials and Methods

The stock and culture techniques used for this study is mentioned previously (3). The larvae were reared at 18°C/under LD 10 : 14 or 16 : 8. The last instar larvae in the former condition were regarded to be committed diapause and the latter not. Three items were studied as follows.

1) *The supercooling point at every developmental stage*

In order to confirm whether diapausing larvae have stage-specific ability to tolerate low temperatures, the supercooling point, a widely accepted criterion to study the cold resistance of poikilotherms, was compared at every developmental stage. The insect attached to the tip of a cooper-

[1] Received for publication November 30, 1981. Contribution No. 2408 from the Inst. Low Temp. Sci.

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constantan thermocouple coated with a bit of silicone grease was placed in a glass vial, 3.5 cm diameter, 6.5 cm height. The vial was placed in a plastic flask, 6 cm diameter, 10 cm height, and was put into a freezer held at −50°C. The body temperature was measured by an electric recorder connected to the thermocouple. The supercooling point was read from the cooling curve under the cooling rate of about 2°C/min. After recording the supercooling point, the larvae was restored in a culture vial. Under each developmental condition the frost damage was examined by the survival ratio of about 20～40 individuals employed.

2) *Effects of chilling on food intake and defecation*

In experiment I the third instar larvae (20～25 days after being oviposited under 18±1°C/LD 10 : 14 and 16 : 8) were allowed to feed on the culture medium containing 0.01% Trypanblau, which was daily renewed, at temperatures, 5±1°C, 2±1°C and 0±1°C under LD 10 : 14 photoperiodic regime. In each condition 30 individuals were used. The food intake was daily examined by observing the larval digestive tract through the transparent body wall under a stereoscopic microscope. The feeding condition were classified into three classes : ++ the gut is nearly entirely filled with stained food and dark colored, + the gut partly contains stained food and is faintly colored, − the gut does not contain any stained food. In experiment II the third instar larvae of the condition as in I were first allowed to take the Trypanblau-medium at 18±1°C for two days under LD 10 : 14 and 16 : 8, respectively. After examinig the feeding condition of all larvae, they were separately kept with the stained food at temperatures, 5±1°C, 2±1°C and 0±1°C under LD 10 : 14. The condition of digestive tract was daily examined for eight days and classified according to the criteria mentioned above. In each combination of experimental condition 30 individuals were used.

3) *Effects of inoculator intake, chilling and dehydration on supercooling point*

Diapausing larvae (20～25 days after being oviposited at 18±1°C/LD 10 : 14) were allowed to take the food consisting of soil 1, culture medium 1, for 24 hr at 18±1°C. The inoculator fed larvae were separated into several groups each consisting from 20～30 individuals. The first group was used for the measurement of supercooling point to examine the influence of inoculator feeding. The second was transferred to an incubator kept at 2±1°C/LD 10 : 14 for one week before measuring the supercooling point. For comparison, the nondiapausing larvae (20～25 days after being oviposited at 18±1°C/LD 16 : 8) fed with inoculator containing medium were also examined in the same way. Other groups were placed each on an open petri dish without giving culture medium and kept in a dessicator for 0～48 hr. Supercooling point was measured after a given interval. In order to examine the survival of dried larvae, ten individuals were removed from

each sample consisting of about 30 individuals to be used for the measurement of supercooling point, restored in culture medium and further development was observed. For comparison, the survival of 50 nondiapausing larvae (20～25 days after being oviposited at 18±1°C/LD 16 : 8) was studied under dehydrated conditions. For the examination of the water content the larvae were weighed after freezing (nondiapausing larvae without freezing), then maintained at 105°C overnight. Thereafter, the larvae were kept in a dessicator with silicagel at room temperature for about 6 hr to completely remove the water. Then, they were again weighed and water content was calculated. For examining the influence of inoculator feeding on dehydration and supercooling point, the diapausing larvae fed with the inoculator free culture medium were studied in the same way.

Results

Supercooling point

Supercooling points of various developmental stages ranged approximately from −15 to −25°C except −25 to −30°C in eggs (Fig. 1). There was no significant difference in supercooling ability between immatures reared under LD 10 : 14 and LD 16 : 8 (Table 1) in every developmental stage. All the individuals except the third instar larvae in LD 10 : 14 died after being frozen. In the short photophase, nearly all third instar larvae aged 24 days survived after freezing and thawing, but their movement and locomotion

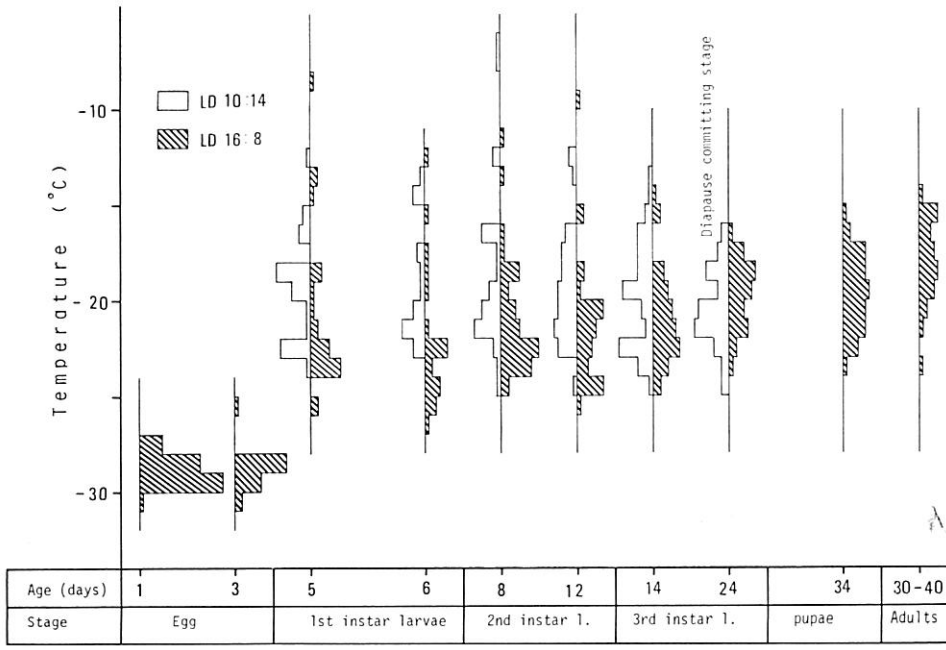

Fig. 1. Distribution of supercooling points in each developmental stage in two different photocycles, LD 10 : 14 and 16 : 8 under 18±1°C

Table 1. Supercooling points of larvae in two diffrent photophases at 18°C

Instar	Age (days)	LD 10 : 14	*n*	*P	LD 16 : 8	*n*
1st	5	−19.2±1.7	31	=	−22.6±2.8	26
	6	−19.8±2.1	20	>	−22.3±2.7	22
2nd	8	−19.7±2.8	30	=	−21.6±1.5	39
	12	−20.1±1.9	32	=	−21.4±2.8	33
3rd	14	−20.7±1.8	42	=	−21.2±1.3	34
	24	−20.8±1.3	39	=	−19.8±1.1	30

*P 0.05

were seriously damaged and most of them (29/30) failed to pupate. Further, the diapausing larvae did not exhibit any stage-specific high supercooling ability (Fig. 1).

Effects of chilling to food intake and defecation

Most feeding larvae in experiment I transferred from 18°C to 5°C continued food intake. On the other hand, nearly all individuals transferred to 3°C or 0°C were unable to feed by inactivation under low temperatures. There was little difference in feeding response between diapausing and nondiapausing larvae (Table 2). In experiment II, almost all individuals terminated their feeding at about 2°C or below as in I. But defecation of diapausing larvae seemed to continue at 2°C. Their digestive tracts became nearly vacant by continuous excretion. Nondiapausing larvae, however, did

Tbble 2. Number of larvae exhibiting gut conditions at low temperatures
(#) digestive tract dark stained by feeding
(+) faintly stained
(−) unstained

		(A) Diapausing larvae					(B) Nondiapausing larvae				
	Day	1	2	3	4	5	1	2	3	4	5
5°C	#	10	17	25	29	27	25	27	27	27	29
	+	13	8	2	1	1	2	0	0	3	1
	−	8	5	3	0	1	4	4	3	1	1
2°C	#	0	0	0	0	0	0	1	0	0	1
	+	0	1	2	0	1	0	0	1	2	2
	−	30	29	28	30	29	29	28	28	27	26
0°C	#	0	0	0	0	0	0	0	0	0	0
	+	0	0	0	0	0	0	0	0	0	0
	−	30	30	30	30	30	30	30	30	30	30

Larvae of 20–25 days age were used in both diapause committing and free conditions.

not show such differential responses as to food intake and defecation under low temperature. Their excretion was also terminated at about 2°C. Thus the nondiapausing larvae still maintained their gut contents at low temperatures. At 5°C, both diapausing and nondiapausing larvae did not exhibit rapid excretion (Fig. 2).

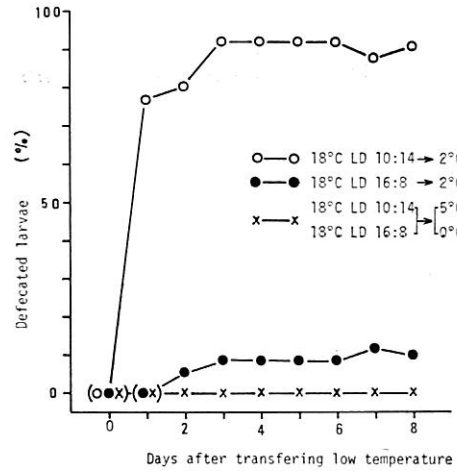

Fig. 2. Effects of transfering 20–25 day Trypanblau-medium feeding larvae from 18±1°C to low temperatures, 5±1°C, 2±1°C and 0±1°C on the defecation

Effects of inoculator intake, chilling and dehydration on supercooling point

The supercooling points of larvae which fed with inoculator containing medium ranged from −8 to −16°C (Fig. 3). These are fairly high compared with those of the

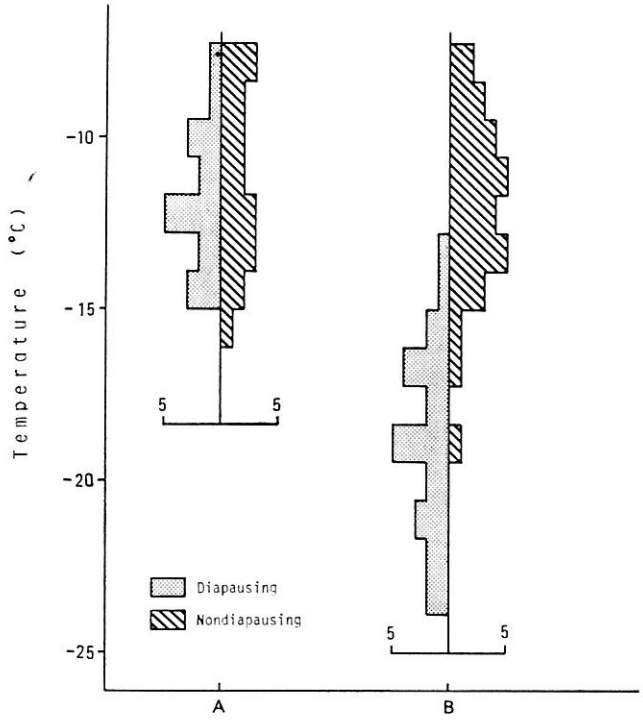

Fig. 3. Distribution of supercooling points of larvae fed on the nucleator containing medium. A: larvae fed on the nucleator-medium for 24 hr. at 18±1°C B: larvae acclimated at 2±1°C for one week after having fed nucleator-medium

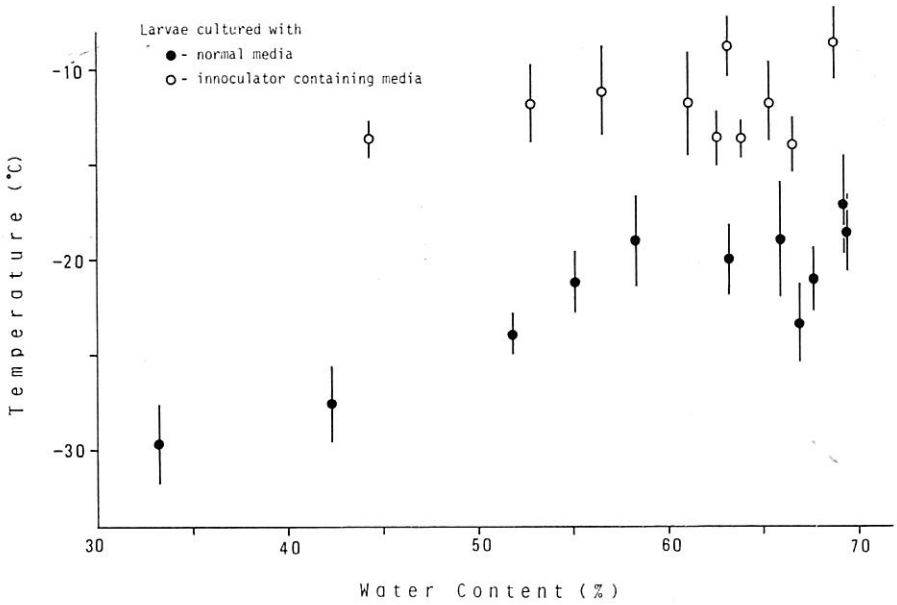

Fig. 4. Change of supercooling points of the diapause committed third instar larvae either dehydrated or not. Vertical bars show SD.

Table 3. Survival of the dehydrated larvae committed diapause

Nondehydrated						
Water content (%)	69.3	69.2	67.6	66.8	65.8	63.2
Survival	10/11	7/10	10/10	8/10	9/10	11/11
Dehydrated						
Water content (%)	58.3	55.1	51.8	42.2	33.1	
Survival	10/10	14/14	11/13	8/11	9/10	

A/B A : The number of individuals completed pupation
B : The number of larvae examined

Table 4. Survival of the dehydrated nondiapausing larvae

Nondehydrated					
Water content (%)	68.5	67.8	64.3	62.8	62.8
Survival	11/11	10/10	8/10	10/10	10/10
Dehydrated					
Water content (%)	55.5	48.7	45.8	42.3	30.4
Survival	10/10	8/11	9/10	7/11	2/10

A/B A : The number of individuals completed pupation
B : The number of larvae examined

larvae fed on the normal culture medium. After one week of chilling, the diapausing larvae froze at temperatures lower than those not committed chilling (mean supercooling point 18.1±2.5°C $n=24$ in chilled, 11.7±1.8°C $n=17$ in not chilled). In non diapausing larvae, however, such shift of supercooling points was not detected (12.0±2.5°C $n=29$ in chilled, 11.4±2.3°C $n=18$ in not chilled). The supercooling points of the larvae which fed on the normal culture medium were variable according to their water content, especially at the range of 30~60%. Without dehydration, larvae maintained the water content at about 63~70% and froze at about −20°C. The dehydrated larvae gradually lowered their supercooling points with decrease of water content. The diapause committed larvae dehydrated to about 33% froze at about −30°C. On the other hand, larvae fed on the inoculator containing culture medium froze at high temperatures compared with the larvae of same water content but fed on the normal culture medium. Further, the variation of supercooling points owing to the water content was less distinct in this group (Fig. 4). The survival ratio of dehydrated diapausing larvae was fairly high. The larvae committed dehydration to 33% water content still survived and maintained the ability to complete further development (Table 3). The nondiapausing larvae, on the other hand, were more susceptible to the dehydration more than 30% water loss (Table 4).

Discussion

Chy. costata was proved to be frost susceptible at any developmental stage. Therefore, the frost avoidance may be the main way for survival during winter as far as their microhabitats give no protection against low temperatures. It has been frequently reported in other insects that ingestion of various materials enhances the ice nucleation in the digestive tract (10, 11), and consequently decreases the cold hardiness in frost susceptible species. *Chy. costata* also shows the ice nucleation by ingesting the soil containing culture medium at higher temperatures. In this regard, the differential response of feeding and excretion of the diapausing larvae at a low temperature may be pertinent in avoiding the ice nucleation in the digestive tract. In fact, while the diapausing larvae fed on inoculator containing medium significantly depressed their supercooling points during one week of chilling at 2°C, the nondiapausing larvae did not. Although information on the pre-wintering habits in natural condition is unavailable, this physiological feature is to be pertinent in the process of acclimation or preparation of overwintering properties just before arrival of the freezing season.

The supercooling points are sometimes reported to correlate with water contents of insects (6). The present experiment, however, did not show such typical trend especially in the larvae feeding on the inoculator containing medium. Such tendency was only seen in individuals of low water con-

tents ranging from 30 to 60% and having fed inoculator-free medium. This may be due to the stochastic nature of supercooling points. The initiation of freezing is usually followed by freezing of the entire body. But it is the molecular environment around the initial ice nucleus that determines the supercooling point, the temperature at which freezing is started. Thus the crude water content would provide no sound forecast for the supercooling ability. This may be true especially in the inoculator fed larvae, in which the initial freezing would occur in the digestive tract, a part that might contain inoculators much more than in other body parts, providing a more heterogeneous molecular environment for nucleators. However, the high viability at advanced dehydration in the diapausing larvae and resulting decrease of supercooling points in the inoculator-free larvae may play a positive role in enhancing the frost avoiding ability. For full understanding of their cold resistance, studies of other aspects such as presence or absence of cryoprotectants (2, 4) in haemolymph, behavioural traits that enable the larvae to escape from the danger of freezing, and especially the actual observations of their winter life under natural condition are required.

Acknowledgments

I express my sincere thanks to Prof. Shôichi F. Sakagami for correcting the manuscript, and to Drs. Kouzou Tanno and Kimio Shimada for their pertinent guidance. Further, I emphasize my gratitude to Mr. M. J. Toda and Dr. M. T. Kimura for their valuable suggestions. Mrs. I. Numasa kindly helped me in preparing the manuscript.

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