Photoperiod-sensitive developmental delay in *facet* mutants of the drosophilid fly, *Chymomyza costata* and the genetic interaction with *timeless*

Kimio Shimada*

Institute of Low Temperature Science, Hokkaido University, Sapporo 060-0819, Japan

Abstract

Previously, we demonstrated that the biological clock gene, *timeless* (*tim*), is potentially involved in photoperiodic diapause induction in the larvae of the drosophilid fly *Chymomyza costata*. Suppression of *tim* transcription was inevitably associated with the loss of diapause induction even under diapause-promoting short-day conditions. In the present paper, I report a novel gene, *facet* (*fa*), which may genetically interact with *tim* in photoperiodically controlled larval development in this species. I demonstrated the effect of photoperiod on the development of *fa* morphological mutants. Developmental delay was remarkable in *fa* larvae under a diapause-preventing photoperiod, 16 hr light: 8 hr dark/day, in which the wild-type individuals showed normal and synchronous development. The delay was recovered by extension of the light phase of daily light/dark cycles or the introgression of a deficient *tim* allele.

Keywords: facet gene, photoperiodism, circadian clock, *timeless* gene, diapause.

* Corresponding author. Fax: +81-117067142. E-mail address: kshimada@pop.lowtem.hokudai.ac.jp

Introduction

Diapause is essential for the survival of insects living in seasonally unfavourable environments. Early observers assumed that diapause was a pathological state induced by depriving the insect of food or exposing it directly to harsh environmental conditions. However the programming of diapause is not so unpredictable. The environmental factors that induce diapause are not the environmental obstacles themselves, but rather environmental tokens that reliably predict the coming of an unfavourable season. Daylength is certainly the most widely exploited environmental token (reviewed by Danilevskii, 1965; Sauders, 1976; Tauber et al., 1986).

How does an insect measure the length of the day or the night and respond to unfavourable seasons. Since the 1920s, an enormous volume of work on insect photoperiodism has been carried out using a 'black box' approach. The traditional black box model includes a clock, an input pathway through which the clock receives light signals to synchronize the clock with the environmental day/night cycles, and an output pathway through which the clock controls development or diapause. Recent studies in this field, however, reveal some cellular and molecular systems inside the black box (Saunders et al., 2004). Involvement of clock-related genes (such as *cryptochrome, period, timeless, clock* and *cycle*) in photoperiodic induction of diapause have been examined in the flesh fly *Sarcophaga crassipalpis* (Goto and Denlinger, 2002), in the drosophilid fly *Chymomyza costata* (Kostal and Shimada, 2001; Pavelka et al., 2003), and the linden bug *Pyrrhocoris apterus* (Hodkova et al., 2003; Syrova et al., 2003). However,

most cellular and molecular elements of the insect photoperiodism still remain in the black box.

Previously, we demonstrated that the essential circadian clock gene, *timeless* (*tim*) is potentially involved in the photoperiodic diapause induction of *C. costata* (Pavelka et al., 2003). Suppression of *tim* gene expression was inevitably associated with the loss of diapause induction under diapause-promoting photoperiodic conditions. In the present paper, I report a novel gene, *facet*, which may play an important function in photoperiodically controlled larval development of this species.

Materials and methods

All strains of *Chymomyza costata* (Diptera: Drosophilidae) were maintained on an artificial agar medium as described before (Lakovaara, 1969). The wild-type (WT) strain was derived from flies collected in the suburbs of Sapporo (43°N, 141°E), Japan in 1983.The laboratory-cultured WT strain had the critical daylength of 14.75 hr at 18°C for diapause induction. The strain was maintained at 18°C under 16 hr light: 8 hr dark (16L: 8D)/day cycles.The *non-photoperiodic-diapause* (*npd*) mutant which was isolated by Riihimaa and Kimura (1988) from flies collected at Tomakomai (42°30'N, 141°30'E) was maintained at 18°C under 10L:14D photoperiodic cycles. The *npd* mutant has lost the ability to respond to photoperiod for diapause induction and carries a deficient *timeless* (*tim*) allele (Pavelka et al, 2003). Thus, the genotype of the *npd* fly was designated as tim^{-}, in this paper. An X-linked recessive *facet* (*fa*) mutant having small rough eyes and nicked wing veins was isolated from flies collected at Olekminsk (60°N, 120°E), Russia in 1993. The critical daylength of *fa* mutants was 19 hr at 18°C. Under 16L: 8D at 18°C, all individuals enter larval diapause. No larvae pupariated within 2 months after oviposition.

The *fa* mutant strain was maintained at 18°C in constant light. The *fa* males were back-crossed for more than 15 generations and bred into a WT genetic background. The established *fa* males did not enter diapause under 16L: 8D at 18°C, but unlike the WT strain, the larval development was remarkably retarded as described later. They were mated with WT virgin females. F1 hybrids were reared at 18°C in constant light to prevent diapause. F2 progeny was produced by intercrosses between F1 siblings. Newly hatched F2 larvae were transferred to various photoperiodic conditions at 18°C. About 1 month after the transfer, adult flies began to eclose from their pupal cases. The date of eclosion, sex and phenotype in each individual were scored. To examine the genetic interaction between *fa* and *tim*, *fa- tim* double mutant males (fa^{-};tim^{-}) were mated with tim^{-} virgin females. F2 progeny produced by intercrossing between F1 siblings was reared at 18°C in various photoperiodic conditions to count the day of adult eclosion and phenotype. Relative cumulative frequencies in male eclosion time were statistically analysed by the Kolmogorov-Smirnov two-sample test (Sokal and Rohlf, 1995). Females were not analysed because of their slight delay in eclosion time.

Results

Wild-type (WT) flies carrying genotype of ($fa^{+/+}$; $tim^{+/+}$) in female or ($fa^{+/y}$; $tim^{+/+}$) in male began to eclose 34 or 33 days after oviposition, when they were reared at 18°C, 16L:8D in the preceding larval stage (Fig. 1A, Table 1). Around 3 days after the first eclosion, 50 % of the individuals had emerged as adult flies. The relatively rapid and synchronous eclosion continued until 42 days after oviposition, and it was followed by occasional eclosion. Within 2 months, almost all (99.8 %) embryos completed their larval

and imaginal development. The S-shape cumulative eclosion curve was common among the eclosion curves obtained with various photoperiodic and genetic conditions (Fig. 1).

Mating between WT (*fa* $^{+/+}$; *tim*$^{+/+}$) females and *facet* (*fa*) males (*fa*$^{-/y}$; *tim*$^{+/+}$) and the following intercrossing produced F2 progenies carrying 4 different genotypes: (*fa* $^{+/+}$; *tim*$^{+/+}$), (*fa* $^{-/+}$; *tim*$^{+/+}$) females and (*fa*$^{+/y}$; *tim*$^{+/+}$), (*fa*$^{-/y}$; *tim*$^{+/+}$) males. The *fa*$^{-}$ phenotype was always linked with male flies and the phenotypic ratio between WT and *fa*$^{-}$ was nearly 3:1, suggesting that *fa* is a recessive allele on the X-chromosome that shows Mendelian inheritance. Among the F2 progenies, *fa*$^{-}$ males (*fa*$^{-/y}$; *tim*$^{+/+}$) showed a peculiar response to photoperiod. Even under16L:8D at 18°C which promoted rapid and synchronous eclosion in the WT flies, the mutant larvae required a long growth time to complete their development (Fig.1B, Table 1). The first mutant fly emerged 34 days after oviposition. It was almost the same as the WT male fly. However, eclosion of mutant flies was seldom during the following 5 days. At this period, over 60 % of WT individuals had emerged as adult flies. Dominant eclosion occurred during the period between 40 and 65 days after oviposition, showing 50 % eclosion at 49 days after oviposition. It was approximately 10 days later than in WT males. The Kolmogorov-Smirnov test indicated that the difference in the relative cumulative frequencies of eclosion time between WT males and *fa*$^{-}$ males was very large and statistically significant (the largest unsigned difference between the relative cumulative frequencies, D_{max}=0.69617; the critical value for a 5 % probability, $D_{.05}$=0.15572; for a 1 % probability, $D_{.01}$=0.18662, P<<0.01).

The developmental delay was recovered by extension of the light phase in daily light/dark cycles. Under 18L:6D (2 hr extension of light phase), eclosion of *fa*$^{-}$ males started at 34 days and ended 50 days after oviposition (Fig. 1C, Table 1). The median

eclosion date was 37.55 days, one day later than in WT males. Statistical tests still indicated a significant difference between the two (D_{max}=0.23026, $D_{.05}$=0.15140, $D_{.01}$=0.18145, P<0.01). Increase of the light phase to 20 hr/day brought a decrease in the difference of eclosion pattern between WT and *fa*$^{-}$ males (D_{max}=0.11829, $D_{.05}$=0.11832, $D_{.01}$=0.14180, P=0.05) (Fig. 1D, Table 1). Under constant light, the difference was statistically not significant (D_{max}=0.10651, $D_{.05}$=0.11478, $D_{.01}$=0.13756, P>0.05) (Fig. 1E, Table 1).

The developmental delay of *fa* mutants under a long-day photoperiod of 16L: 8D was also recovered by the introgression of a deficient *tim* allele into *fa*$^{-}$ progeny (Fig. 1F, Table 1). In *fa-tim* double mutants, the eclosion started at 33 days and ended 55 days after oviposition. 50% of the male flies (*fa*$^{-}$; *tim*$^{-}$) had completed their eclosion around 37 days after oviposition. These developmental traits were statistically different from those of *tim* single mutant males (D_{max}=0.19868, $D_{.05}$=0.15675, $D_{.01}$=0.18786, P<0.01). But the difference between the D_{max} and the critical value ($D_{.01}$) was small.

Discussion

In the present study, I demonstrated the effect of photoperiod on larval development in the *facet* (*fa*)morphological mutants of the drosophilid fly, *Chymomyza costata*. Developmental delay was remarkable in *fa* mutant larvae under a diapause-preventing long-day photoperiod of 16L: 8D in which the wild-type (WT) flies showed rapid and synchronous eclosion. The delay was recovered by extension of the light phase of daily light/dark cycles or the introgression of a deficient *timeless* (*tim*) allele.

Developmental delay in mutant strains has been well demonstrated in the congeneric species, *Drosophila melanogaster* (see Lindsley and Zimm, 1992). As in the *ecdysoneless*,

some developmental delays caused by mutation are temperature-sensitive: the deficient phenotypes are hidden by decreased or increased temperature. However, photoperiod-sensitive mutants for developmental delay are rare in literature. Photoperiod-sensitive or photoperiod-regulating developmental delay itself is not unusual among insect species. The duration of nymphal or larval stages in certain species is frequently regulated by photoperiod. It is prolonged with the initiation and maintenance of diapause and shortened with termination or avoidance (Danilevskii, 1965; Danks, 1987). In some crickets, the developmental delay at the nymphal stage is controlled by photoperiod without a commitment to diapause (Masaki and Walker, 1987). The molecular mechanism of these developmental delays, however, remains largely unsolved. Thus the present *fa* mutant may provide a useful tool for the genetic and molecular analysis of insect photoperiodism.

The *fa* gene of *C. costata* may be involved in the differentiation of the ectoderm and possibly in the activation of the prothoracic gland, as suggested by its effect on the eye and wing morphology and on the larval growth rate. The expression of *fa* may be negatively controlled by the *tim* protein (TIM), because extension of the light phase of daily light/dark cycles or the introgression of a deficient *tim* allele was effective in recovery of the developmental delay. TIM is known to be unstable in the presence of light (Myers et al., 1996). CRYPTOCHROME-mediated ubiqutination is assumed to promote the degradation of TIM in response to light (Ceriani et al., 1999). Thus, extension of the light phase might be effective in recovery of the developmental delay by reducing the suppressive activity of TIM on the *fa* gene or its transcript.

As described above, *fa* is a single recessive gene on the X-chromosome. On the same chromosome, the presence of gene controlling the critical photoperiod was suggested by

Riihimaa and Kimura (1989). Previously, I reported that the gene termed as *critical photoperiod* (*cpp*) is not identical with the clock gene, *period* (Shimada, 1999). The genetic linkage between *cpp* and *fa* is still unknown. However, these two genes have a common trait for genetic interaction with the *tim* gene. Larval developmental delay associated with fa^{-} phenotypes was recovered by the introgression of a deficient *tim* allele. In the larvae carrying a wild-type *cpp* allele, their critical daylength was sifted to a shorter daylength with the $tim^{-/+}$ genetic background (Riihimaa and Kimura, 1989). In both cases, the reduction in *tim* expression seems to promote continuous development without short-term (in fa^{-}) or long-term (in WT) delay. Future studies will be required to distinguish the relative roles of these two genes.

In general, larval diapause of insects is thought to be caused by a shutdown of the brain-prothoracic gland axis which would lead to a reduction in ecdysone synthesis (Denlinger, 1985). Thus, the molecular analysis of the biological clock in the brain and its output pathway(s) terminating in ecdysone secretion is needed for further understanding of insect diapause. In this context, the present results demonstrating the genetic interaction between *tim* (a biological clock component) and *fa* (a cell differentiation factor) may provide a key information on the molecular basis of the clock output pathway(s).

References

Ceriani, M.F., Darlington, T.K., Staknis, D., Mas, P., Petti, A.A., Weitz, C.J., Kay, S.A., 1999. Light-dependent sequestration of TIMELESS by CRYPTOCHROME. Science 285, 553-556.

Danilevskii, A.S., 1965. Photoperiodism and Seasonal Development of Insects. Oliver and Boyd, Edinburgh.

Danks, H.V., 1987. Insect Dormancy: An Ecological Perspective. Biological Survey of Canada, Ottawa.

Denlinger, D.L., 1985. Hormonal control of diapause. In: Kerkut, G.A., Gilbert, L.I. (Eds), Comprehensive Insect Physiology Biochemistry and Pharmacology, vol. 8. Pergamon Press, Oxford, pp. 353− 412.

Goto, S.G., Denlinger, D.L., 2002. Short-day and long-day expression patterns of genes involved in the flesh fly clock mechanism: *period*, *timeless*, *cycle* and *cryptochrome*. Journal of Insect Physiology 48, 803-816.

Hodkova, M., Syrova, Z., Dolezel, D., Sauman, I., 2003. *Period* gene expression in relation to seasonality and circadian rhythms in the linden bug, *Pyrrhocoris apterus* (Heteroptera). European Journal of Entomology 100, 267-273.

Kostal, V., Shimada, K., 2001. Malfunction of circadian clock in the non-photoperiodic-diapause mutants of the drosophilid fly, *Chymomyza costata*. Journal of Insect Physiology 47, 1269-1274.

Lakovaara, S., 1969. Malt as a culture medium for Drosophila species. Drosophila Information Service 44, 128.

Lindsley, D.L., Zimm, G.G., 1992. The Genome of *Drosophila melanogaster*. Academic Press, New York.

Masaki, S., Walker, T.J., 1987. Criket life Cycles. Evolutionary Biology 21, 349-423.

Myers, M.P., Wager-Smith, K., Rothenfluh-Hilfiker, A., Young, M.W., 1996. Light-induced degradation of TIMELESS and entrainment of the *Drosophila* circadian clock. Science 271, 1736-1740.

Pavelka, J., Shimada, K., Kostal, V., 2003. TIMELESS: A link between fly's circadian and photoperiodic clocks? European Journal of Entomology 100, 255-265.

Riihimaa, A.J., Kimura, M.T., 1988. A mutant strain of *Chymomyza costata* (Diptera: Drosophilidae) insensitive to diapause-inducing action of photoperiod. Physiological Entomology 13, 441− 445.

Riihimaa, A.J., Kimura, M.T., 1989. Genetics of the photoperiodic larval diapause in *Chymomyza costata* (Diptera; Drosophilidae). Hereditas 110, 193− 200.

Saunders, D.S., 1976. Insect Clocks. Pergamon Press, Oxford.

Saunders, D.S., Lewis, R.D., Warman, G.R., Photoperiodic induction of diapause: opening the black box. Physiological Entomology 29, 1-15.

Shimada, K., 1999. Genetic linkage analysis of photoperiodic clock genes in *Chymomyza costata* (Diptera: Drosophilidae). Entomological Science 2, 575-578.

Sokal, R.R., Rohlf, F.J., 1995. Biometry (3rd Edition). Freeman, New York.

Syrova, Z., Dolezel, D., Sauman, I., Hodkova, M., 2003. Photoperiodic regulation of diapause in linden bugs: are *period* and *Clock* genes involved? Cellular and Molecular Life Sciences 60, 2510-2515.

Tauber, M.J., Tauber, C.A., Masaki, S., 1986. Seasonal Adaptations of Insects, Oxford University Press, New York.

Table 1. Effect of diapause-preventing long-day photoperiods on development of *facet* mutants in the drosophilid fly,

Chymomyza costata

Experiments	Parents	Phenotypes	Genotypes	Photoperiods light:dark	No. of individuals	Day of first eclosion	Day of 50% eclosion	Day of last eclosion	Dmax*	P
A	WT female	WT female	fa+/+;tim+/+	16L:8D	548	34	36.95	56		
	WT male	WT male	fa+/Y;tim+/+	16L:8D	567	33	36.27	56		
B	WT female	WT female	fa+/+;tim+/+, fa+/-;tim+/+	16L:8D	348	31	38.54	66		
	fa male	WT male	fa+/Y;tim+/+	16L:8D	168	32	37.38	58		
		fa male	fa-/Y;tim+/+	16L:8D	139	34	49.25	72	0.69617	P<0.01
C	WT female	WT female	fa+/+;tim+/+, fa+/-;tim+/+	18L:6D	317	31	36.8	55		
	fa male	WT male	fa+/Y;tim+/+	18L:6D	171	34	36.42	49		
		fa male	fa-/Y;tim+/+	18L:6D	152	34	37.55	50	0.23026	P<0.01
D	WT female	WT female	fa+/+;tim+/+, fa+/-;tim+/+	20L:4D	574	33	37.7	66		
	fa male	WT male	fa+/Y;tim+/+	20L:4D	262	33	37.65	54		
		fa male	fa-/Y;tim+/+	20L:4D	265	33	37.94	54	0.11829	P=0.05
E	WT female	WT female	fa+/+;tim+/+, fa+/-;tim+/+	LL	630	31	39.51	64		
	fa male	WT male	fa+/Y;tim+/+	LL	298	31	39.31	61		
		fa male	fa-/Y;tim+/+	LL	264	33	39.94	66	0.10651	P>0.05
F	tim female	tim female	fa+/+;tim-/-, fa+/-;tim-/-	16L:8D	296	32	37.44	54		
	fa-tim male	tim male	fa+/Y;tim-/-	16L:8D	158	33	37.56	57		
		fa-tim male	fa-/Y;tim-/-	16L:8D	143	33	36.39	55	0.19868	P<0.01

*D_{max} is the largest unsigned difference between the relative cumulative frequencies illustrated in Fig. 1.

The values were obtained by the Kolmogorov-Smirnov two-sample test.

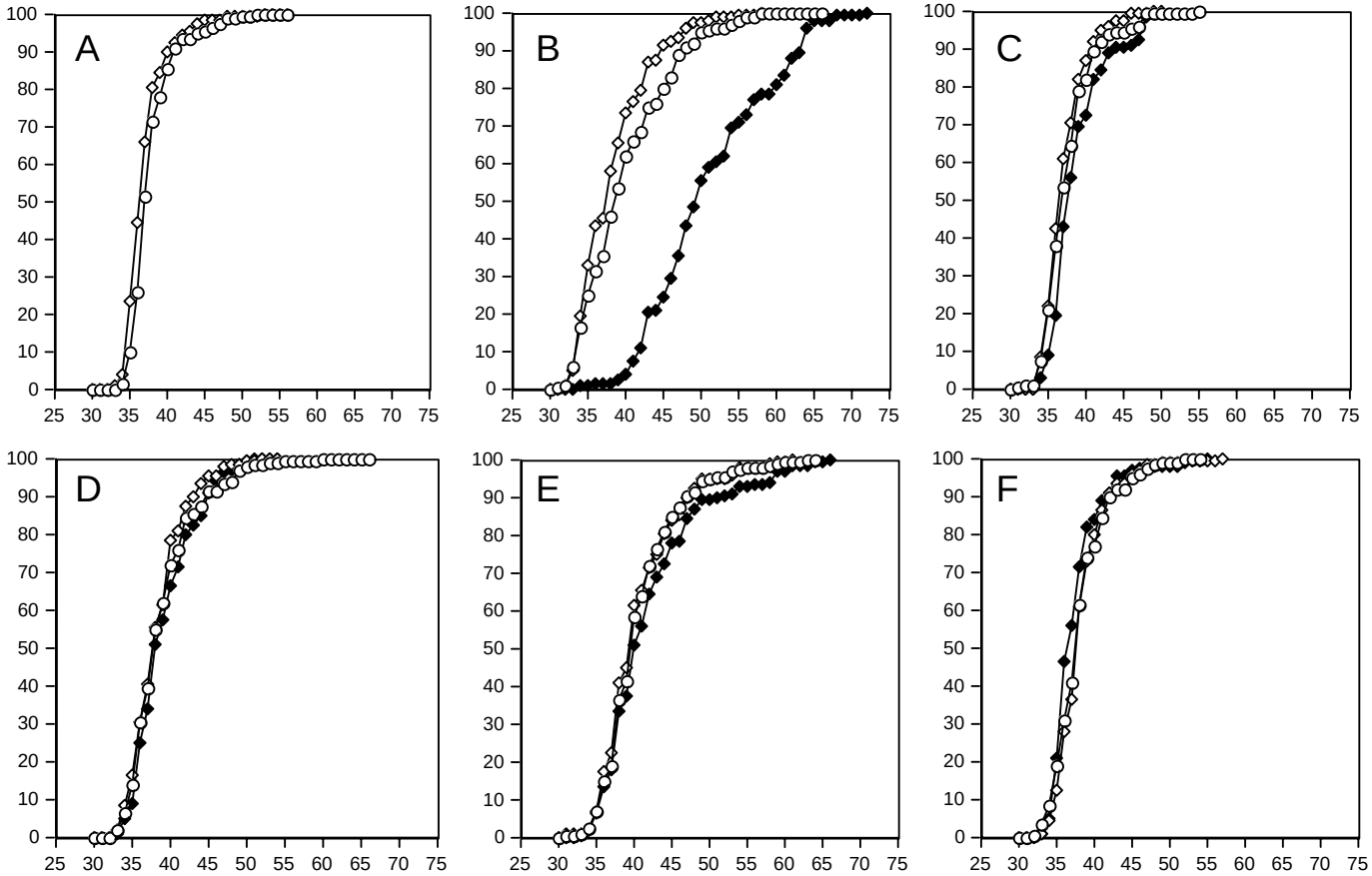

Fig. 1. Effect of diapause-preventing long-day photoperiods on development of *facet* mutants in the drosophilid fly, *Chymomyza costata*. Horizontal axis, time of eclosion (days after oviposition); vertical axis, cumulative eclosion (%); open circle, wild type female (A-E) or *timeless*$^{-}$ female (F); open diamond, wild-type male (A-E) or *timeless*$^{-}$ male (F); solid diamond, *facet*$^{-}$ male (A-E) or *facet*$^{-}$;*timeless*$^{-}$ male (F); A-F, correspond to Experiments A-F in Table 1.