Alteration of the eclosion rhythm and eclosion behavior in the flesh fly, *Sarcophaga crassipalpis*, by low and high temperature stress

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Heat shock (45°C), cold shock (−10°C), and indirect chilling injury (a prolonged exposure to 2°C) did not interfere with the continuation of pharate adult development in the flesh fly, *Sarcophaga crassipalpis*, but such flies failed to eclose properly when the exposure was of sufficient duration. In all three forms of injury, development following the temperature treatments was also retarded. Among flies that were less severely affected and still capable of emerging as adults, the circadian time of adult eclosion shifted from near dawn to near the middle of the photophase, thus suggesting that the neurally-based clock is among the systems most vulnerable to heat-shock and cold-shock injury. Tensiometric records of ptilinum expansion revealed important differences in the nature of the injury caused by the different temperature stresses. Heat-shocked flies and those subjected to indirect chilling injury displayed the two behavioral programs normally associated with adult eclosion, the program for obstacle removal (POR) and the program for forward movement (PFM), but such flies failed to eclose because the muscle contractions generated by these motor patterns were insufficient for successful eclosion. In contrast, cold-shocked flies retained the capacity for strong muscle contraction, but the centrally-generated POR and PFM programs were altered. As the duration of cold shock increased, both patterns became more erratic; the PFM program was then lost completely, and in the most severe cases of cold-shock injury, flies lost the capacity to generate both programs. This suggests that neuronal damage is the likely cause of injury inflicted by cold shock.

Eclosion  Circadian rhythm  Heat shock  Cold shock  Chilling injury

**INTRODUCTION**

The thermal history of an insect can have profound effects upon subsequent developmental stages of that insect. At the extremes, death is immediate, but at less severe temperatures death may occur much later or the insect may in some other manner be affected. For example, pupal exposure to supraoptimal temperatures causes wing deformation in *Drosophila melanogaster* (Milkman, 1962), *Ephesia cautella* and *Plodia interpunctella* (Arbogast, 1981). Supraoptimal temperature exposure during the pupal stage of *Trogoderma granarium*, *Tribolium castaneum*, *Callosobruchus chinesis*, *E. cautella* and *P. interpunctella* reduces fecundity or caused complete sterility (Arbogast, 1981; Saxena et al., 1992). Larvae of *Aedes sierrensis* that are genetically male are feminized when reared at 30–31°C (Horsfall et al., 1964). Heat shocking pupae or pharate adults of *D. melanogaster* (Mitchell and Lipps, 1978) and *Sarcophaga crassipalpis* (Delinger et al., 1991) does not interfere with pharate adult development, but the heat-shocked flies fail to emerge from the puparium.

Similar effects are apparent in response to suboptimal temperatures (Sehnal, 1991). Cooling larvae of *Eurosta solidaginis* to −40°C increases mortality at subsequent developmental stages (Bale et al., 1989). Egg viability in *Musca domestica* is reduced by exposing pharate adults to −7°C (Coulson and Bale, 1992), and pharate adults of *S. crassipalpis* that have been cold shocked at −17°C continue to develop to the completion of the pharate adult stage but fail to eclose (Lee and Denlinger, 1985). Clearly, the effects of nonoptimal temperature exposure are not always immediate but may be manifested, subtly or catastrophically, at a later point in the insect's life.

In this study, we examine the nature of the injury inflicted by temperature stress in the flesh fly, *S. crassipalpis*. We compare the effects of heat shock (injury caused by a brief exposure to high temperature), cold
shock (nonfreezing injury caused by a brief exposure to a low temperature that is above the supercooling point), and indirect chilling injury (injury caused by a prolonged exposure to a less severe low temperature). All three of these forms of injury have some common elements (Denlinger et al., 1991). In S. crassipalpis, heat shock (Chen et al., 1991; Denlinger et al., 1991), cold shock (Joplin et al., 1990), and indirect chilling injury (Chen and Denlinger, 1992) during pharate adult development permit the continuation of development, but in each case, adult flies fail to eclose properly from the puparium. Moreover, both heat shock (Joplin and Denlinger, 1990) and cold shock (Joplin et al., 1990) elicit the synthesis of stress proteins, and injury at both temperature extremes can be prevented by first exposing the flies to a less severe high or low temperature (Chen et al., 1987, 1991; Lee et al., 1987; Yocum and Denlinger, 1992). But, some differences in the responses are apparent. Glycerol is elevated by low temperature (Lee et al., 1987) but not by high temperature (Chen et al., 1990), and, although stress proteins are synthesized in response to both temperature extremes, some of the stress proteins are different (Joplin et al., 1990).

Though both temperature extremes result in injury that is manifested at the time of adult eclosion, it is not clear whether the different forms of injury have the same cause. We search for clues to the nature of the injury by comparing details of adult eclosion. Do the flies injured by these different methods die at exactly the same stage? Does injury alter the duration of pharate adult development or the circadian rhythm of eclosion? Eclosion in the higher Diptera involves a set of behaviors that enable the fly to escape from the puparium and crawl free to the surface of the substrate. By contracting abdominal and thoracic muscles the fly increases its hemocoelic pressure and thereby expands its ptilinum to open the operculum. When the fly extended its ptilinum the shot was dislodged and fed via a funnel into a fraction collector (Isco model 1850) set to collect 30 min fractions. Pharate adults (N = 100) were used for each treatment and control, and each experiment was replicated three times. All eclosion experiments were conducted under a 15L:9D regime at 25°C.

**Measurement of eclosion time**

Individual pharate adults were placed in test tubes (5 mm i.d.), and a color-coded metal ball (air gun shot, 4.5 mm) was inserted on top. Each tube was nearly horizontal, with the metal ball resting against the anterior cap of the puparium. When the fly extended its ptilinum the shot was dislodged and fed via a funnel into a fraction collector (Isco model 1850) set to collect 30 min fractions. Pharate adults (N = 100) were used for each treatment and control, and each experiment was replicated three times. All eclosion experiments were conducted under a 15L:9D regime at 25°C.

**Tensiometric measurement**

Eclosion behavior was recorded by a tensiometric apparatus that measured ptilinal movements caused by hemocoelic pressure fluctuations reflecting contractions of the abdominal and thoracic muscles (Zdarek et al., 1986). A pharate adult still within the puparium was placed into a plastic cone (disposable Eppendorf pipette tips), the narrow tip of which was cut off to create an opening wide enough to allow the fly to expand the ptilinum but not to escape. A tensiometric sensor for a force-displacement transducer FTO3C (Grass Instruments, Quincy, MA, U.S.A.) was placed in front of the ptilinum to detect movements and transform them into electrical signals that were amplified and recorded. Flies most seriously injured by heat or cold shock failed to open the operculum by themselves; the puparial cap of such defective flies was removed so that the head of the fly was in direct contact with the sensor.

**Statistics**

The medians of eclosion time were analyzed by a nonparametric χ²-test (Siegel, 1956). Controls from all 12 experiments were pooled for statistical analysis and presentation.

**RESULTS**

**Heat-shock injuries**

Heat-shock injury inflicted by exposure to 45°C during larval or pharate adult development in S. crassipalpis is manifested at adult eclosion by several problems: (1) failure of adult eclosion, (2) delay in the day of eclosion, (3) delay in the circadian timing of eclosion, and (4) alteration of the behavioral program associated with extrication from the puparium.

First, eclosion failure represents a gradation of injury.
For example, a 70 min exposure of pharate adults (red-eye stage) to 45°C permitted nearly half of the flies to successfully emerge [Fig. 1(A)]. Most of the others treated for 70 min could expand the ptilinum but failed to emerge. An increase of exposure time increased the severity of the injury, and at the extreme time tested (120 min), none emerged and most were incapable of even expanding the ptilinum.

Secondly, flies that were heat shocked at 45°C as pharate adults [Figs 2(B) and (C)] or as wandering larvae [Fig. 2(E)] for short periods that were nonlethal (15–60 min) were developmentally delayed. Adult eclosion is a gated response [Fig. 2(A)], and eclosion for the control flies was fairly equally divided between two peak days of emergence, beginning 14 days after pupariation at 25°C. But in the heat-shocked groups, fewer flies eclosed during the first gate. The majority of flies heat shocked as pharate adults eclosed during the second gate [Figs 2(B) and (C)], and among flies heat shocked as wandering larvae, many flies delayed eclosion until the third gate [Fig. 2(F)]. Pharate adults can be made tolerant of a 1 h exposure to 45°C by first exposing them to 40°C for 2 h (Yocum and Denlinger, 1993). When pharate adults were manipulated in this manner to generate thermotolerance, the delay in eclosion was also eliminated [Fig. 2(D)].

Thirdly, the circadian pattern of eclosion was dramatically altered by heat shock. A circadian rhythm of eclosion is well recognized in flesh flies (Saunders, 1976, 1979), and in S. crassipalpis reared under a 15L:9D cycle, eclosion begins late in the scotophase and reaches a peak shortly after the beginning of the photophase [Fig. 2(A), median eclosion 1 h after lights-on]. A circadian pattern persisted in the heat-shocked flies, but the time of eclosion was delayed in a dose-dependent manner. Among pharate adults heat shocked at 45°C for 30 min, very few flies eclosed during the scotophase, and the median emergence was 4 h (gate 1) or 3 h (gate 2) after lights-on [Fig. 2(B)]. For pharate adults heat shocked for a longer period, 60 min, median emergence was delayed until 7 h (gate 1) or 6.5 h (gate 2) after lights-on. A slight delay in the circadian time of eclosion was evident in the flies that were heat shocked for 15 min as wandering larvae [Fig. 2(E)]. Generation of tolerance to a 1 h exposure to 45°C by a 2 h pretreatment at 40°C was successful in shifting median eclosion time to the left [Fig. 2(D)], but this treatment did not completely restore the pattern observed in untreated controls [Fig. 2(A)]. In all the above experiments, the heat shock was administered to the pharate adults early in the photophase (2–3 h after lights-on). The same effect was noted when the flies were heat shocked at the end of the photophase (data not shown).

To test if the shift in eclosion time was simply the result of the heat-shocked flies struggling for a longer time before they succeeded in opening the operculum, a subset of pharate adults heat shocked for 60 min was visually observed. The operculum was removed from the puparium late in pharate adult development, and the onset of ptilinum expansion was noted. The delay was reflected in the onset of ptilinum expansion (data not shown), thus indicating that the entire behavioral program was shifted in time.

The final aspect of heat shock injury that we examined was its effect on the patterns of extrication behavior. Two distinct behavioral patterns characterize the normal extrication process (Ždárek et al., 1986): a program for obstacle removal (POR) that is essential for removal of the operculum from the puparium and a program for forward movement (PFM) that allows the fly to extricate itself from the puparium and to move upward through the soil. Tensiometric recordings of the ptilinum movements readily distinguish these two patterns in normal flies [Fig. 3(A)]. Heat-shocked flies that failed to open the
FIGURE 2. The adult eclosion pattern in *S. crassipalpis* that were thermally stressed during pharate adult (red-eye) development (B, C, D, F, G), or as third instar (wandering) larvae (E). Nonstressed control (A), 30 min at 45°C (B), 60 min at 45°C (C), 2 h at 40°C then transferred immediately to 45°C for 1 additional h (D), 15 min at 45°C (E), 30 min at -10°C (F), 60 min at -10°C (G). Shaded area indicates scotophase. *Indicates the median time of eclosion; N, total number of flies that eclosed; %, percentage of flies eclosing within each 24 h period. All treatments are significantly different from the controls (A): $P < 0.05$ for second day of eclosion of B and G; for all others, $P < 0.001$; days with less than 10% eclosion were not tested statistically. Within the heat shock comparison, C is significantly different from B and D ($\chi^2; P < 0.001$), but B is not different from D ($\chi^2; P > 0.05$). The cold shock treatment F is not significantly different ($\chi^2; P > 0.005$) from G.

operculum were monitored tensiometrically by opening the operculum and placing the sensor directly against the ptilinum. Clearly, both the POR and PFM programs were expressed in the heat-shocked flies, but the amplitude of the muscular contractions responsible for these behavioral patterns was much lower as a result of heat.
FIGURE 3 (Caption opposite.)

A. Control, 25°C

B. 45°C, 80 min

C. -10°C, 45 min

D. -10°C, 60 min

E. -10°C, 75 min

F. 0°C, 10d
shock [Fig. 3(B)]. The intensity of the muscular efforts, as revealed by the amplitude of the ptilinal movements, progressively decreased with increased severity of heat shock from a mean value of 0.46 mm (N = 3) obtained from flies exposed for 70 min to less than 0.1 mm in flies exposed for 90–120 min (N = 10). Mean value of control flies was 0.89 mm (N = 6).

Cold-shock injuries

A set of experiments parallel to the above heat-shock experiments examined injury inflicted by cold shock, a brief exposure to −10°C. At this temperature, the body water in S. crassipalpis is supercooled, but does not freeze.

As observed with heat shock, increasing exposure time to the temperature stress, −10°C in this case, increased the severity of the injury. When pharate adults were exposed to −10°C for 45 min, nearly half the adults succeeded either in emerging or partially emerging from the puparium [Fig. 1(B)]. Interestingly, partial emergence was prevalent in this cold-shock treatment and in other unreported experiments we have completed at this temperature, but none of the heat-shocked flies were in that category [Fig. 1(A)]. With longer exposure to −10°C, emergence success decreased and fewer individuals were capable of expanding the ptilinum. When cold-shocked flies that failed to extricate themselves from the puparium were freed manually, they were unable to reorient their legs to the walking position. Instead, they remained in the “pupal” posture jerking their legs and displaying tremors.

As observed with the heat-shocked flies, development was delayed when pharate adults were cold shocked. Though adult eclosion occurred over a 3 day period, relatively few emerged during the first gate; most emerged during the second gate [Figs 2(F) and (G)].

Cold shock administered to pharate adults also shifted the peak of adult eclosion later into the pharate adult development (χ² = 0.005–0.001), although the delay was not as pronounced as that observed with heat shock, nor was the effect dose dependent ([i.e., times of eclosion did not differ for 30 min [Fig. 2(F)] or 60 min [Fig. 2(G)] exposure to −10°C, χ² tests, *P > 0.05*]).

Tensiometric measurements of ptilinal movements revealed abnormal features in the performance of extrication behavior and provided quantitative data on the degree of impairment of muscle strength exerted during extrication. Flies that were least affected (45 min at −10°C) typically displayed both the POR and PFM programs [Fig. 3(C)], but performance of both programs deviated from the normal pattern [Fig. 3(A)]. Though the amplitude of the muscle contractions was approximately as high as in the controls [compare Fig. 3(A) and (C)], the POR cycles were rather irregular and the contractions were twice as fast as in the control flies. The PFM cycles were even more irregular; the pulses of the ptilinum were erratic and at least four times faster than in normal flies. Moreover, the PFM cycles lacked some components characteristic of the peristaltic contractions of the thoracic and abdominal intersegmental muscles. Contraction of these muscles is essential if the fly is to be successful in making the forward movements needed for extrication and digging through the soil (Reid et al., 1987). The absence of these contractions could also be noted visually by the lack of “shoulder” movements that are visible on the thorax of normal flies during extrication.

Interestingly, the quantitative aspects of the two extrication programs, as reflected in the amplitudes of the displacement curves (0.92 mm, *N = 11*), did not differ much from that observed in normal flies (0.89 mm, *N = 6*). This implies that muscle strength was not affected by a 45 min cold shock.

With a more severe cold shock (60 min at −10°C), the POR cycles were still performed, but the intensity of the muscular contractions was reduced (0.14 mm, *N = 5*) [Fig. 3(D)]. The PFM cycles were typically absent or greatly suppressed. Flies that were too weak to even rupture the old pupal cuticle after removal of the operculum [most of the flies exposed to −10°C for 75 min, Fig. 1(B)] failed to display regular patterns of muscular contraction, although very feeble, slow pressure fluctuations could be detected [Fig. 3(E)].

Long-term chilling injury

The third form of temperature injury tested was indirect chilling injury, the form of low temperature injury caused by a long-term exposure to a less severe temperature. In this experiment, pupae [2 days after pupariation at 25°C, Fig. 4(A)] or pharate adults [red-eye stage, Fig. 4(B)] were exposed to 2°C for varying numbers of days and then transferred to 25°C for the remainder of pharate adult development. Pupae readily tolerated up to 20 days at 2°C, but beyond 20 days, fewer flies succeeded in emerging [Fig. 4(A)]. Pharate adults (red-eye stage) were less tolerant: adult eclosion was reduced by a 15-day exposure to 2°C, and very few survived a 25-day exposure [Fig. 4(B)]. Pupae and pharate adults (red-eye stage) were more tolerant of long-term exposure to 2°C than either a later stage of pharate adult development (none at the black-bristle stage survived beyond 20 days) or third instar larvae exposed during the wandering phase or at the time of pupariation (none survived beyond 10 days) (data not shown).

As observed with the heat- and cold-shocked flies, long-term chilling also retarded subsequent development at 25°C, as evidenced by the dose-dependent increase in the amount of time required by such flies to emerge following their transfer to 25°C [Fig. 4(A) and (B)]. Circadian patterns of eclosion were not monitored in this treatment group.

Visual and tensiometric observations were made using pharate adults (black-bristle stage) that had been chilled for 10 days. Most of these flies failed to eclose properly. Some opened the operculum, but extrication from the puparium took much longer (30–50 min) than in controls (< 2 min). By removing the operculum of flies that failed to emerge, extrication behavior could be observed...
in some individuals. Most flies in this category eventually emerged from the puparium after a prolonged (30–60 min) period. Some that did eventually emerge failed to expand their wings and did not properly withdraw their ptilinum. The tensiometric record indicates the flies that were unable to open the operculum were capable of performing normal eclosion behavior, including both the POR and PFM program [Fig. 3(F)], but the muscular effort, expressed by the amplitude of the tensiometric sensor, was only about one-third (0.39 mm, N = 9) of that in control flies (0.96 mm, N = 6).

DISCUSSION

All three forms of temperature-related injury, heat shock, cold shock and indirect chilling injury, enabled the pharate adults to continue developing up to the point of adult eclosion, but many of the flies were unsuccessful in extricating themselves from the puparium. This is a logical bottleneck for developmental problems because eclosion demands the institution of new behavioral programs that require a new expression of coordinated neural and muscular activity. It is a common point of death for a range of developmental problems. Flesh fly larvae, pupae and pharate adults treated with juvenoids or pharate adults exposed to excessive amounts of ecdysteroids also die when they reach this stage (Mitchell, 1966; Lindsley and Poodry, 1977). Injury can be reduced by first exposing the flies to less severe temperatures (Chen et al., 1991; Yocum and Denlinger, 1992), and in this experiment, we noted that thermotolerant flies not only survived the heat-shock temperature but the characteristic delay in development was also prevented.

One of the most striking effects of heat shock and cold shock was the alteration of the eclosion rhythm. Adult eclosion in flesh flies (Saunders, 1976, 1979) and many other flies (Denlinger and Ždárek, 1994) is a circadian event with peak activity occurring near dawn. Median eclosion time for our colony of S. crassipalpis reared under a 15L:9D cycle was 1 h after the beginning of the photophase. The eclosion rhythm clearly persisted after red-eye pharate adults (3–4 days before the expected day of eclosion) were heat shocked or cold shocked, but these treatments shifted the timing of the peak. The most pronounced effect, caused by a 60 min heat shock at 45°C, shifted the peak of adult eclosion to the middle of the photophase. This shift was not the result of an increase in "struggling time" for the fly to escape from the puparium because visual observations verified that the "late" flies made no attempt to expand the ptilinum until the moment of eclosion. The clock controlling eclosion in Drosophila pseudoobscura can be altered by temperature shifts (a shift from a light-dark cycle at 26°C to constant dark at 16°C caused a 12 h shift the next day, but subsequent peaks were only 3 h out of phase with the entrainment cycle) (Pittendrigh, 1954), and a 4 min heat shock at 40°C can cause a 1–4 h delay phase shift under constant darkness (Maier, 1973). Both reports on D. pseudoobscura showed a very immediate response to a change in temperature. In contrast, the effect we observed was manifested several days after the temperature stress was administered, and during this entire period the flies remained under the original light.
entrainment regime. It's unknown how much earlier the temperature shock can be administered and still elicit the shift in eclosion time, but we know that a 15 min shock at 45°C during the wandering phase of the third instar causes only a modest shift. At the time we administered our low and high temperature pulses (wandering larvae or pharate adults), the flies were presumably no longer sensitive to the entraining effects of the light cycle (Saunders, 1979). In all of our experiments the temperature shocks were administered during the photophase. We detected no difference in effect between early and late photophase. The disturbance of the eclosion rhythm is the most subtle consequence of temperature stress we have observed, and this suggests that the neurally-based clock mechanism that controls this rhythm (Saunders, 1982) is among the systems most vulnerable to temperature stress. The biochemical basis for the time-keeping mechanism remains unknown, and quite possibly a number of modulator substances are involved in expression of the rhythm. For example, both serotonin (Page, 1987) and an ecdysteroid agonist RH 5849 (Cymborowski et al., 1993) can elicit phase shifts in insect locomotory activity. Temperature stress could disrupt the normal rhythm by interfering with a number of potential components of the clock and the downstream events dictated by the clock.

The tensiometric recordings of eclosion behavior proved useful for dissecting the effects caused by the different types of temperature injury. The most remarkable finding was that the effects of heat shock and indirect chilling injury are very similar, whereas the effect of cold shock appears to be different. Heat shock and indirect chilling did not affect the eclosion behavior programs: both the POR and PFM programs were intact and readily recognizable. Failure of eclosion for such flies appears to be based on a decrease in strength of muscle contraction. Muscular dysfunction originating either within the neuromuscular system or within the muscles alone could account for the symptoms observed. In contrast, cold shock has a more profound effect on the eclosion behavior pattern than on the strength of muscle contraction. Of the two programs involved in eclosion behavior, the PFM program appears to be the more sensitive to cold-shock injury, and its absence in cold-shocked flies explains why such flies may succeed in opening the operculum, a behavior based on the POR program, but fail to totally emerge from the puparium. Impairment of this program also explains why partial emergence was more prevalent among cold-shocked flies than in flies that had been heat shocked. With increased intensity of cold shock the POR program also was affected. Its expression became more erratic and finally the POR program was not expressed at all. Its absence explains why the most severely cold shocked flies failed to open the operculum. The nervous system appears to be especially vulnerable to injury at low temperature. Mammalian central nervous system function is significantly impaired by low-temperatures exposure (10–30°C) (review; Brooks, 1983). Symptoms of low temperature injury in cultured neuronal cells include dendrosomatic swelling, apparently caused by activation of the glutamate receptor-ion channel complexes (Lucas et al., 1990). The cause of such injuries in insects has not yet been defined.

The results presented here clearly indicate that there are similarities as well as differences in the effects of high and low nonoptimal temperature exposure. Heat shock, cold shock and indirect chilling injury all retarded pharate adult development and interfered with successful adult eclosion. Both heat shock and cold shock shifted the circadian peak of eclosion from dawn to near the middle of the photophase. The effect on eclosion time was expressed several days after the actual shock was administered, and it was evident even under the normal light entrainment regime. Though failure of eclosion was evident in all three forms of injury, we suggest that the causes are different. Muscular dysfunction appears to be mainly responsible for the fly’s failure to execute eclosion behavior following heat shock or indirect chilling injury, whereas damage to the nervous system is the more likely cause of injury following cold shock.

REFERENCES


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