Lethality of Cold to Third Instars, Pupae, and Pharate Adults of the Mexican Fruit Fly (Diptera: Tephritidae)

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ABSTRACT Tolerance of Mexican fruit fly, *Anastrepha ludens* (Loew), 3rd instars and puparial stages to 1.1°C was evaluated. Mexican fruit flies were more susceptible to cold during the first few days after cessation of larval feeding (through the cryptocephalic pupal stage) than feeding 3rd instars. That is a period of intense morphological change for the insect. First-day phanerocephalic pupae, however, were estimated to require twice as much time to achieve $LT_{99.9968}$ at 1.1°C compared with feeding 3rd instars. Although the Mexican fruit fly does not diapause, the phanerocephalic stage is the diapausing stage for tephritids that do diapause. A major implication of this study is that cold-treated fruits are usually packed before treatment, and larvae could conceivably emerge and pupate within the packaging before temperatures are reduced to sufficient levels to stop development. Newly formed phanerocephalic pupae and pharate adults >3 d old are more tolerant of cold than 3rd instars. Therefore, fruits should not be held at temperatures after packaging that allow sufficient time for fruit fly development to the phanerocephalic pupa.

KEY WORDS *Anastrepha ludens,* quarantine treatment, diapause, pupariation, cold treatment

TEMPERATURES OF 0–2.2°C for 10 or more days are commonly used to disinfest fruits of tephritid fruit fly eggs and larvae (Gould 1994). A cold quarantine treatment is almost always applied to packed fruits because the cold treatment does not harm the packaging, and fruits are often treated en route to market. Quarantine treatments against tephritids have only been concerned with efficacy against eggs and larvae because pupae are generally not present in fruit; 3rd instars leave the fruit to pupariate in the soil. However, it is possible when enough time passes between packing and cessation of insect development by cold storage that 3rd instars inside fruits may egress and pupariate inside the fruit containers. Also, techniques used to reduce chilling injury in fruits, such as holding them at temperatures several degrees above the quarantine treatment temperature for several days before cold treatment (Paull and McDonald 1994), favor development of insect stages past the 3rd instar. Puparial stages of the Mexican fruit fly, *Anastrepha ludens* (Loew), reared at 25°C varied considerably in tolerance to 4.2°C with the following order of increasing tolerance per age (in days after pupariation): $2 < 0 < 6 < 8 < 10 < 4$ (Darby and Kapp 1933). There is no information on susceptibility of tephritid stages past the 3rd instar to temperatures at which quarantine cold treatments are done (0–2.2°C).

The objective of this research was to determine the susceptibility of Mexican fruit fly 3rd instars and later stages within the puparium to low temperature and its potential impact on the efficacy of cold quarantine treatments.

Materials and Methods

Mexican fruit flies used in this research originated from wild populations in Montemorelos, Nuevo Leon, Mexico, and had been reared at 26.2 ± 2°C on a semiartificial diet (Spishakov and Hernandez-Davila 1968) at Weslaco, TX, for ≈20 generations when the research was initiated. The entire research was conducted over the span of ≈15 generations. To obtain insects of known ages, larvae were removed from the diet and placed in vermiculite for pupariation. Any larvae that had not pupariated by the following day were discarded. Mexican fruit flies of the following stages were selected for exposure to cold storage: 3rd instars still in diet 1 d before they are usually removed, 3rd instars removed from diet and placed in vermiculite, and insects from 1 to 10 d after pupariation. Some puparia were opened to determine the stage of development before the rest were placed in cold storage.

Tephritids pass through a series of distinct stages once the larva completes feeding (Fraenkel and Bhaskaran 1973). Feeding 3rd instars are still feeding; postfeeding 3rd instars have irreversibly ceased feeding and emptied their crops, but have not yet initiated pupariation. The prepupa has pupariated but not yet formed the pupa. The cryptocephalic pupa comprises the stage between the larval–pupal molt (pupation) and evagination of the head. The phanerocephalic pupa is the stage after the head has everted but before the pupal–adult molt. After the pupal–adult molt but before emergence from the puparium the insect is the pharate adult. The postfeeding 3rd instar, prepupal,

Table 1. Probit analysis of prevention of emergence of normal-appearing adults from Mexican fruit fly 3rd instars and puparial stages at 1.1 ± 0.9°C

Stage[a]	n	Probability density function	Slope ± SE	Lethal time (95% CL), days			χ^2
				LT_{50}	LT_{99}	$LT_{99.9968}$	
Feeding 3rd instar	9,575	Normal	0.73 ± 0.087	3.9 (2.7–4.7)	7.1 (6.3–8.6)	9.4 (8.1–12.2)	14.6**
		Gompertz	0.71 ± 0.020	4.3 (4.2–4.4)	7.0 (6.9–7.1)	10.5 (10.2–10.7)	3.5
Postfeeding 3rd instar	15,992	Normal	1.16 ± 0.20	4.4 (3.6–4.8)	6.4 (5.9–7.7)	7.9 (6.9–10.5)	169**
		Gompertz	0.95 ± 1.1	4.4[b]	6.4[b]	9.0[b]	235**
Prepupa	9,217	Normal	0.98 ± 0.43	3.2[b]	5.6[b]	7.3[b]	556**
		Gompertz	1.09 ± 0.50	3.6[b]	5.2[b]	7.4[b]	359**
Cryptocephalic pupa	9,237	Normal	0.91 ± 0.13	1.4 (0–2.0)	4.0 (3.4–6.4)	5.9 (4.6–11.3)	44**
		Gompertz	0.81 ± 0.077	1.4 (0.4–1.9)	3.7 (3.4–4.4)	6.8 (5.6–9.5)	14.4*
Phanerocephalic pupa, day 1	13,385	Normal	0.37 ± 0.065	7.1 (2.9–8.7)	13.3 (12.1–15.8)	17.8 (15.5–24.2)	154**
		Gompertz	0.32 ± 0.060	7.1 (1.8–8.8)	13.0 (12.1–14.9)	20.8 (17.6–30.9)	133**
Phanerocephalic pupa, day 2	9,943	Normal	1.68 ± 0.045	7.0 (7.0–7.1)	8.4 (8.4–8.5)	9.4 (9.3–9.5)	1.7
		Gompertz	1.48 ± 0.045	7.0 (7.0–7.1)	8.3 (8.3–8.3)	10.0 (9.8–10.1)	0.007
Pharate adult, day 1	7,485	Normal	1.11 ± 0.069	5.8 (5.4–6.0)	7.9 (7.6–8.4)	9.4 (8.8–10.4)	7.3*
		Gompertz	0.88 ± 0.029	5.6 (5.6–5.7)	7.8 (7.7–7.9)	10.6 (10.4–10.9)	0.21
Pharate adult, day 2	7,700	Normal	1.38 ± 0.036	5.2 (5.2–5.2)	6.9 (6.8–7.0)	8.1 (8.0–8.2)	0.081
		Gompertz	1.16 ± 0.12	5.2 (4.5–5.6)	6.8 (6.4–7.6)	9.0 (8.0–11.3)	28.4**
Pharate adult, day 3	8,199	Normal	0.89 ± 0.11	5.2 (4.1–5.7)	7.8 (7.1–10.0)	9.7 (8.3–14.0)	48.1**
		Gompertz	0.81 ± 0.049	5.2 (4.7–5.5)	7.5 (7.2–8.0)	10.6 (9.7–12.1)	10.2*
Pharate adult, day 4	12,808	Normal	0.58 ± 0.17	7.1 (0-8.6)	11.2 (10.2–40.5)	14.1 (11.9–107)	126**
		Gompertz	0.41 ± 0.11	6.5 (0-6.2)	11.1 (10.3–18.7)	17.1 (13.8–66.2)	101**
Pharate adult, day 5	9,189	Normal	0.59 ± 0.081	6.9 (5.2–7.5)	10.8 (10.1–12.4)	13.6 (12.1–17.3)	33.2**
		Gompertz	0.44 ± 0.053	6.4 (4.6–7.2)	10.7 (10.1–11.8)	16.3 (14.3–20.8)	24.9**
Pharate adult, day 6	6,742	Normal	0.38 ± 0.028	4.7 (3.8–5.4)	10.9 (10.6–11.2)	15.3 (14.6–16.3)	4.2
		Gompertz	0.26 ± 0.022	3.7 (2.4–4.7)	10.9 (10.7–11.1)	20.2 (18.8–22.2)	2.5

* $P \leq 0.05$; $P \leq 0.01$.
[a] 24 hours between successive stages; terminology follows Fraenkel and Bhaskaran (1973).
[b] Fit too poor for estimation of confidence limits and did not improve by trying log 10 of dose or Gompertz function.

and cryptocephalic pupal stages each last ≈1 d at 25°C in the Mexican fruit fly; whereas, the phanerocephalic pupal stage lasts ≈2 d. The pharate adult stage lasts ≈2 wk.

About 200 insects were mixed with ≈100 ml of vermiculite and placed in 130-ml clear plastic containers with many small holes in the lids. The containers were placed in an environmental chamber (Percival Scientific model I-35VL, Boone, IA). Thermocouples (Omega, Type T, #36 gauge, Stamford, CT) were positioned in the chamber and in the plastic containers and read with a temperature logging system (Gaffney Engineering, GETC, Gainesville, FL). For improved accuracy, thermocouples were made from the same spool of wire, readings were calibrated with a thermometer certified according to National Institute of Standards and Technology procedures, and unused junctions were shorted with copper wire. Temperatures within the plastic containers were 1.1 ± 0.9°C, which is a common cold treatment mean target temperature. Relative humidity was ≈70% inside the chamber. The containers cooled down to the upper limit of this temperature range in ≈40 min.

Dose–response curves were generated by removing containers from the chamber after different numbers of days and placing the insects and vermiculite in 330-ml plastic containers with screen lids at ≈25°C for continued development of survivors and emergence of adults. Systematic bias was eliminated by randomizing treatment order for both insect ages and cold treatment durations. Data points were concentrated at the upper end of the mortality curve because the objective involved estimating high levels of mortality. Only 18% of the points had <50% mortality, whereas 43% had >99% mortality. Also, larger numbers of insects were subjected to the longer exposures to increase accuracy at the high end of the curve. For example, data points <50% mortality were generated with a few hundred total insects, whereas points >99% mortality used several thousand. Probit analysis (PROC PROBIT, SAS Institute 1988) was used to estimate times (lethal time) that would provide 50, 99, and 99.9968% mortality based on nonemergence of adults with normal-looking wings. $LT_{99.9968}$ is often the level of efficacy demanded of a quarantine treatment against tephritids (Shannon 1994).

Results

Estimates of lethal times using the normal and Gompertz probability density functions are presented in Table 1. Several stages did not significantly fit either function, although there was no consistent deviation from the probit model (Fig. 1). Estimation of confidence limits on lethal time values for the prepupal stage was not possible, although the mean lethal time-point estimates are likely close given that a 6-d treatment of prepupae resulted in 99.957% mortality. Also, confidence limits for day 4 of the pharate adult were wide because of a poor fit. Again, the lethal time-point estimates are likely close because an 11-d treatment resulted in 99.32% mortality.

The general trend was for Mexican fruit flies to decrease in cold tolerance up to the 3rd d after pupariation (1st d of the phanerocephalic pupa) when

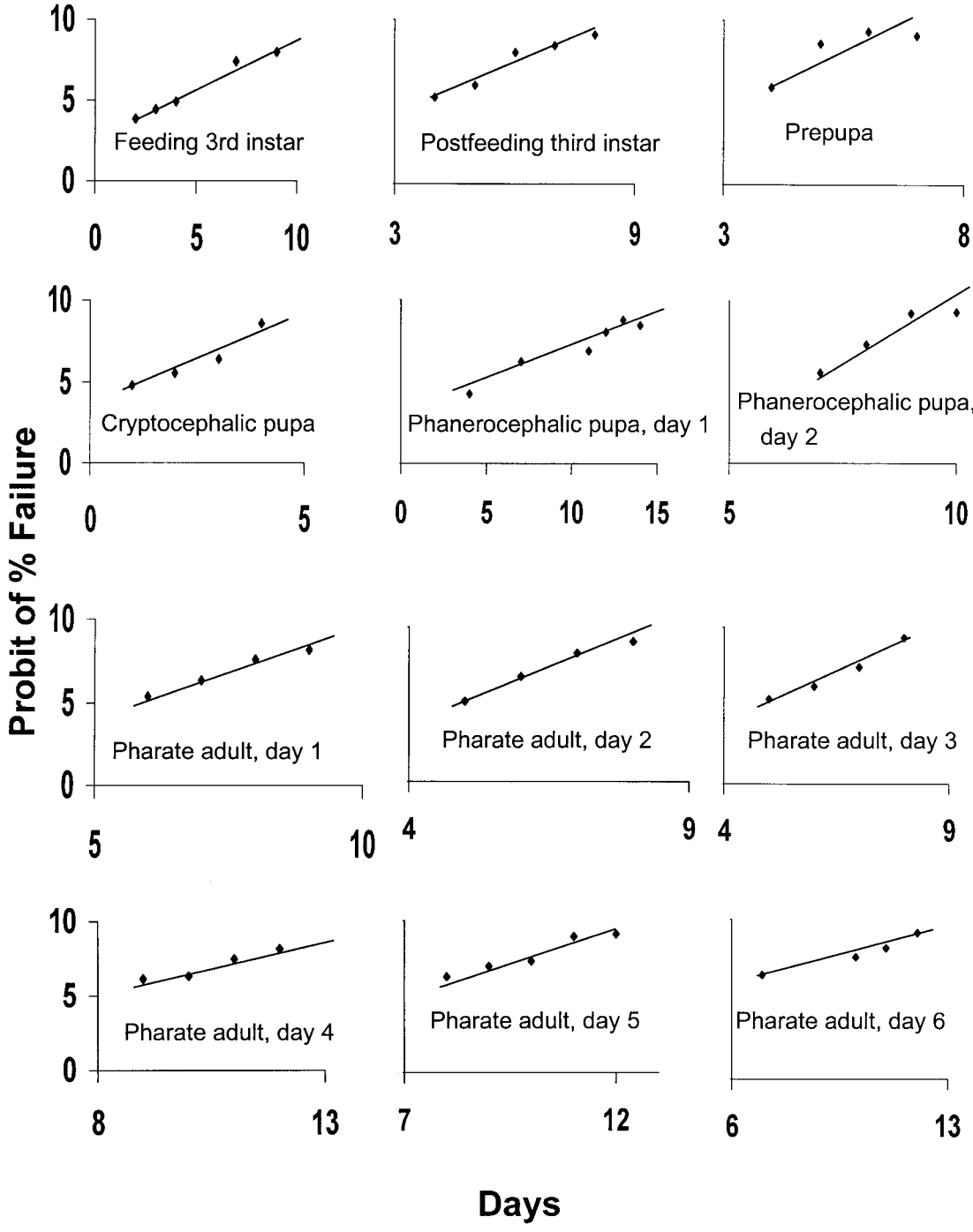

Fig. 1. Probit of percentage of Mexican fruit fly stages failing to emerge as normal-looking adults after holding at 1.1°C for various days. Line is best fitting model between normal and Gompertz probability density functions (Table 1).

there was a 3-fold increase compared with insects treated the previous day (Fig. 2). Cold tolerance dipped to almost feeding 3rd-instar levels after the 3rd d after pupariation (2nd d of the phanerocephalic pupal stage), but then increased to levels not quite as high as the 1st d of the phanerocephalic pupal stage by the 8th d after pupariation (≈3 d after formation of the pharate adult).

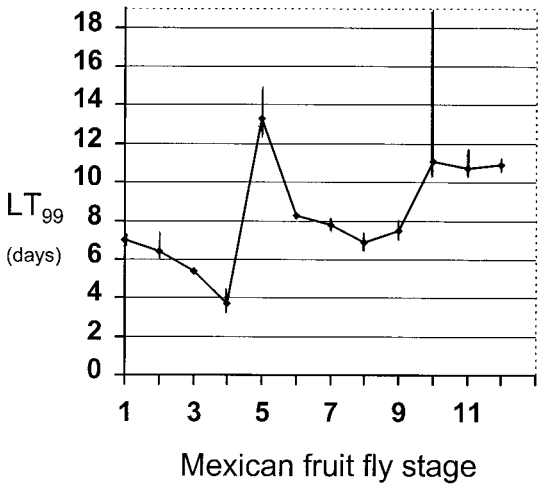

Fig. 2. LT_{99} estimates (in days including 95% CL) using best fit between normal and Gompertz probability density functions (Table 1) for consecutive days of Mexican fruit fly stages exposed to 1.1°C. Day 1, feeding 3rd instar; day 2, postfeeding 3rd instar; day 3, prepupa; day 4, cryptocephalic pupa; days 5 and 6, phanerocephalic pupa; and days 7–12, pharate adult. Confidence limits for day 3 were not estimable because of poor fit.

Discussion

The irregular changes in tolerance of Mexican fruit fly puparial stages to 1.1°C agreed substantially with the observations of Darby and Kapp (1933) at 4.2°C: cryptocephalic pupae were most susceptible, followed by prepupae, late phanerocephalic pupae and early pharate adults, middle-aged pharate adults, and the most tolerant were early phanerocephalic pupae (Fig. 2). In Darby and Kapp (1933), the phanerocephalic ("pronymph") stage occurred at ≈4 d at 25°C, and there was virtually no mortality in this stage after 15 d at 4.2°C, whereas all 2-d-old puparia were dead at 7 d. This divergence in results was more extreme than my observations with the 2 stages (Table 1). More or less continuous development occurs in nondiapausing immature insects; perhaps Darby and Kapp (1933) happened to select phanerocephalic pupae closer to the particular age that was most tolerant to cold compared with pupae used in my experiment, which would indicate that at some precise stage the phanerocephalic Mexican fruit fly may be very tolerant to cold. The relative differences found between the 2 studies could also be caused in large part by the different temperatures used.

Cyclorrhaphous Diptera undergo considerable transformation during the 1st quarter of their puparial life, changing from a coarctate larva to a phanerocephalic pupa, and it is during this period that tolerance of Mexican fruit fly to cold was reduced relative to previous and later stages. Tolerance to cold increased dramatically on the 1st d of the phanerocephalic pupa, which is the stage in which tephritids enter diapause. This suggests that either a natural tendency for the early phanerocephalic stage to be relatively more cold-tolerant was exploited by tephritids as the stage that developed into the diapausing stage or increased cold-tolerance of the phanerocephalic pupa is simply a relic of earlier development of diapause in the ancestral lineage of the subtropical, nondiapausing Mexican fruit fly.

The Mexican fruit fly appears to be more tolerant of cold than other *Anastrepha,* because a longer cold quarantine treatment interval is required for the former (Gould 1994). Gould and Hennessey (1997) estimated the $LT_{99.9968}$ to be 7.9 d (95% CL, 6.4–10.9 d) for Caribbean fruit fly, *A. suspensa* (Loew), feeding 3rd instars that were cooled to 1.1°C in 40–45 min, which was the same cooling temperature and rate as my study in which the $LT_{99.9968}$ for Mexican fruit fly feeding 3rd instars was estimated to be 9.4 d (95% CL, 8.1–12.2 d). Although there are differences in methodology and measurement of mortality between the 2 studies, the largely overlapping confidence limits for the 2 species indicates that the Mexican fruit fly is not much more cold tolerant, if at all, compared with the Caribbean fruit fly. Another possible indication that the 2 fruit flies have similar cold tolerance is that the northern distributional limit of both species coincides roughly with the northern limit of the plant hardiness zone 9b (average annual minimum temperature of −1.2 to −3.8°C), which encompasses the 5 southernmost counties of Texas (Mexican fruit fly) and, together with zone 10, the southern half of Florida (Caribbean fruit fly) (Anonymous 1990). Therefore, it would be worthwhile to determine if cold treatment temperatures against the Mexican fruit fly could be reduced from the current 20 d at 1.1°C. Grapefruits and carambolas in Florida are currently disinfested of Caribbean fruit fly with 12 d at 1.1°C (Ismail 1989, Gould and Hennessey 1997).

Finally, the risk that Mexican fruit flies in the 1st d of the phanerocephalic stage might not be entirely controlled by present cold treatment regimes suggests that limits should be placed on the amount of time that packed fruit hosts can be held at ambient temperatures before cold quarantine treatments are applied. This may not seem important given that the $LT_{99.9968}$ for 1st-d phanerocephalic pupae was estimated to be 17.8 d (Table 1), which is under the 20-d requirement for Mexican fruit fly hosts at 1.1°C (Gould 1994). However, the cooling rate in this experiment was faster than is accomplished commercially with fruits. Gould and Hennessey (1997) found a 40% reduction in time estimated to achieve the $LT_{99.9968}$ when Caribbean fruit fly 3rd instars in carambolas were cooled to 1.1°C in 40–45 min versus a more normal commercial cool-down time of >24 h. Applying that magnitude of reduction in cold tolerance to the Mexican fruit fly (if it were shown to occur) would result in an estimated $LT_{99.9968}$ of 29.5 d for 1st-d phanerocephalic pupae but only 15.6 d for feeding 3rd instars when cooled to 1.1°C over a longer period more akin to commercially applied treatments. Most fruit hosts are not at temperatures that allow significant insect development for very long after packing; however, some, such as citrus, may be in commercial situations. This

concern should also be examined for other fruit fly species.

Leyva-Vázquez (1988) estimated the lower temperature threshold for development of Mexican fruit fly 3rd instars and puparial stages to be 9.3 and 7.8°C, respectively. From his data one can estimate that the shortest time required for 3rd instars inside of fruits to emerge and develop into phanerocephalic pupae would be ≈6.3, 4.5, 3.7, 3.2, and 2.8 d at temperatures of 17.7, 20.7, 23.7, 26.7, and 29.7°C, respectively. Ismail (1989) recommends preconditioning grapefruits destined for transport by ship to Japan at 15.6°C for 7 d before initiating cold quarantine treatment against fruit flies. This period together with the time lags caused by packing and placement in preconditioning storage, cool down to 15.6°C, loading of ships, and cool down to quarantine treatment temperatures allows sufficient time for late instar larvae to be in the early phanerocephalic or even pharate adult stages at the time cold treatment begins. This is certainly a theoretical concern, but whether fruit fly pupae are actually found in packaging and what proportion of those might be in relatively cold-tolerant stages when cold treatment is initiated has not been determined. Most puparia found in packaging will probably contain insects that are of equal or less tolerance to cold (postfeeding 3rd instars, prepupae, cryptocephalic pupae, late phanerocephalic pupae, early pharate adults) as feeding 3rd instars.

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