MORBIDITY OF THE PUPAL STAGE OF THE MEXICAN AND WEST INDIAN FRUIT FLIES (DIPTERA: TEPHRITIDAE) INDUCED BY HOT-WATER IMMERSION IN THE LARVAL STAGE

DONALD B. THOMAS AND ROBERT L. MANGAN
USDA-ARS Subtropical Agriculture Research Laboratory
Weslaco, TX 78596

ABSTRACT

Heat treatments are used to disinfest fruit exported from regions where *Anastrepha* fruit flies are indigenous. Larvae that survive the heat treatments typically form misshapen puparia. The assumption that all of these puparia will die before eclosion of the adult is shown to be incorrect. Two types of malformed puparia are typically induced by hot-water immersion. A larviform puparium is seldom viable with an eclosion rate of <1% in both the Mexican and West Indian fruit flies. However, a bottle-nosed puparium will produce an adult about 50% of the time. It should be assumed that if any larvae survive treatment to form puparia, some will give rise to adults.

Key Words: *Anastrepha*, morbidity, puparial malformations, heat treatments, quarantine, Tephritidae.

RESUMEN

Los tratamientos con agua caliente se utilizan en regiones donde se encuentran moscas del género *Anastrepha* para desinfestar las frutas que van a ser exportadas. Las larvas que sobreviven los tratamientos de calor producen pupas malformadas. Se demostró que la suposición de que todas las pupas mueren antes de la eclosión del adulto es incorrecta. Dos tipos de puparios malformados se pueden inducir con el tratamiento de agua caliente: larviformes y con nariz en forma de botella. Los puparios larviformes raramente son viables, con una tasa de eclosión menor del 1% tanto en la mosca mexicana como en la mosca de las frutas de las Indias Occidentales. Sin embargo, las pupas con nariz en forma de botella producen adultos en aproximadamente el 50% de los casos. Debe asumirse que si algunas larvas sobreviven al tratamiento y forman puparios también darán lugar a adultos.

The Mexican fruit fly, *Anastrepha ludens* (Loew), and the West Indian fruit fly, *Anastrepha obliqua* (Macquart), are quarantined insect pests. Importation of host fruits to the United States from countries where these flies are indigenous requires a disinfestation treatment. For example, Mexican mangoes are given a hot-water immersion for 1 h at 46°C, a treatment reported by Sharp (1988) to disinfest the fruit of tephritid immatures. USDA-APHIS quarantine restrictions mandate a treatment which will cause mortality of the infesting insects at the Probit 9 level, equivalent to 99.9968% mortality (Baker 1939; see also Chew 1994, Robertson et al. 1994, Shannon 1994, for recent discussion).

In reviewing the published studies on hot-water immersion treatments of various combinations of host fruits and infesting insects, we noted that in many cases, malformed puparia were counted in the mortality figures (Sharp 1986; Sharp et al.

1989a,b,c; Sharp & Hallman 1992). We were concerned that scoring malformed puparia as dead insects without supporting data might include an undefined risk of disinfestation failure.

We, therefore, undertook experiments emphasizing marginally sublethal temperature levels designed to produce large numbers of malformed puparia. The purpose of these experiments was to identify the kinds of malformations associated with hot-water immersion treatments and to quantify the frequency of these malformations and their specific mortality rates.

MATERIALS AND METHODS

All experiments were conducted at the USDA Subtropical Agriculture Research Laboratory in Weslaco, Texas. Ten-day-old, third-instar larvae of *A. ludens* and *A. obliqua* were obtained from colonies of these insects maintained at this laboratory using rearing procedures described by Rhode (1957) and Rhode & Spishakoff (1965) for the Mexican fruit fly, and by Mangan & Ingle (1992) for the West Indian fruit fly. Under laboratory conditions at room temperature, 10-day-old larvae are sufficiently mature to pupariate and metamorphose successfully to adults. The colonies of both species were founded with material originating in Mexico, the *A. obliqua* colony dating from 1987 and the *A. ludens* colony originating in 1953.

All larvae, except controls, were immersed in a circulating water bath controlled to ± 0.02°C by a computer driven temperature control system. The computer software program "Water Troll" was developed by J. J. Gaffney, USDA-ARS, Gainesville, FL. The water baths consisted of a series of four 11-liter capacity stainless steel water banks; each equipped with an electric stirrer motor with shaft and propeller, a 1,000 Watt electrical resistance heater and a Hart 1006 precision thermometer. The larvae were free within a 150 cc capacity, organdy mesh basket.

The experimental temperatures used were 38, 40, 41, 42, 43 and 44°C. The water baths were brought to the selected temperature before immersion of the larvae, and all immersions were for a duration of 1 h. The number of replicates performed varied among temperatures and between species because of the difference in the numbers of malformed puparia produced at the different temperatures and the necessity of using those temperatures which produced the most malformations. Lower temperatures which produced few malformations were replicated as a check against the possibility that the malformations could have been caused by immersion in water alone. With *A. ludens* there were eight replicates from two rearing batches at 38°C; five replicates from five rearing batches at 40°C; nine replicates from six rearing batches at 41°C; 16 replicates from four rearing batches at 42°C; 23 replicates from eight rearing batches at 43°C, and six replicates from three rearing batches at 44°C. With *A. obliqua* there were 12 replicates from three rearing batches at 38°C, five replicates from five rearing batches at 40°C; 59 replicates from 20 rearing batches at 41°C; 12 replicates from three rearing batches at 42°C; 14 replicates from five rearing batches at 43°C, and four replicates from one rearing batch at 44°C. The numbers of larvae varied among replicates because of the necessity to avoid unnecessary handling and possibly detrimental effects to the larvae prior to pupariation. Thus, batches of larvae were tested as they became available and the precise numbers tested were counted after treatment during the immobile puparial stage. The mean treatment size was 289.6 larvae per replicate with a total of 61 replicates for *A. ludens*, and 154.7 larvae per replicate with a total of 106 replicates for *A. obliqua*.

Immediately following the hot-water immersion, both treated and untreated control larvae were transferred to 10 cm wide, 250 cc capacity, plastic containers with

screened lids. The containers were half-filled with clean, slightly moistened vermiculite (the pupariation medium) and held in an incubator at 25 ± 2°C temperature, 75 ± 5% humidity and 12:12 L:D cycle. After 3-4 days the puparia were sifted from the pupariation medium and scored as normal or malformed and separated. Dead larvae were counted and removed. The puparia were then returned to the slightly moistened vermiculite, placed in the incubator and held for emergence. After adult eclosion, the puparia were examined to determine the percent eclosion for each type of malformation.

In order to determine the viability of the adults from the malformed puparia, some of the emergent flies were held and tested for fertility. Adults (males and females) from eleven replicates each of *A. ludens* and *A. obliqua* were held in screen cages at 25°C and ambient light cycle with a diet of sugar and hydrolyzed yeast until an age of 10 days. The treated, but normal, puparia were segregated from the malformed puparia. Thus, males from malformed puparia mated with females from malformed puparia, and males from treated, but normal, puparia mated with the corresponding females. Untreated controls from the same rearing batches were also tested.When the flies were ten days old, surviving females (up to 10 if available) from each test group were placed in cages with an artificial gel oviposition medium and left overnight. The numbers of eggs oviposited were counted on the following day. These eggs were held and re-examined three days later to determine the percent hatch.

Differences in mean mortality between treatment and control groups were tested using the t-test for paired comparisons (Sokal & Rohlf 1973). The relationship between temperature and mortality and between temperature and frequency of malformation was computed with the product moment correlation coefficient (Sokal & Rohlf 1973). The mean eclosion rate from normal vs. malformed puparia was tested using single classification analysis of variance (Model I ANOVA) (Sokal & Rohlf 1973). The probability value *P* for each *F*, *t* and *r* statistic was calculated with the software program Speakeasy (Speakeasy Computing 1987).

Results and Discussion

A one-h exposure to a water bath at 44°C resulted in 100% total mortality in four replicates of *A. obliqua* and 99.8 ± 0.57% mean (±SE) mortality in six replicates of *A. ludens*. A total of two adults were produced from 1,132 *A. ludens* larvae tested, and none from the 624 *A. obliqua* larvae tested. However, at 43°C, mean mortality was 79.2 ± 12.5% in 23 replicates of *A. ludens* (n = 6,105) (Table 1). *A. obliqua* was more susceptible to these temperatures; at 43°C only one adult was produced from 2,619 treated larvae (14 replicates), a mean mortality rate of 99.96 ± 0.15%. At 42°C, only 5 adults were produced from 2,480 treated larvae, a mean mortality rate of 99.8 ± 0.35% (12 replicates). At 41°C, however, there was appreciable survival to the adult stage, with a reduction in mean mortality to 75.6 ± 12.0% (58 replicates) in *A. obliqua* (Table 2).

Significant mortality was produced at all water temperatures tested except the lowest. At the lowest test temperature, 38°C, there was 69.6 ± 8.4% mean survival in 12 replicates of *A. obliqua* larvae (n = 2,526), whereas 85.9% of the controls from all tests produced adults (Table 1.) The difference in mean survival, defined as the percentage of adults emerging from puparia, was statistically significant using a pair-wise t-test (t = 3.98; df = 11; P = .001). But, for *A. ludens*, the difference in survival at the low temperature was much less. When subjected to 38°C for 1 h, mean survival was 84.5 ± 13.6%, compared to a mean survival of 84.5 ± 12.9% in controls.

TABLE 1. NUMBERS OF DEAD LARVAE, MALFORMED PUPARIA, NORMAL PUPARIA AND ECLOSED ADULTS AFTER HEAT TREATMENTS ON THE 3RD INSTAR LARVAE OF *ANASTREPHA LUDENS*.

			Normal		Malformed			
Test Temp	Total n	Dead Larvae	Total Pupae	Adults Eclosed	Bottle-nose	Larvi-form	Peanut Pupae	Adults Eclosed
C	6059	110	5917	5500	25	3	4	13
38°	2362	106	2172	1913	82	2	0	44
40°	1154	68	1049	863	31	2	4	25
41°	1911	258	1488	1085	164	0	1	72
42°	5017	530	3834	3576	576	72	5	447
43°	6105	2668	2281	1090	924	228	4	197
44°	1132	1104	1	0	4	23	0	2

The difference in survival was not significant with a pair-wise t-test ($t = 0.18$; $df = 7$; $P = 0.43$).

Larval Mortality

Most of the mortality produced by the hot-water treatment was to the larval stage: 57% of total deaths in *A. ludens* and 55% of total deaths in *A. obliqua*. Larvae immersed in hot water stretched to their full length and became immobile. Surviving larvae recovered mobility within 1-2 h and most pupariated eventually. Those which did not recover mobility turned black and shriveled within 1-2 days.

At the lowest test temperature, 38°C, there was a mean mortality of 11.4 ± 1.9% in *A. obliqua* larvae and 4.2 ± 2.9% in *A. ludens* larvae. However, even this low level of mortality was significantly greater than for the untreated controls in which failure to pupariate was 3.5 ± 2.3%. Using a pair-wise t-test to compare larval mortality at

TABLE 2. NUMBERS OF DEAD LARVAE, MALFORMED PUPARIA, NORMAL PUPARIA AND ECLOSED ADULTS AFTER HEAT TREATMENTS ON THE 3RD INSTAR LARVAE OF *ANASTREPHA OBLIQUA*.

			Normal		Malformed			
Test Temp	Total n	Dead Larvae	Total Pupae	Adults Eclosed	Bottle-nose	Larvi-form	Peanut Pupae	Adults Eclosed
C	3455	73	3291	2945	73	15	6	24
38°	2526	285	2078	1704	152	7	4	51
40°	2454	176	2058	1354	179	13	4	81
41°	7831	1846	2193	1001	2898	891	3	765
42°	2480	1935	225	3	204	116	0	2
43°	2619	2590	1	1	14	14	0	0
44°	624	624	0	0	0	0	0	0

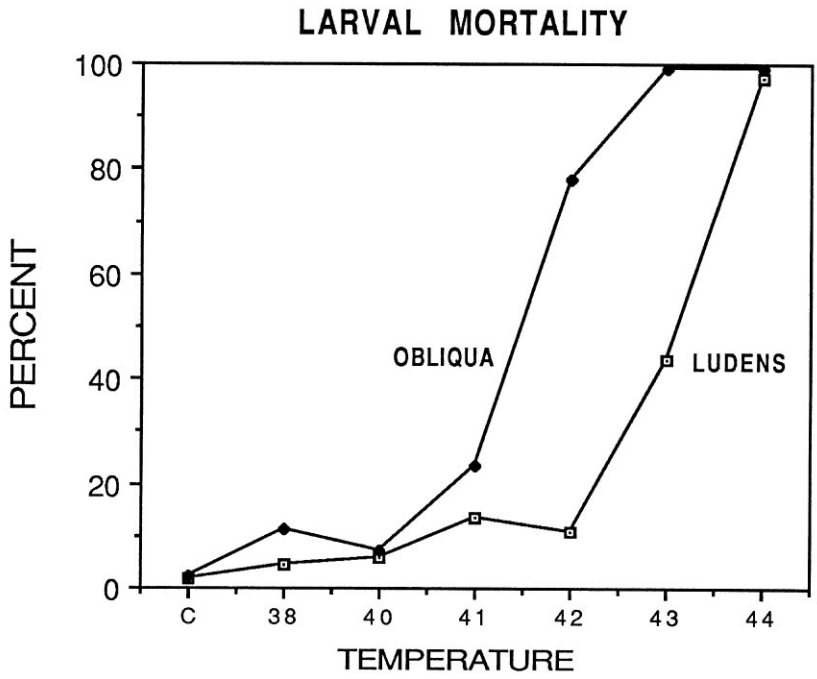

Fig. 1. Mortality in late third-instar larvae of *Anastrepha ludens* and *A. obliqua* following one h exposure in hot-water bath at 38-44°C, and non-exposed controls (c).

38°C, the t-value for *A. ludens* was 7.19 ($df = 7$, $P = 8.5^{e-5}$); and for *A. obliqua* $t = 7.41$ ($df = 11$, $P = 6.73^{e-6}$). Larval mortality rate increased sharply at 1 h exposures to 42°C for *A. obliqua* and at 43°C for *A. ludens* (Fig. 1).

Pupal Mortality

There was a 100% failure of larvae to pupariate at only one test temperature, 44°C, and for only *A. obliqua*. Some adults enclosed in all cases in which at least some larvae pupariated. Pupal mortality was significantly correlated with temperature in both species. The correlation coefficient (r) between temperature and mortality in *A. obliqua* was 0.94 ($P = .0026$). Mortality rose sharply at 41°C to 69.5 ± 12.2% and at test temperatures of 42°C, mean mortality was 99.2 ± 1.5% in this species. For *A. ludens* the correlation between temperature and pupal mortality was not as rigid but still significant ($r = 0.785$, $P = .032$). The test temperatures did not induce high pupal mortality in *A. ludens* until 43°C and above (Fig. 2).

Puparial Malformations

Pupariation in cyclorrhaphous Diptera is a four-step process (Zdarek & Frankel 1972, 1987). First, the anterior segments invert. Second, flexion of the integumental musculature constricts the body into a barrel-shape. Third, the cuticle shrinks (mainly by dehydration), and fourth, the cuticle becomes sclerotized through mela-

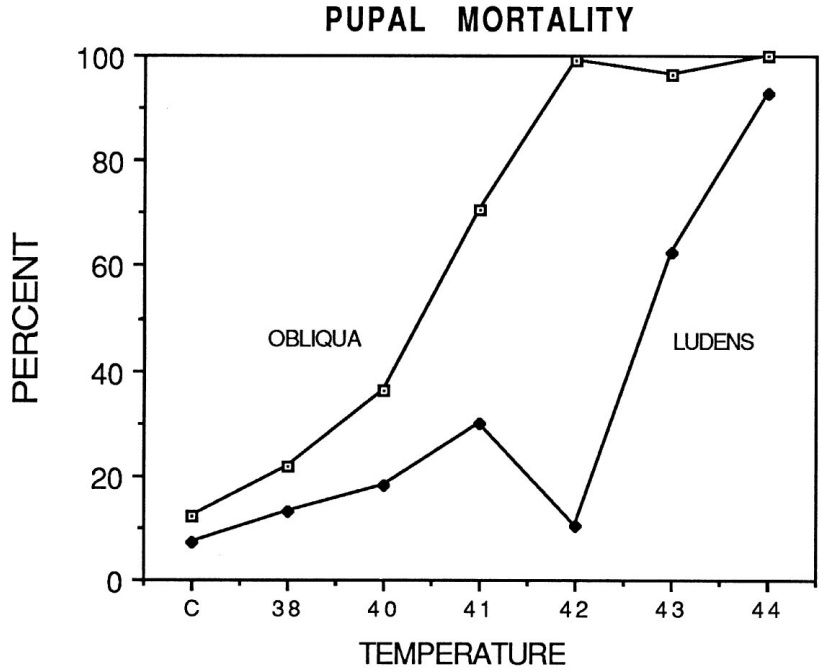

Fig. 2. Mortality in the pupal stages of *Anastrepha ludens* and *A. obliqua* from larvae subjected to hot-water immersion, and non-treated control larvae (c).

nization. Puparial malformations can be explained as dysfunctions of one or more of these mechanisms (Thomas & Mangan 1990).

Three types of malformations were observed in the test groups of puparia. The rarest form, a peanut-shaped puparium, was found in both treated and untreated groups. This malformation was characterized by a mild constriction in the middle segments. Its immediate cause was not determined but did not appear to be induced by, or associated with, hot-water immersion. A total of 17 cases were seen in *A. obliqua* (6 among the controls); and 18 cases in *A. ludens* (4 among controls). From the 35 peanut-shaped puparia, 15 adults emerged. These numbers were too small to treat statistically.

Larviform (Fig. 3a) and bottlenose (Fig. 3b) puparia were found to be associated with hot-water immersed larvae in both species. The larviform malformation was characterized by a failure to constrict into a typical barrel shape, and a failure of the head segments to invert. Of the 1,056 larviform puparia of *A. obliqua*, none produced an adult (100% mortality). However, of the 330 larviform puparia induced in *A. ludens*, two adults eclosed (99.1% total mortality). Both were from the same treatment cohort subjected to 42°C. There was a significant correlation between temperature and the percent frequency of larviform puparia in *A. obliqua* ($r = 0.88$, $P = .0245$). For *A. ludens*, the correlation was not significant at the 95% confidence level ($r = 0.64$, $P = .086$). The lower mathematical correlation seemed to result from the abrupt increase in this type of malformation at the highest treatment temperature (Fig. 4), as opposed to the linear increase in frequency seen in *A. obliqua* (Fig. 5).

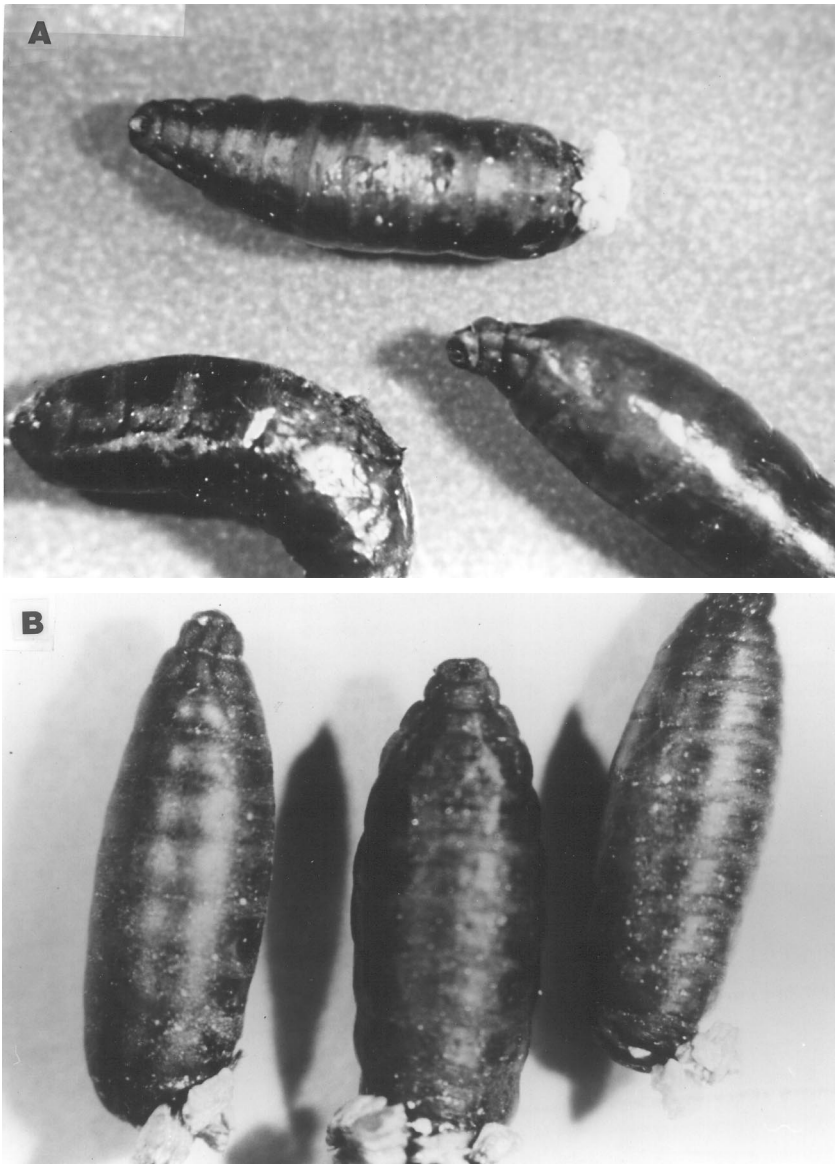

Fig. 3. Puparial malformations in *Anastrepha ludens* and *A. obliqua*: subjected to hot-water immersion. (a) larviform puparia; (b) bottlenose puparia.

In the bottlenose malformation, the anterior-most segment of the puparium is abnormally constricted, but otherwise morphologically asymptomatic (Fig. 3b). The frequency of the bottlenose malformation was significantly correlated with temperature in both species: $r = 0.77$ ($P = .037$) for *A. ludens*; $r = 0.84$ ($P = .038$) for *A. obliqua*. The greatest numbers of bottlenose puparia were produced at 40-41°C (*A. obliqua*) and

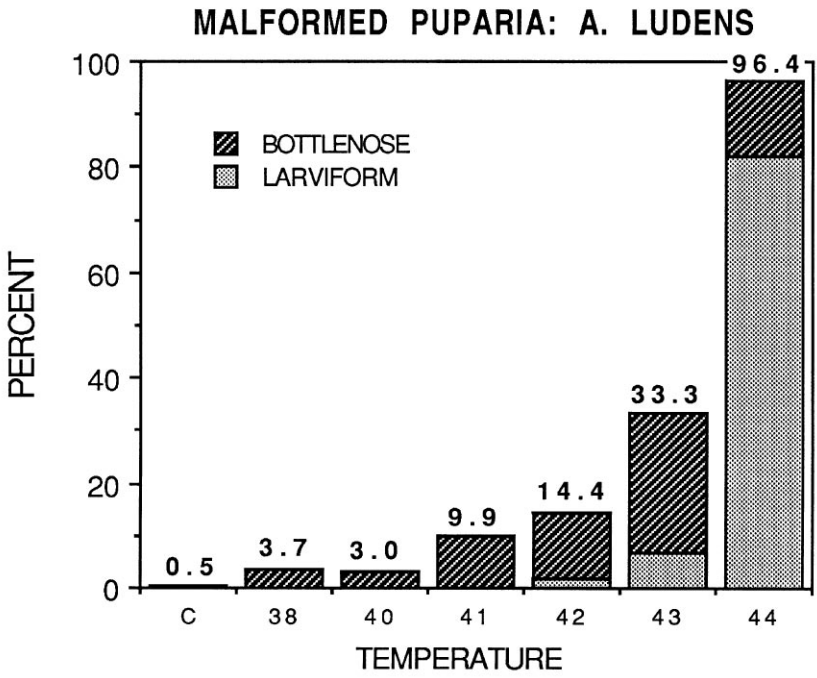

Fig. 4. Frequency of larviform or bottlenosed puparia following exposure of late third-instar *A. ludens* larvae to one-h hot-water bath at 38-44°C, and non-exposed controls (c).

41-42°C (*A. ludens*). The survival of the pupal stage to eclosion as an adult was significantly less in the bottlenose puparia compared to the normal puparia at each test temperature for both species. With *A. obliqua*, eclosion rate at 40°C was 75.9 ± 14.5% for the normal puparia, but only 51.9 ± 15.1% for the bottle-nose puparia. The difference was significant tested by ANOVA (F = 9.19; df = 1, 12; P = .01). Eclosion rate at 41°C was 37.4 ± 13.5% for the normal puparia, but only 25.4 ± 8.9% for the bottlenose puparia, a significant difference (F = 7.67; df = 1, 26; P = .01). With *A. ludens*, adult eclosion rate at 41°C was 73.4 ± 14.2% for the normal puparia, but only 50.5 ± 20.4% for the bottlenose puparia (F = 7.59; df = 1, 16; P = .01). At 42°C the adult eclosion rate from the normal puparia was 92.7 ± 5.3%. In comparison, the eclosion rate was lower, 78.7 ± 12.7% for the bottlenose puparia, but the difference was not significant at the 95% confidence limit with ANOVA (F = 4.21; df = 1, 6; P = .09).

Morbidity

Morbidity is the proportion of sick individuals in a population (Lapedes 1976). Hot-water immersion of late third-instar larvae at sublethal temperatures resulted in significant numbers of individuals which turned black and failed to pupariate or which formed misshapen puparia in both *A. ludens* (Fig. 6) and *A. obliqua* (Fig. 7). Adults failed to eclose from most of the malformed puparia. However, the results of this study strongly suggested that morbidity can be equated with mortality only if the

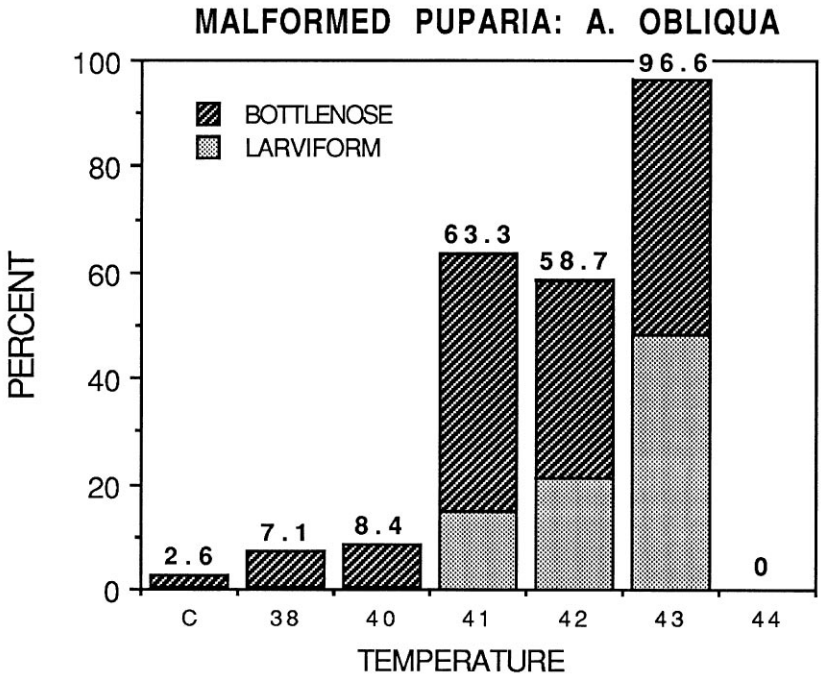

Fig. 5. Frequency of larviform or bottlenosed puparia following exposure of late third-instar *A. obliqua* larvae to one-h hot-water bath at 38-44°C, and non-exposed controls (c).

puparia are scored for the class of malformation. The larviform type of malformation was lethal at a rate in excess of 99%. Conversely, a high percentage of the bottlenose malformations are viable. In these experimental treatments, it was found that some of the larvae that survived to pupariate eventually reached the adult stage, even under conditions in which the mortality of the larval stages was greater than 99% and all of the puparia were malformed.

Followup tests with the adults that survived the hot-water treatments demonstrated that they were capable of attaining reproductive age, mating and ovipositing at normal levels. This was true for adults from treated but normal puparia, as well as for those emerging from malformed puparia. Mature *A. ludens* females (n = 66) from normal puparia oviposited a mean of 26.6 ± 25.0 eggs, of which 75.9% hatched. Females (n = 69) from bottlenosed puparia oviposited a mean of 14.9 ± 14.5 eggs, of which 65.3% hatched. Control females (n = 35) laid a mean of 13.8 ± 10.8 eggs, of which 77.5% hatched. Mature *A. obliqua* females from normal puparia oviposited a mean of 13.5 ± 10.8 eggs (n = 66), of which 61.2% hatched. Females (n = 65) from bottlenosed puparia oviposited a mean of 8.0 ± 6.9 eggs, of which 64.5% hatched. Control females (n = 67) oviposited a mean of 12.3 ± 11.5 eggs, of which 65.0% hatched.

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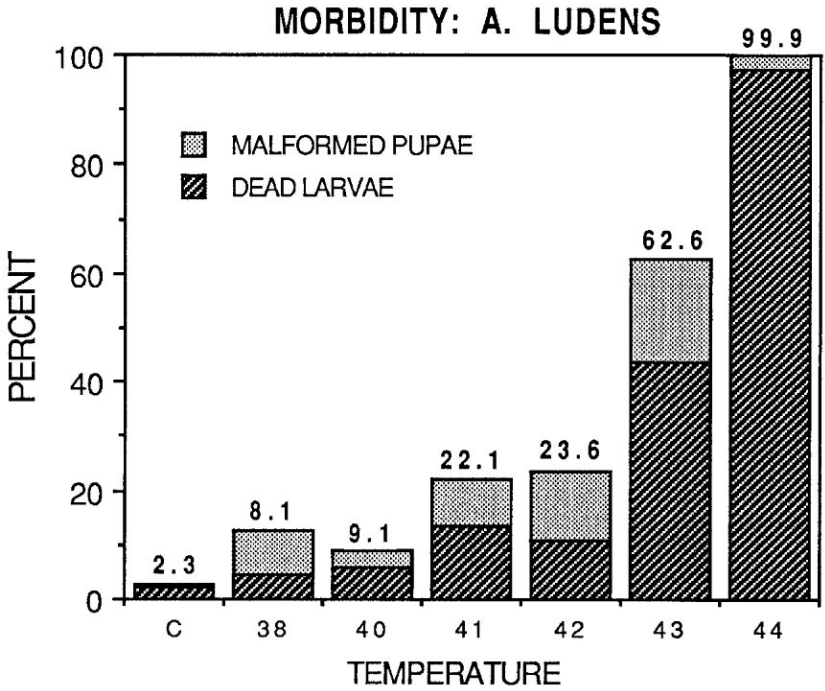

Fig. 6. Morbidity: larval death or puparial malformation following exposure of late third-instar *A. ludens* larvae to one-h hot-water bath at 38-44°C, and non-exposed controls (c).

the test insects and for data collection. Guy Hallman and Felix Guerrero provided valuable reviews of the manuscript.

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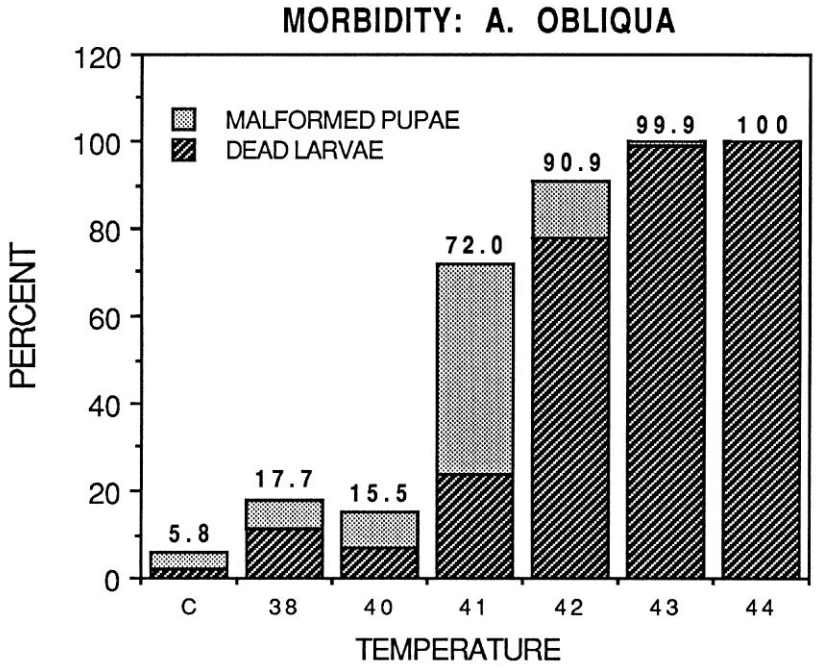

Fig. 7. Morbidity: larval death or puparial malformation following exposure of late third-instar *A. obliqua* larvae to one-h hot-water bath at 38-44°C, and non-exposed controls (c).

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