Thermal Death of Third Instars of the Caribbean Fruit Fly (Diptera: Tephritidae) Treated in Different Substrates[1]

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Abstract Eight substrates (air, water, artificial diet, and fruit pulp blends of guava, grapefruit, orange, mango, and orange) were examined for their impact on survival of third-instar Caribbean fruit fly, *Anastrepha suspensa* (Loew), exposed to 40°C for 67 min. Survival was the greatest for larvae heated in air (92.9 ± 1.1%) and lowest in water (12.1 ± 1.6%). Mortality was intermediate to high and similar among larvae heated in the fruit blends and artificial rearing diet. Percentage water and oxygen of each substrate, pH, and specific gravity did not affect larval mortality, but the type of substrate (air, water, fruit pulp blend) was significant. The upper thermal limits of a quarantine treatment can be screened for all pest life stages by testing time-temperature regimes using air as a substrate.

Key Words Survival, heat, quarantine, fruit pulp blend

Thermal treatments increasingly are being investigated as alternative quarantine treatments to methyl bromide fumigation to satisfy state and federal tephritid fruit fly quarantine regulations. Agricultural commodities that have received approved heat treatments before importation into the United States include: pineapple (*Ananas comosus* [L.] Merr.), papaya (*Carica papaya* L.), mango (*Mangifera indica* L.), various citrus, bell pepper (*Capsicum annuum* L.), eggplant (*Solanum melongena* L.), tomato (*Lycopersicon lycopersicum* [L.] Karst ex Farw.), and squash (*Cucurbia* spp.) (Animal Plant Health Inspection Service 1998). Additional commodities that have been examined for potential thermal treatments include: carambola (*Averrhoa carambola* L.) (Hallman 1990, 1991, Hallman and Sharp 1990, Sharp and Hallman 1992), guava (*Psidium guajava* L.) (Gould and Sharp 1992), and stone fruits (Sharp 1990). These commodities have a wide range of chemical and physical differences which may affect pest mortality when the commodities are heated.

Fruit fly mortality has been examined in the laboratory following exposure to different treatment durations at set temperatures using different substrates ranging from water (Hallman 1994, 1996, Heard et al. 1991, Jang 1986, Rodriguez et al. 1989, Sharp and Chew 1986), artificial rearing diet (Hansen and Sharp 1994, 1997, 1998), fruit juice (Hallman 1996), fruit pulp (Darby and Kapp 1933), or intact fruit (Baker 1939, Hansen et al. 1990). None of these studies provided a measure of the physical properties of the substrate, such as thermal conductivity, yet the studies attempted to infer time-temperature effects on insect survival. Comparisons of larval mortality rates

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are poorly known for identical temperature regimes with different types of host material.

The objective of this study was to examine if different types of substrate affect insect survival after being subjected to the same temperature exposure on mature third instars of the Caribbean fruit fly, *Anastrepha suspensa* (Loew). Mature third-instar Caribbean fruit fly larvae were selected because they infest more than 80 different fruits (Swanson and Baranowski 1972), are easily handled without injury, and their time to pupation is shorter than that of other larval stages. Also, the results from this study can be compared with data from similar studies with the same life stage (Hansen and Sharp 1994, 1997, 1998).

Materials and Methods

Experimental design. Larvae were reared to late third instar in the laboratory on an agar-based diet (Burditt et al. 1974) with wheat germ substituted for defatted wheat germ (Hennessey 1994). Before each test, mature larvae were placed on top of the substrate contained inside conduit metal tubes (5 cm long × 2.5 cm diam) filled mid-point with the test substrate and the open ends sealed with rubber stoppers (Hansen and Sharp 1994, 1997, 1998).

A stainless steel water bath (Model 228, Seco-Ware, St. Louis, MO) was heated with electric thermal elements and the water circulated with stirrers (Model 3M563B, Dayton Electric Mfg., Chicago, IL). Temperatures were monitored within tubes and water baths by using thermocouples (no. 36-gauge, type t, copper-constantan); a thermocouple was inserted into the test substrate for each test. Data were recorded using a data logger (Model 2285, John Fluke, Orlando, FL).

Previous experiments indicated that increased larval density caused increased mortality rates (Hansen and Sharp 1997). Thus, larval density for all tests was standardized to 25 larvae per tube. Hansen and Sharp (1994) demonstrated extensive, but not complete, mortality rates at this density when using agar-based rearing diet as a substrate in tubes submerged in a 40.5°C water bath, maintained at 40°C for 67 min, then hydrocooled (<10 min) in a 25°C water bath. The same treatment was used in this study to obtain a sufficient survivorship range among the tested substrates. The treatment began when the substrate reached 40°C, then continued for 67 min at that temperature. The bath temperature was 0.5°C higher than the treatment temperature to reduce runup time and to compensate for energy lost from bath agitation.

Each treatment replicate had five tubes for every tested substrate and two sets of controls with each having five tubes of agar-based rearing diet. One set of controls indicated larval quality and viability, and the other control set was the check for the treatment. Both controls were maintained at ambient temperature (≈25°C) with one set submerged in a water bath (the treatment control), whereas the other remained out of water (control for insect rearing quality). Survival rates for both sets had to average >85% before the replicate was considered valid.

After treatment, larvae were removed from the substrate and placed on vermiculite where development continued to adult emergence. Within 1 wk after treatment, the numbers of puparia and dead larvae were counted for each sample. Larval mortality was determined by failure to pupariate (to form puparia). Survival was defined as the percentage of live larvae after treatment, % survival = (100%)(total larvae - dead larvae)/total larvae.

Substrates. The substrate was the material in which the larvae were tested. The agar-based rearing diet was the substrate used to compare with other substrates because of its extensive use as the control substrate in previous studies (Hansen and Sharp 1994, 1997, 1998). Other substrates examined separately were fruit pulp from whole fruits processed by using a blender, excluding the inedible portions such as the peel and seed, and distilled water (Table 1). Fruits used to make the blended substrates were grapefruit ('Marsh'), orange ('navel'), guava, carambola, and mango. Using whole fruits would increase the variables such as size and shape, which would be difficult to make comparisons across substrates. Also, the location of larvae within fruits would not be the same. However, the fruit blends had similar texture, which standardized the fruit substrates, and did not show detectable discoloration, such as due to oxidation. The pH was determined for the the rearing diet, distilled water, and processed fruit substrates using a pH meter (Fisher Scientific, Model 915, Pittsburgh, PA). Specific gravity for each substrate was calculated by the ratio of its mass with that of an equal volume of water. Percentage oxygen saturation was determined using a calibrated oxygen meter (ICM, Model 3100, Hillsboro, OR). Tests were also done using empty tubes (air substrate). At least five replicates were used for each substrate.

Data analysis. Data were analyzed with the Statistical Analysis System (SAS Institute 1988). PROC MEANS was used for data summary. Acute survival after treatment in a substrate was analyzed by two methods. First, nonparametric tests were used to determine significant differences by first arranging data by PROC RANK, then conducting the equivalent to the Kruskal-Wallis *k*-sample test by using PROC GLM with LSD means separation (Zar 1974, SAS Institute 1988). Second, the average survival for each replicate was transformed by Abbott's formula (Abbott 1925), originally used to correct for natural mortality in field insecticide tests, then

Table 1. Physical parameters and percentage oxygen saturation of test substrates used in substrates exposed to 40°C for 67 min*

	Physical parameters			% oxygen saturation	
Substrate	% water	pH	Specific gravity	Mean ± SE	No. tests
Guava	78	3.9	1.07	45 ± 3abd	3
Grapefruit	89	3.3	0.81	54 ± 5ab	3
Mango	81	5.6	1.02	29 ± 2ce	3
Diet	—	4.4	—	—	—
Carambola	—	3.9	0.91	20 ± 2ef	3
Orange	87	4.1	0.96	35 ± 4bcdf	3
Water	100	7.0	1.00	54 ± 2ab	16

The water composition of substrate contain only edible portions (no peel or seed) of fruits (Biale 1960). Values followed by the same letter in each column are not significantly different at the 5% level as determined by the Kruskal-Wallis k-sample test.

* —Indicates no data

nonparametric tests (the equivalent to the Kruskal-Wallis *k*-sample tests) were conducted using PROC RANK with PROC GLM. The first nonparametric test was also used for the oxygen saturation data.

Results

The temperature profiles of the substrates were consistent during treatment among replicates. After treatment, the larvae were found in different locations depending on the substrate. Larvae were generally interspersed throughout each of the fruit blends, were on top of the artificial rearing diet, and were on the bottom of the tubes with distilled water and no substrate. Oxygen saturation was significantly different among the substrates ($F = 16.05$; df = 5, 25; $P < 0.01$) and was the highest in grapefruit and water (Table 1).

Larval survival was similar among the controls for the treatment substrates although larvae from the orange blend substrate had the lowest survival (Table 2). Survival of the treated larvae was significantly different among the different substrates, including air, when using the pooled data ($F = 86.36$; df = 7, 467; $P < 0.01$) or the Abbott transformed values ($F = 20.84$; df = 7, 85; $P < 0.01$). The similarity in survival rates among replicates for each treated substrate, as shown by their respective standard errors of means (SEM), indicates consistency among replicates in effects of treatment parameters (Table 2). Larvae exposed to distilled water had the

Table 2. Acute survival of third instar Caribbean fruit fly, *Anastrepha suspensa*, in substrates exposed to 40°C for 67 min. Mean and SE calculated from pooled data of replicated tests (5 tubes per test and 25 larvae per tube) and the Abbott-transformed values for treated larvae derived from the averages of each test

	% survival					
	Control		Treated		Abbott transformed	
Substrate	Mean ± SE	No. larvae	Mean ± SE	No. larvae	Value	No. reps.
None	94.9 ± 0.7a	3,875	92.9 ± 1.1a	4,000	98.0a	31
Guava	94.6 ± 1.3ab	625	72.6 ± 3.0b	625	76.5b	31
Grapefruit	90.2 ± 2.6bc	625	71.4 ± 2.8b	625	80.2b	5
Mango	90.4 ± 2.6bc	625	68.9 ± 3.5b	625	76.7b	5
Diet	90.8 ± 0.8bc	4,000	64.4 ± 1.8bc	4,000	70.3b	5
Carambola	97.1 ± 0.9a	625	64.3 ± 1.8bc	625	66.1b	5
Orange	89.1 ± 2.0bc	625	54.7 ± 5.0c	625	61.1b	5
Water	97.5 ± 0.6a	750	12.1 ± 1.6d	750	12.4c	6

Values followed by the same letter in each column are not significantly different at the 5% level as determined by the Kruskal-Wallis k-sample test.

lowest percentage of survival (12.1%), whereas larvae treated in air had the highest percentage of survival (92.9%). Among the blended substrates, guava had the highest larval survival (72.6%) while orange had the lowest survival (54.7%) and was significantly different from the other fruit substrates except carambola. Larval survival in carambola was not significantly different than that in any other fruit blend. When Abbott-transformed survival values were analyzed, none of the fruit blends and the rearing diet were significantly different from each other (Table 2).

Discussion

Larval survival was influenced by the type of treated substrate. The greatest larval survival was with air, whereas the lowest larval survival was with water as the substrate (Table 2). The rearing diet and the blended fruit substrates were intermediate. Based on differences in thermal capacity, the total amount of energy to which the larvae were exposed varied between air and the other substrates. The thermal capacity of air, $c_p \simeq 0.241$ cal/g°C (Salisbury 1950), is less than that of water, $c_p = 0.998$ cal/g°C (Weast 1986). Although standards for the thermal capacity of tropical fruit blends have not been established, the blended substrates were water-based (Table 1) so that their thermal capacity values would be expected to be similar to water as are those of other fruit blends (Choi and Okos 1986). Thus, the larvae in the water-based substrates were exposed to more energy than those larvae treated in air. Although the fruit blends did not completely represent the conditions in their respective fruits, they were closer biochemically to the true fruit hosts than to water or rearing diet.

Water as a substrate was particularly detrimental to the larvae during the thermal treatments. In a previous study, we found that distilled water in heated (40°C) tubes only retained ≃ 33% oxygen (Hansen and Sharp 1998). Furthermore, the maximum amount of oxygen dissolved at ambient temperature (≃ 25°C) is 8.3 mg/liter, whereas when heated to 40°C, it is 6.4 mg/liter (Mortimer 1981). Hansen and Sharp (1998) discussed why fruit fly larvae should not be considered as aquatic insects and that they do not have adaptations for extracting oxygen from water. Moreover, the thermal death point for most aquatic insects is below 40°C (Wallace and Anderson 1996). A major contributing factor is the "Q_{10} Rule" where the metabolic rate doubles for every 10°C increase in water temperature (Reid 1961) while oxygen saturation is simultaneously decreasing. Hallman (1996) reported higher mortality for Caribbean fruit fly third instars treated at 43°C in grapefruit juice than in distilled water. This test is not analogous to our study because fruit juice does not have the same oxygen retention capacity as fruit blends and no measurements were given for the amount of oxygen in the water or juice (Hallman 1996).

In this study, however, oxygen saturation was greater in water than in any of the fruit blends (Table 1), yet survival was significantly less (Table 2). There may be other factors contributing to lower survival in water than in fruit blends. First, most aquatic insects have closed or sealed tracheal systems which prevent the tracheae from collapse under water pressure as oxygen is consumed (Chapman 1971); fruit fly larvae lack such a system (Tesky 1981). Second, the oxygen in the water may not be accessible. The fruit blends form an emulsion, like whipped cream, containing "entrained" oxygen among the fruit particles. Presumably, all the fruit blends had similar microstructure and capacity for oxygen retention. Although the amount is less than in water, the oxygen is in a form that can be extracted by the insect, thus prolonging

survival. This was observed regardless of specific gravity of the blend (Table 1). Third, the distilled water may have produced a lethal osmotic stress. Aquatic insects have active osmotic regulation processes to maintain a proper internal salt and water balance while in fresh water (Wallace and Anderson 1996). Because fruit fly larvae are not aquatic insects, they may not have hyperosmoregulation with active ionic transport. Fourth, fruit fly larvae normally inhabit an acidic environment (Table 1). A neutral pH may adversely influence biochemical operations. Finally, differences in exposure to energy may be discounted because both the fruit blends and the water have similar thermoconductivity (Choi and Okos 1986).

The difference in larval survival between the grapefruit and orange substrates is inexplicable (Table 2). Previous field studies indicated that the Caribbean fruit fly and other *Anastrepha* spp. infested grapefruits more frequently than oranges (Eskafi 1988, von Windeguth et al. 1973, 1976). Low larval survival in the orange substrate may be due to volatile oil components; higher concentrations are found in the flavedo of orange than of grapefruit (Greany et al. 1983). However, the citrus substrates used in this experiment were composed of blended endocarp with only an inner layer of albedo; the amounts of volatile oils should have been relatively low for both. Future research is needed to determine if the same oil relationship in the peel also occurs in the pulp.

The rearing diet was composed of ingredients required for adequate larval development. None of these components should be toxic at the amounts used. Although survival in the rearing diet was statistically no different from that in the fruit blends, the larvae remained on top of the substrate during treatment, which precludes respiratory obstruction as a cause of death. Perhaps a volatile by-product is produced during the heat treatment that has a detrimental effect during the exposure period, but there was no evidence for this assumption.

Although the substrate tests were conducted at temperatures inadequate for a quarantine treatment, this experiment demonstrated that the degree of efficacy of a treatment can be influenced by the type of substrate. Under commercial situations, commodity differences may also show the same variability. Because of biochemical interactions occurring between the larvae and substrate, the severity of these interactions depended on the type of substrate. For example, benzyl isothiocynate in papaya is highly toxic to fruit fly eggs and first instars (Seo and Tang 1982). Thus, a generic treatment based on the disinfestation of a single type of commodity is unsuitable. Even closely related commodities, such as types of citrus, may show variable results as they did in this study.

An outcome of this study, however, suggests a procedure to determine the initial experimental exposure in the development of thermal treatments. If importing countries demand the most effective treatment based on the most tolerant life stage of a pest (Ouye and Gilmore 1985), treating insects without substrate may be used to estimate the upper thermal limit for efficacious quarantine treatments. Any exposure above this barrier would be assumed to be effective regardless of the fruit treated. Because this barrier is beyond what is needed for quarantine security, treatment temperature or exposure would be lowered by determining the influence on pest mortality of heated substrates for each fruit type. Furthermore, all pertinent life stages could be screened by using no substrate to determine the highest thermal tolerance.

Finally, care must be taken when interpreting mortality in experiments with laboratory-reared flies. The biointergrity of fly varies with generations and routine laboratory procedures. In this study, a separate control was used to ensure viability of the

test cohort. Furthermore, because of the variable conditions wild flies are exposed to in the field, wild flies may have preadapted resistance to thermal treatments. For example, Krebs and Loeschcke (1994) found that *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) adults, subjected to short non-lethal durations of 40°C, formed heat shock proteins, and these researchers hypothesized that flies in warm climates would have greater thermal resistance than those in cooler ones. Also, Hallman (1994) found that Caribbean fruit flies were more tolerant of heat when reared at higher temperatures than at lower temperatures inside the range of 20 to 30°C.

In commodity disinfestation, the efficacy of a heat treatment depends not only on temperature duration but also on the surrounding biochemical environment. Additional research is warranted to determine if compounds toxic to pests are present or released in any heat treated commodity. With commodities that are easily damaged by exposure to thermal treatments, perhaps the toxic compounds can contribute to controlling the insects at lower temperatures.

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