Phytosanitary Cold Treatment for Oranges Infested With *Bactrocera zonata* (Diptera: Tephritidae)

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ABSTRACT The peach fruit fly, *Bactrocera zonata* (Saunders), attacks a wide range of tree fruits in countries from Egypt to Vietnam and is occasionally trapped in the United States. Phytosanitary treatments may be required to export fruit hosts of this insect from countries where it is endemic to countries where it is absent but could become established. This research describes comparative studies to determine if *B. zonata* could be phytosanitarily controlled by cold treatment schedules existing for *Ceratitis capitata* (Wiedemann) and *Anastrepha ludens* (Loew), and the development of a cold treatment of 18 d at 1.7°C for *B. zonata* infesting oranges. Fruit were infested by puncturing holes in oranges and allowing tephritids to oviposit in the holes. The treatments were initiated when the larvae reached late third instar because previous research had shown that stage to be the most cold-tolerant. *B. zonata* was not found to be confidently as or less cold tolerant than *C. capitata*; therefore, treatment schedules for the latter are not supported by this research for the former. *B. zonata* was found to be more susceptible to 1.7°C than *A. ludens*; therefore, the use of treatment schedules for *A. ludens* is supported by this research for *B. zonata*. However, the treatment for *A. ludens* requires 22 d. A shorter treatment was verified for *B. zonata* when 36,820 third instars reared from the eggs in oranges were stored at 1.7°C for 18 d with no larvae moving on examination 24 h after removal from the cold treatment chamber.

KEY WORDS cold treatment, quarantine treatment, phytosanitary treatment, peach fruit fly, Mediterranean fruit fly

The peach fruit fly, *Bactrocera zonata* (Saunders), poses an obstacle for the export of fruit from countries where it is endemic, such as Egypt, India, Pakistan, and Vietnam, into countries where it does not occur but could become established, such as the United States (Mohamed and El-Wakkad 2009). Detections of the pest in California in 2006 (Western Farm Press [WFP] 2006) and Florida in 2010 (Steck 2010) highlight the importance of developing treatments that can be used not only for imports into the United States and other countries but where parts of countries become quarantined until the pest is eradicated from them.

As with many tropical tephritids, *B. zonata* has a wide host range including apple, guava, mango, peach, and orange (Steck 2010). Phytosanitary treatments are often required to export potential commodities that may carry invasive species to ecosystems where the species are not endemic but could become established (Heather and Hallman 2008).

In an effort to establish efficacy of existing phytosanitary cold treatments for application to *Bactrocera invadens* Drew, Tsuruta & White and *B. zonata*, Hallman et al. (2013) compared cold tolerance of those two species with *Ceratitis capitata* (Wiedemann) in oranges at 0.94°C and found that *B. zonata* and *C. capitata* were similar. Further research would be needed to determine if cold treatment schedules for *C. capitata* could be used for *B. zonata*. Hashem et al. (2004) found that *B. zonata* was more susceptible to 1.7°C than *C. capitata* in four fruits; however, the measure of efficacy was prevention of pupariation, and plant protection organizations (PPOs) may not accept that as an end point for efficacy of cold treatments because it allows for larvae to be alive for some time after treatment. A PPO normally inspects fruit on arrival at a port of entry, dissecting the fruit in the case of tephritids. If fruit are cold when they are examined and any larvae found are the color of live larvae, the PPO may hold the larvae for a rather brief time until

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they warm to check for movement. However, they will normally not hold up a consignment until nonmoving larvae are given sufficient time to pupariate, which could be days. Regardless, if the PPO has reason to believe that a larva might be alive (whether or not it moves), the consignment could be refused.

If it could not be reasonably determined by comparison that *B. zonata* was not more cold tolerant than *C. capitata*, other options for developing a cold treatment for the former are 1) comparison with a species that had longer times for cold treatment schedules or 2) develop a treatment for *B. zonata* independently via large-scale confirmatory testing that would require treating ≈30,000 insects at one time–temperature combination with no survivors (Heather and Hallman 2008). The number of days required for phytosanitary cold treatments for *C. capitata* and *Anastrepha ludens* (Loew) at 1.7°C are 17 and 22, respectively (Animal and Plant Health Inspection Service [APHIS] 2013b). If the cold schedule for *C. capitata* would not suffice for *B. zonata*, probably the one for *A. ludens* would.

This research has two objectives: 1) compare relative cold tolerance of *B. zonata* with *C. capitata* and *A. ludens* in fruit to determine if treatment schedules for the latter two species could be used for *B. zonata*, and 2) determine the shortest possible treatment time that would provide quarantine security for *B. zonata* in oranges at 1.7°C. In this case, quarantine security is defined as a cold treatment that will prevent movement or subsequent pupariation (if some larvae were not seen to move but pupariated later) of at least 30,000 third instars infesting oranges throughout the entire larval period and treated in the oranges.

Materials and Methods

Tephritids. The *B. zonata* used in this research originated from Mauritius and was reared for 1 yr on diet. *C. capitata* was from a 5-yr-old laboratory strain originating from wild-infested oranges in Argentina. *A. ludens* was from a 2-yr-old colony from Mexico. Voucher specimens were collected and kept at the FAO/IAEA Insect Pest Control Laboratory (IPCL) at Seibersdorf, Austria.

The three species were reared under similar conditions at the IPCL. Adults were maintained in transparent plastic and muslin cages at 25.0 ± 0.5°C, 65 ± 5% relative humidity (RH), and a photoperiod of 14:10 (L:D) h, and fed water and a 3:1:1 dry mixture of sucrose-hydrolyzed yeast-wheat germ ad libitum.

A small amount of guava juice in plastic bottles (0.1 liter) with the sides punctured all around with ≈200 <0.5-mm diameter holes was placed inside separate cages housing *B. zonata* overnight for egg collection; females oviposited into the bottles through the holes. Eggs were collected from *C. capitata* by allowing adults to oviposit through a fine-meshed side wall of their cage into a trough of water. A container of water with its base replaced by cloth mesh pasted with black silicone was placed on top of the cage of *A. ludens*, and females oviposited through the top of the cage and the silicone into the water.

Eggs of *B. zonata* and *C. capitata* were seeded onto standard Seibersdorf larval diet based on wheat bran as the bulking agent (Braga Sobrinho et al. 2006). The larval diet for *A. ludens* used dried grated carrot instead of wheat bran. Diets with developing tephritids were held at 25 ± 0.5°C until the larvae were ready to pupariate, at which time the puparia were separated from the diet and placed in adult cages with food and water to continue the rearing cycle for the insects that were used to lay eggs into the oranges used in tests.

Previous research had determined that the third instar was the most cold-tolerant stage for *B. zonata* and *C. capitata* (Powell 2003, Hashem et al. 2004, Mohamed and El-Wakkad 2009), although Hallman et al. (2013) note that studies done only in Australia show that the second instar is the most tolerant to cold. Unpublished research (G.J.H.) demonstrated that the third instar was the most cold-tolerant stage of *A. ludens*. The third instar was used in all trials with all tephritids.

Infestation of Oranges. Oranges (cultivar 'Valencia') in two size classes (mean wgt = 144.2 ± 1.1 and 237.8 ± 3.2 g) imported to the IPCL from Egypt were stored overnight to acclimate to room temperature (≈24°C), washed, and then allowed to air dry. Holes were made in the fruit peel to facilitate oviposition. To reduce contamination with air-borne *Penicillium*, the fruit arranged in rows of six oranges placed on their side (cheek) in 7- by 40-cm plastic trays were tightly wrapped with a double layer of low-density polyethylene film. Six holes (0.3 mm in diameter) were made into the side that was facing up to a depth just below the peel of each fruit with fine-tipped forceps dipped in 95% ethanol to sterilize the tips, and two to three trays were placed into each cage with 1,000–5,000 adults of each of the three tephritid species. Female flies oviposited into the fruit only through the punctures made through the plastic wrap, thus reducing fungal contamination of the fruit. Exposure times varied from 45 to 120 min, depending on the age and number of flies available; that is, when many flies of ideal reproductive age were present, the fruit were left in for less time. The objective was an infestation rate of ≈30 larvae per fruit. After infestation, the plastic wrap was removed from the trays, and the trays with oranges were placed in 25 by 25 by 45 cm cages inside fine-mesh (Terylene) bags to prevent *Drosophila* spp. from infesting the fruit during the larval developmental period.

The fruit was held in the cages at ≈26°C until the majority of larvae had developed to the third instar (11–16 d); *A. ludens* required longer periods to develop to the third instar than the other two species. Approximately 10% of the infested oranges were randomly selected as controls (untreated), dissected, and the number of live and dead larvae recorded, whereas the rest of the infested fruits were placed into the cold treatment chamber.

Cold Treatment Chamber. A 1.22 by 1.22 by 1.32 m (inside dimensions) environmental chamber (Thermotron Industries, Holland, MI; model SE-2000–4) was used to treat the infested oranges. The tempera-

ture operating range of the chamber is −35–180°C. Four adjustable grill shelves allow for uniform distribution of fruit boxes within the chamber and unobstructed airflow through the shelves. Airflow within the chamber was ≈28 m^3/min. Behind the insulated exterior door, the chamber has a an interior glass door with four flexible iris ports (0.15 m in diameter) to allow fruit and thermocouple manipulation inside the chamber with minimal exposure to the exterior atmosphere and temperature.

Temperature within the chamber was set via a thermocouple placed on top of a box of oranges near the center of the chamber. Temperatures inside the chamber were recorded every 10 min by using an independent type-T thermocouple system (model S8TC, GEC Instruments Gainesville, FL) that was checked for accuracy before experimentation in an ice slurry of reverse osmosis water (accurate to ± 0.03°C). The system consisted of eight thermocouples placed in the center of noninfested oranges (at the same initial temperature as infested oranges) that were introduced into and removed from the chamber when infested fruits were introduced and removed.

Cold Treatment Tests. When mostly third instars were present in the oranges, but before they began forming emergence holes, the oranges were placed into cardboard boxes (0.30 by 0.22 by 0.22 m) lined on the bottom with paper towels to absorb any leakage that might occur during the treatment and placed in the cold treatment chamber at 1.7°C for 8–20 d. Oranges infested with the three species were treated at the same time. Fruit that had begun obvious decomposition were not used in the tests. On removal from the chamber, the oranges were allowed to equilibrate at ≈24°C for 24 h before being dissected and all larvae counted. Larvae that were found moving were noted, and any that did not look obviously dead (i.e., were the cream color of live larvae) were placed in containers with a small amount of moisture for observation (observed several times per day) until they were found to have moved, pupariated (in any form), or were obviously dead. Larvae that moved or pupariated were counted as survivors, regardless of subsequent condition, because inspectors of importing PPOs generally count moving larvae as failures for any treatment except irradiation. Larvae that pupariated were obviously alive whether or not they were observed moving.

Large-scale confirmatory testing was conducted for third-instar *B. zonata* in infested oranges at 18 d until a minimum of 30,000 larvae were exposed to the cold treatment at 1.7°C. Five percent of the fruit were kept as untreated controls to observe movement of untreated larvae.

Means are reported with ± SE. Probit regression (normal probability density function, Log_{10} of dose) was used to analyze dose–mortality relationships for the three species of tephritids, and slopes and intercepts were compared by using likelihood ratio tests to compare relative cold tolerance between species (SAS 9.3, SAS Institute, Cary, NC). Controls were included in the analyses. Lethal dose ratios (Robertson and Preisler 1992) for the estimated level of efficacy that has historically been used by the United States for quarantine security against tephritids, 99.9968% ("probit 9"), were tested for significance (95% CI) by using a probit analysis program (PoloPlus, LeOra Software, Petaluma, CA).

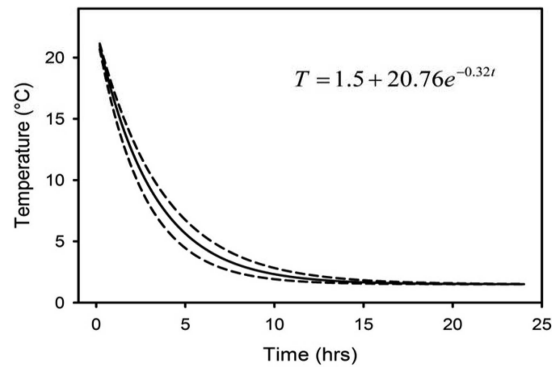

Fig. 1. Temperature (T in °C) decline over time (t in hours) in the center of oranges placed at 1.7°C.

Results

Temperatures in the chamber during the research were quite stable at 1.67 ± 0.001°C. The cooling curve for oranges at the center is presented in Fig. 1; the fruit required almost 24 h to stabilize at ≈1.7°C. The time required for a load to cool down to the prescribed temperature of a cold treatment was not counted as part of the treatment. Therefore, 1 d was subtracted from all of the treatment times.

Movement in the control was 98.4, 99.5, and 99.6% for *A. ludens*, *C. capitata*, and *B. zonata*, respectively. No nonmoving larvae (at 24 h examination) that were saved for further observation were observed to move or pupariate later. Mean larvae per fruit were 12.75 ± 3.91, 25.63 ± 3.82, and 70.81 ± 9.01 for *A. ludens*, *C. capitata*, and *B. zonata*, respectively.

The results of cold treatment of oranges infested with third-instar *C. capitata*, *A. ludens*, and *B. zonata* are presented in Table 1. *B. zonata* appears very similar in cold tolerance to *C. capitata*, whereas *A. ludens* appears more cold tolerant than both based on comparison of percentage not moving at several different dose levels. Although 99% prevention of larval movement 24 h after removal from cold could be achieved at 10 d for both *B. zonata* and *C. capitata*, several more days would be required to prevent the last 1% of the larvae from moving. A similar observation was made for *A. ludens*, which was controlled to the 99% level at 15 d.

The probit model to compare slope and intercept across species showed a significant effect of slope (df = 2; $\chi^2 = 10.14$; $P<0.006$), but none for intercept (df = 1; $\chi^2 = 2.14$; $P = 0.14$). Therefore, the species term was removed and the model was reanalyzed with a common intercept to compare slopes. Maximum likelihood estimates indicated that the *A. ludens* model

Table 1. Third instars of three tephritid species not moving (mean ± SEM) 1 d after being subjected to 1.7°C for 8–20 d in oranges

Dose (d)	Tephritid					
	Ceratitis capitata		*Anastrepha ludens*		*Bactrocera zonata*	
	No. treated	% not moving	No. treated	% not moving[a]	No. treated	% not moving
8	721	96.99 ± 3.01	—	—	4,449	98.03 ± 0.73
9	791	99.43 ± 0.33	—	—	1,387	97.39 ± 2.61
10	519	99.70 ± 0.30	—	—	1,247	99.71 ± 0.29
11	497	99.60 ± 0.40	84	95.23	5,682	99.56 ± 0.14
12	1,305	100.00 ± 0.00	—	—	3,717	99.46 ± 0.05
13	2,395	99.84 ± 0.16	41	95.12	6,830	99.86 ± 0.08
14	3,327	99.99 ± 0.01	264	98.86	5,377	99.87 ± 0.13
15	2,396	99.97 ± 0.02	170	99.67 ± 0.33	10,920	99.89 ± 0.08
16	—	—	821	99.90 ± 0.10	6,849	99.99 ± 0.01
17	—	—	522	99.63 ± 0.37	—	—
18	—	—	2,544	99.94 ± 0.04	7,124	100.00 ± 0.00
19	—	—	500	99.79 ± 0.21	—	—
20	—	—	473	99.46 ± 0.55	—	—

[a] Treatments where the mean % not moving is not followed by a SE were not replicated.

was significantly different from both *B. zonata* (df = 1; $\chi^2 = 9.68$; $P < 0.002$) and *C. capitata* (df = 1; $\chi^2 = 5.29$; $P<0.015$). No differences in the models for *C. capitata* and *B. zonata* occurred (df = 1; $\chi^2 = 0.01$; $P = 0.94$). However, at the 99.9968% level of control (prevention of larval movement or pupariation ≥24 h after removal from cold treatment), there was a significant difference (95% CI) in lethal dose ratios among the three species, with *A. ludens* = *B. zonata* > *C. capitata*, providing evidence that *B. zonata* was significantly more cold tolerant than *C. capitata* at the high levels of control required for phytosanitary treatments.

In the 18-d confirmatory tests, 36,820 *B. zonata* larvae were treated in 1,208 navel oranges over 37 replicates (22–38 fruit per replicate), with no larvae moving 24 h after removal from the cold chamber (Table 2). Mean number of larvae per fruit was 30.88 ± 4.08. In 61 control fruit across 37 replicates, 1,786 live and 29 dead larvae were recovered. Mean number of larvae per fruit was 29.75 ± 7.89.

Using equation 1 adopted from Couey and Chew (1986):

$$P_c = (1 - C)^{1/n} \quad [1]$$

Where P_C is the probability of efficacy, C is the one-tailed CI, and n is the number of insects treated with no movement. Calculating the probability of efficacy with a 95% CI by using the number of insects tested to solve for P_c yields 0.999919, or 81 moving larvae per million treated after an 18-d cold treatment. Mortality (failure of movement or subsequent pupariation) in the untreated control in the large-scale 18-d confirmatory tests with *B. zonata* was 1.67% (29 of 1786 larvae).

During the large-scale confirmatory tests, a technical problem with the cold chamber resulted in a temperature increase that brought fruit pulp temperature above 2.5°C for ≈6 h (maximum temperature reached was 5.98°C). There were six replicates (of the 37 total) with 8,940 larvae; however, no moving larvae were found in any of the infested fruit that were in the chamber during that period.

Discussion

Previous cold-treatment research comparing *C. capitata* with *B. zonata* by using lower numbers of insects and comparisons made at lower treatment times (5–10 d) than those used in the present research did not find significant differences in lethal dose ratios at the estimated 99.9968% level of control between *C. capitata* and *B. zonata* (Hallman et al. 2013). Despite overlapping 95% fiducial limits between estimates of 99.9968% control in this study (Table 2), lethal dose ratios for *C. capitata* and *B. zonata* at that extreme level of control were significantly different, highlighting the value of lethal dose ratio testing (Robertson and Preisler 1992) in comparing quarantine pest species and phytosanitary treatments. Payton et al. (2003) noted that comparison of the overlap of fiducial limits is an overly conservative approach to determining differences between means. The fact that differences in

Table 2. Probit analysis of numbers of third instars of three tephritid species not moving 1 d after being subjected to 1.7°C for 8–20 d in oranges

Tephritid	Slope[a]	ED[b] (95% fiducial limits) in days		
		ED_{95}	$ED_{99.9}$	$ED_{99.99682}$
Ceratitis capitata	0.261 ± 0.058	7.84 (6.90–8.45)	12.63 (11.74–14.18)	15.64 (14.11–18.45)
Anastrepha ludens	0.178 ± 0.103	12.56 (9.40–15.38)	18.25 (15.42–23.73)	21.83 (18.31–29.88)
Bactrocera zonata	0.258 ± 0.091	6.23 (1.80–7.95)	14.55 (12.86–18.86)	19.79 (16.55–28.97)

[a] Slope and intercept parameters followed by the same letter do not differ significantly (likelihood ratio tests; $P < 0.05$).
[b] ED = effective dose to prevent 95, 99.9, and 99.99682 (probit 9) % of third instars from moving or pupariating 24 h after removal from cold.

lethal dose ratios at the estimated 99.9968% level of control were found when higher doses were used and numbers of insects at those higher doses highlights the value in concentrating research at the high levels of control near those required for quarantine security.

Large-scale confirmatory testing supports a treatment time at 1.7°C of 18 d for *B. zonata* beginning once interior fruit temperatures have decreased to 1.7°C. The measure of efficacy used in this research was very conservative: no third-instar larvae moving 24 h after removal from cold treatment. In all of the treatments resulting in ≥99% prevention of movement (e.g., ≥10 d for *B. zonata*), movement was minimal, much less than the controls, and all larvae died before pupariation. In addition, the treatment was developed against third instars, whereas most tephritids infesting harvested fruit are egg or early instar, which are more susceptible to cold than third-instar *B. zonata* (Mohamed and El-Wakkad 2009). Therefore, there is a very low risk that *B. zonata* could survive 18 d at 1.7°C to result in an infestation.

The temperature spike up to 4.3°C higher than the target temperature observed during part of the confirmatory testing provides evidence that the treatment is adequately robust to provide efficacy even with a short period (≈6 h) of temperature increase caused by a system malfunction. The results of this research were used to schedule a cold phytosanitary treatment at 1.7°C for 18 d for oranges and tangerines from Egypt exported to the United States (APHIS 2013a).

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