Development of Phytosanitary Cold Treatments for Oranges Infested With *Bactrocera invadens* and *Bactrocera zonata* (Diptera: Tephritidae) by Comparison With Existing Cold Treatment Schedules for *Ceratitis capitata* (Diptera: Tephritidae)

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ABSTRACT Phytosanitary cold treatments were tested for *Bactrocera invadens* Drew, Tsuruta, and White and *Bactrocera zonata* (Saunders) using comparisons with *Ceratitis capitata* (Wiedemann). Oranges were infested by puncturing holes in the peel 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 for all three species. Results show that *B. invadens* is not more cold tolerant than *C. capitata* and *B. zonata* at 1.0 ± 0.1°C and lend support to the use of *C. capitata* cold treatment schedules for *B. invadens*. It cannot be concluded that *B. zonata* is not more cold tolerant than *C. capitata*.

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

Tephritid fruit flies are the most important family of quarantine pests restricting trade in fresh fruits because 1) most have wide host ranges, 2) they have high reproductive capacity, 3) they attack hosts of relatively high value late in the season when the majority of the costs of production have been expended, 4) many countries have quarantines against their hosts, and 5) their cryptic nature of feeding renders attempts to cull infested fruits during harvest and packing only partially successful.

Phytosanitary treatments are used to reduce the risk that fruit from quarantined areas will result in an infestation becoming established in a noninfested importing area. Often phytosanitary treatments are not available when tephritid hosts from an area are quarantined because a novel fly species is found in that area. Various areas of the world have a history of repeated tephritid finds, with some resulting in quarantines that prevent export of host commodities until the pest is declared eradicated. Sometimes the pest becomes established, as is happening with *Bactrocera invadens* Drew, Tsuruta, and White in central Africa (De Meyer et al. 2010).

The development and approval of a new phytosanitary treatment to address a quarantined pest can require considerable resources and a year or more of research. To avoid this problem, treatments for pests that have a high potential to become established or that pose a high risk to agricultural commodities are sometimes developed proactively; plant protection organizations have dedicated significant resources toward this end. However, a tremendous amount of work and cost would be required to develop proactive phytosanitary treatments for all reasonable possibilities.

A faster and more cost-effective alternative is to compare species for which treatments are needed with species for which sound treatments already exist. This effort would eventually culminate in broad generic treatments that would be applicable across groups of pests and commodities, including some for which no research was done (Hallman 2012). For example, generic cold treatments for all species of *Anastrepha* have been approved, although research was not done for all (U.S. Department of Agriculture [USDA] 2013).

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Nuclear Techniques in Food and Agriculture (FAO and IAEA) and the USDA–Animal and Plant Health Inspection Service, Plant Protection and Quarantine (PPQ), entered into an agreement to develop proactive phytosanitary treatments for tephritids, taking advantage of the ability of the FAO/IAEA laboratories in Seibersdorf, Austria, to maintain colonies of a number of economically important tropical tephritids.

Hallman et al. (2011) found that third instar *B. invadens* was no more cold tolerant than third instar *Anastrepha ludens* (Loew), *Bactrocera dorsalis* (Hendel), and *Ceratitis capitata* (Wiedemann) in vitro at 0.94 ± 0.65°C. That information was used to allow cold treatment schedules for these three species to be used for *B. invadens* on an emergency basis (USDA 2013). The objective of this research was to determine whether *B. invadens* was not more cold tolerant than *C. capitata* in fruit and also compare cold tolerance of *Bactrocera zonata* (Saunders) with *C. capitata* and *B. invadens* in fruit.

Materials and Methods

Tephritids. *B. invadens* was from a 2-yr-old laboratory colony that originated in Kenya from wild-infested mangoes. *B. zonata* was from a 1-yr-old colony originating from wild flies infesting fruit in Mauritius. *C. capitata* was from a 5-yr-old laboratory strain originating from wild-infested oranges in Argentina.

All tephritid species were reared under similar laboratory conditions. Adults were maintained in Perspex and muslin cages at 25 ± 0.5°C and 65 ± 5% relative humidity (RH) under 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 each *Bactrocera* spp. 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.

Bactrocera spp. and *C. capitata* eggs were placed onto standard Seibersdorf diet based on wheat bran as the bulking agent (Braga Sobrinho et al. 2006). Diets with developing tephritids were held at 25 ± 0.5°C until the larvae were ready to pupariate, and puparia were collected and placed in adult cages with food and water to continue the rearing cycle.

Previous research was used to determine that the third instar was the most cold-tolerant stage for all three tephritids (Powell 2003, Mohamed and El–Wakkad 2009, Grout et al. 2011a, Ware et al. 2012). Therefore, that stage was used in all trials.

Infestation of Oranges. Oranges ('Valencia'; mean weight = 127.5 g; mean diameter = 8.0 cm) imported from Spain were infested by the three tephritids via oviposition in cages. Fruit was stored overnight in the infestation room to acclimate to room temperature (≈23°C), then washed and allowed to air dry. To reduce contamination with air borne *Penicillium*, the fruit was arranged on their sides in rows of six oranges in 7 by 40-cm plastic trays, which were wrapped with a double layer of plastic film. Six holes (0.3 mm in diameter) were made into the cheek facing up to a depth just below the peel of each fruit through the peel with fine tipped sterilized forceps, and the trays were placed into cages with 1–5 thousand adults of each tephritid species. This arrangement constrained female flies to oviposit into the fruit only through the punctures made through the plastic wrap, thus reducing microbial contamination of the fruit. Exposure times varied from 45 to 120 min, depending on the age and number of flies available, that is, if flies were few and young or old, fruit were exposed for more time. The goal was to achieve an infestation rate of ≈30 larvae per fruit. After the infestation period, the plastic wrap was removed and 2–3 trays were placed in each 25 by 25 by 45-cm cage. The cages were then placed inside fine mesh polyester 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 (11–12 d). One fruit in 10–12 was randomly selected as a control (untreated), dissected, and the number of live (moving) and dead (dark-colored, nonmoving) larvae recorded while the rest of the fruits were placed into the cold treatment chamber. The controls were used to determine natural mortality in nontreated fruit.

Cold Treatment Chamber. A cold treatment chamber (model SE-2000–4, Thermotron Industries, Holland, MI; inside dimensions: 1.22 by 1.22 by 1.32 m) with temperature and humidity controls was used to treat the infested oranges. Airflow within the chamber was ≈28 m^3/min. Four adjustable grill shelves allowed for uniform distribution of fruit containers in the chamber and unobstructed airflow through the shelves. The chamber has a vertical center-parting glass inner door with four flexible iris ports (0.15 m in diameter) so that fruit and thermocouples inside the chamber could be manipulated with minimal exposure to the exterior atmosphere and temperature.

Temperature within the chamber was set using a thermocouple that was placed on top of a box of oranges near the center of the chamber. Temperatures inside the chamber were measured and recorded every 10 min with a 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 and found to be accurate to ±0.03°C. The eight thermocouples were 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 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 with paper towels and placed in the cold treatment cham-

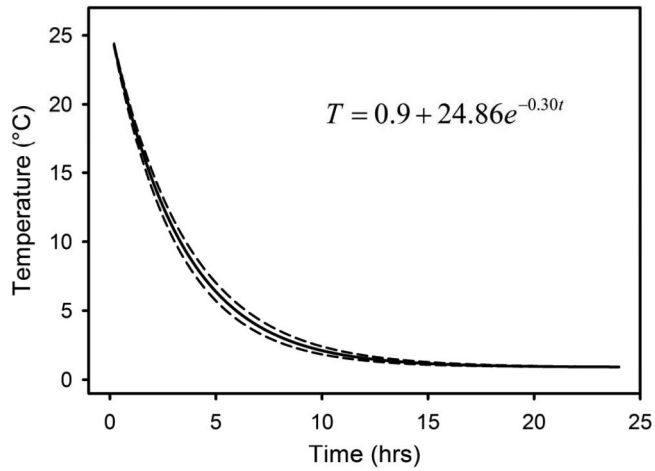

Fig. 1. Temperature (T in degrees Celsius) decline over time (t in hours) in the center of oranges placed at 0.94°C.

ber at 0.94°C for 6–11 d. Fruit that had begun obvious decomposition were not used in the tests. Fruit cooling rates were measured by inserting a thermocouple into the center of a single fruit in each replicate as the fruit was loaded into the chamber. Upon removal from the chamber, the oranges were allowed to equilibrate at ≈24°C for 24 h before being dissected and all larvae counted. Any larvae that were moving were noted and any that did not look obviously dead (e.g., were the cream color of live larvae) were placed in containers with a small amount of moisture for observation until they were found to move (on being observed several times per day), 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 plant protection organizations count moving larvae as failures for any treatment except irradiation. Insects that pupariated were obviously alive whether or not they were observed moving.

Temperatures in the chamber during the research were quite stable at 0.94 ± 0.01°C. The cooling curve for oranges at the center is presented in Fig. 1; mean fruit pulp temperature before cooling was 25.7 ± 0.1°C, and mean time to the treatment temperature of 1.1°C was 15.9 ± 0.5 h. 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, treatment time was defined as the number of days the fruit was held in the chamber minus one day to account for the cooling period.

Statistics. Fruit cooling rates were assumed to follow Newton's law of cooling (the rate of change in temperature of an object is proportional to the difference between its temperature and the ambient temperature). Mean initial fruit pulp temperature, time to treatment temperature, and 95% CIs were used to calculate cooling constants k, and establish fruit pulp cooling rates with 95% CIs.

Dose–mortality relationships were established for each of the three species treated using probit analysis (SAS Institute 2011) based on the number of days of cold treatment after the fruit cool down period. Normal and Gompertz probability distribution functions were both evaluated to determine the best fit. Differences in susceptibility to cold treatment were determined by comparing slope and intercept parameters using likelihood ratio tests. The lethal dose ratio test (PoloPlus, Petaluma, CA; Robertson et al. 2007) was used to calculate statistical significance of differences among the tephritid species at 95, 99, and 99.9968% mortality. Unless otherwise noted means are presented ±SE.

Results

Movement of control larvae upon removal from fruit was 98.4 ± 0.9, 99.5 ± 0.6, and 99.6 ± 0.5%, respectively, for *B. invadens*, *C. capitata*, and *B. zonata*, so it was not necessary to correct for control mortality. None of the larvae that were found not to be moving at the 24-h examination that were saved for further observation were observed to move or pupariate later.

The results of cold treatment of oranges infested with third instar *C. capitata*, *B. invadens*, and *B. zonata* are presented in Table 1. *B. invadens* appeared to be the most susceptible to cold, with 100% mortality after 9 d. Larvae of *C. capitata* and *B. zonata* were similarly tolerant with one or more larvae moving after 9 and 10 d, respectively. Probit regression models showed a slightly better fit using normal probability density function models. Models to compare slope and intercepts showed significant effect of dose (days cold) and dose × species (slope); however, no significant effect of species (intercept) was found. Likelihood ratio tests to compare slopes between species using a common intercept showed the slope for *B. invadens* to be significantly greater (more cold susceptible) than the other two species (Table 2). Lethal dose ratio comparisons showed that among the three species at 95, 99, and 99.9968% mortality, *C. capitata* and *B. zonata* were statistically indistinguishable (although *B. zonata*

Table 1. Third instars of three tephritid species not moving 1 d after being subjected to 0.94°C for 5–10 d in oranges

Dose (days)	Tephritid								
	C. capitata			*B. invadens*			*B. zonata*		
	No. treated	No. not moving	% not moving	No. treated	No. not moving	% not moving	No. treated	No. not moving	% not moving
5	50	37	74.0	346	259	74.9	135	85	63.0
6	114	99	86.8	299	285	95.3	119	96	80.7
7	239	211	88.3	912	909	99.67	574	540	94.1
8	309	308	99.68	994	993	99.90	2,698	2,665	98.8
9	254	253	99.61	475	475	100	430	427	99.30
10	—	—	—	—	—	—	266	263	98.9

tended to seem more cold tolerant) while both species were more cold tolerant that *B. invadens* at the 95% confidence level, with the difference nominally increasing with increasing levels of mortality (Table 3).

Discussion

The literature is not consistent concerning the most tolerant stage of *C. capitata* to phytosanitary cold treatments. Grout et al. (2011b) concluded that the most cold-tolerant stage was the second instar based on a commodity group research report from South Africa (Ware et al. 2005). Hallman et al. (2011) cited Powell's (2003) analysis of historical cold treatment data for *C. capitata* as supporting the third instar to be the most cold-tolerant stage. A summary of studies of most cold-tolerant stage of *C. capitata* follows.

Back and Pemberton (1916) studied cold tolerance among eggs and all three instars of *C. capitata* in apples from 0 to 4.4°C in Hawaii and found that third instar was more tolerant than second instar in all seven tests. Most of Powell's (2003) analysis was based on Back and Pemberton (1916). Hashem et al. (2004) in Egypt found that the third instar was the most cold-tolerant stage at 1.7 and 4°C when *C. capitata* was reared in guava, mango, and orange ("Navel " and Valencia). Ware et al. (2005) did not test distinct instars but evaluated eggs and 6- and 8-d-old larvae (reared at 26°C) at 1°C in grapefruit, orange, and lemon. They concluded that there was no statistical difference in mortality although in orange (but not grapefruit or lemon) survival was nominally higher for 6 than 8-d-old larvae. The data for orange are curious in that survival of 8-d-old larvae was higher up to 5 d at 1°C (25.3%) than in the nontreated control (17.6%).

Three relevant studies with *C. capitata* were done in Australia. Hill et al. (1988) compared tolerance of eggs, a mixture of first and second instars, and mostly third instars to 1.5 ± 0.5°C in oranges and found both larval groups to be very similar, with the younger instars showing a very slight advantage in survival. Jessup et al. (1993) found that the second instar was the most tolerant to 1 ± 0.2°C in two cultivars of lemons. De Lima et al. (2007) in Australia found that the second instar was more tolerant than the third in five types of citrus fruit at 2 and 3°C.

It is possible that different populations of *C. capitata* vary in relative tolerance of the different stages to cold. Diamantidis et al. (2011) found that geographically isolated populations of *C. capitata* vary in a number of traits (e.g., reproductive patterns, survival, developmental rate, and intrinsic rate of increase). From the literature, it seems that the most cold-tolerant stage for most populations of *C. capitata* is the third instar with the only well-documented exception being Australia. Incidentally, Ware et al. (2012) write that Sproul (1976) working in Australia determined that third instar *C. capitata* was the most cold tolerant of the stages in 'Granny Smith' apples when in actuality Sproul (1976) cannot be used to determine most cold-tolerant stage nor does he claim one stage was more tolerant than the others.

These results demonstrate in a fruit that *B. invadens* is not more cold tolerant than *C. capitata* at ≈1°C and lend support to the use of *C. capitata* cold treatment schedules for *B. invadens.* Furthermore, given the higher mortality rate of *B. invadens* (demonstrated by the statistically significant greater slope in Table 2), it may be possible to develop shorter cold treatments than those used for *C. capitata.* However, in large scale testing at 1.1°C, Grout et al. (2011a) found one survivor (moving larva 1 d after termination of cold treatment) of 22,449 tested at 13 d, indicating that the 1.1°C treatment schedule of 14 d for *C. capitata* cannot be

Table 2. Slope and dose (days in cold storage) estimates for mortality from probit models for three tephritids subjected to 0.94°C for 5–10 d in oranges

Tephritid	Slope[a]	Estimated days to achieve % mortality (95% CI)		
		95%	99%	99.9968%[b]
B. invadens	0.743 ± 0.16a	6.0 (5.8–6.2)	6.7 (6.5–7.0)	8.5 (8.0–9.0)
B. zonata	0.631 ± 0.10b	7.1 (5.8–8.4)	8.3 (7.4–12.2)	11.2 (9.3–23.6)
C. capitata	0.628 ± 0.11b	7.3 (6.0–20.0)	8.6 (7.4–50.3)	11.8 (9.2–126.1)

[a] Mean slopes followed by the same letter indicate no significant difference ($P > 0.05$).
[b] Probit 9.
Common intercept = −2.872 ± 0.682.

Table 3. Lethal dose ratio comparisons among third instars of three species of tephritids infesting oranges subjected to 0.94°C

Species comparisons	Lethal dose ratio limits[a] (95% CI) at		
	LD_{95}	LD_{99}	$LD_{99.9968}$
C. capitata vs *B. invadens*	1.15–1.28*	1.21–1.44*	1.31–2.01*
C. capitata vs *B. zonata*	0.97–1.1	0.97–1.15	0.91–1.40
B. invadens vs *B. zonata*	0.81–0.87*	0.76–0.84*	0.62–0.78*

[a] Limits not including one are significantly different (95% CI). An asterisk indicates significance.

lowered for *B. invadens.* Cool-down time during that test required 3 d; it is possible that the slower cool-down allowed for greater physiological acclimation and possible increased cold tolerance compared with the present test where cool-down was achieved in 1 d (Heather and Hallman 2008). Ware et al. (2012) found that >15 d was required to kill *B. invadens* in avocado at 1.5°C. Treatment time required by Animal and Plant health Inspection Service (APHIS) for *C. capitata* at 1.6°C is 16 d, further indicating that treatment times shorter than those for *C. capitata* may not be possible for *B. invadens.* Again, cool-down times were 2–3 d in Ware et al. (2012) as opposed to 1 d in the current study. From this research it cannot be concluded that *B. zonata* is not more cold tolerant than *C. capitata.*

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