RAPID COMMUNICATION

Comparison of In Vitro Heat and Cold Tolerances of the New Invasive Species *Bactrocera invadens* (Diptera: Tephritidae) With Three Known Tephritids

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ABSTRACT *Bactrocera invadens* Drew, Tsuruta & White (Diptera: Tephritidae) is spreading throughout central Africa attacking a variety of fruit; quarantines are placed on fruit from this region that are considered hosts. The only phytosanitary treatment that is commercially available is an ionizing irradiation treatment for all Tephritidae at 150 Gy. The development of other treatments, such as heat, cold, or fumigation, usually requires testing tens of thousands of insects at a dose that provides efficacy and may take several years. It may be possible to shorten the time required to develop treatments by comparing tolerance of a new quarantine pest to tolerances of pests with similar behaviors and modes of infestation for which treatment schedules are available. Cold and heat tolerance of *B. invadens* was compared with tolerance of *Anastrepha ludens* (Loew), *Bactrocera dorsalis* (Hendel), and *Ceratitis capitata* (Wiedemann) in vitro. Third-instar *B. invadens* was no more cold tolerant than the other species when treated in diet at 0.94 ± 0.65°C and no more heat tolerant than *C. capitata* when immersed in vials in water at 44.7 ± 0.1°C. The data at 0.94 ± 0.65°C was used to include *B. invadens* in a USDA cold treatment schedule for citrus fruit from Africa so that trade would not be interrupted while protecting U.S. agriculture from this invasive pest.

KEY WORDS cold treatment, hot water immersion, heat treatment, quarantine treatment, phytosanitary treatment

Since 2003 *Bactrocera invadens* Drew, Tsuruta & White (Diptera: Tephritidae) has been rapidly spreading throughout central Africa and attacking a wide variety of fruit, such as mango (*Mangifera indica* L.), guava (*Psidium guajava* L.), *Citrus* spp., papaya (*Carica papaya* L.), tomato (*Lycopersicon esculentum* L.), and banana (*Musa* spp.) (Drew et al. 2005). The pest is closely related to *Bactrocera dorsalis* (Hendel) and is thought to have originated in tropical Asia.

As the geographical distribution of the invasive pest increases, fruit in infested areas are placed under quarantine, prohibiting their shipment to markets that do not have the fly if no acceptable phytosanitary measures exist. Thus, there is an urgency to develop efficacious phytosanitary treatments for the pest to prevent interruptions in trade. The development of a phytosanitary treatment for a new pest requires time-consuming, large-scale testing to confirm that the potential treatment is efficacious to a high degree (Heather and Hallman 2008). Ducamp-Collin et al. (2008) preliminarily explored hot water immersion as a phytosanitary treatment for mango infested with *B. invadens* and concluded that a treatment might be feasible.

One route for developing phytosanitary treatments is to compare susceptibility of a pest to treatments already developed for similar species, and if that pest is no more tolerant to the treatment than the other species it can be argued that the treatments are also efficacious for the pest in question. That strategy was successfully used to develop a radiation phytosanitary treatment of 70 Gy for *Anastrepha obliqua* (Macquart) without conducting large-scale confirmatory tests by comparing it in vitro with *Anastrepha ludens* (Loew) for which large-scale testing in fruit was done (Hallman and Worley 1999, Hallman and Martinez 2001, FAO 2009).

Only 11 treatments for quarantine pests (all using ionizing radiation) are currently adopted in the International Standard for Phytosanitary Measures listing phytosanitary treatments (FAO 2009), nevertheless the U.S. Department of Agriculture approves various phytosanitary treatments for a variety of tephritids (APHIS 2010). Among these are heat and cold treatments, which are broadly accepted and technologically accessible throughout the world (Heather and Hallman 2008). Tropical tephritids are known to vary in tolerance to cold as exemplified by the treatment schedules in Table 1. Differences among species in heat tolerance also have been reported (Table 2).

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Table 1. Cold treatment schedules for commodities infested with three tephritids (APHIS 2010)

Schedule	Fly	Temp (°C)	Time (d)
T 107-b	*A. ludens*	0.6	18
		1.1	20
		1.7	22
T 107-j	*B. dorsalis*	1.0	15
		1.4	18
T 107-a	*C. capitata*	1.1	14
		1.7	16
		2.2	18

The objective of this research was to compare in vitro cold and heat tolerance of *B. invadens* to three species of tropical tephritids for which treatment schedules are available (APHIS 2010) to explore the possibility of using a heat or cold treatment against *B. invadens.*

Materials and Methods

Tephritids. *Ceratitis capitata* (Wiedemann) was from a 3-yr-old laboratory strain originating from wild-infested orange in Argentina. *B. invadens* was from a 5-yr-old laboratory colony that originated in Kenya from wild-infested mango. *Bactrocera dorsalis* (Hendel) was from a 3-yr-old laboratory strain originated in Thailand in wild-infested mango. *A. ludens* was from a Mexican laboratory strain originally reared from wild-infested mango and transferred to the Food and Agricultural Organization (FAO)/International Atomic Energy Agency (IAEA) Agriculture and Biotechnology Laboratory in Seibersdorf, Austria, where this research was done, two months before the research was initiated.

All species used in these experiments were reared under similar laboratory conditions: Adult flies were housed in Perspex and muslin cages at 25 ± 0.5°C and 65 ± 5% RH under a photoperiod of 14:10 (L:D) h. Adults were fed water and a 3:1 mixture of sugar–protein hydrolysate ad libitum.

A 100-ml plastic bottle with lid containing a small quantity of guava juice with its sides punctured all around with ≈200 <0.5-mm-diameter holes was placed inside separate cages housing each *Bactrocera* spp. overnight for egg collection. 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.

Bactrocera spp. and *C. capitata* eggs were seeded onto the standard Seibersdorf diet based on wheat bran as the bulking agent (Braga Sobrinho et al. 2006). The diet for *A. ludens* used dried, grated carrot, *Daucus carota* L., instead of wheat bran. Diets with developing tephritids were held at 25 ± 0.5°C until they were ready to pupariate. Puparia were collected and placed in adult cages with food and water to continue the rearing cycle.

Cold Treatments. Cold treatment experiments were conducted in a 0.3-m^3 cooled incubation chamber (model IR44, LMS, Ltd., Kent, United Kingdom). Temperature was 0.94 ± 0.65°C, which is within the range used for cold treatment schedules to control fruit flies associated with shipments of fruit imported into the United States (Table 1). Air was constantly circulating in the chamber via a small fan. Temperatures were measured inside the chamber and recorded every 10 min by using a type-T thermocouple system (model S8TC, GEC Instruments Gainesville, FL). Before the experiment, the thermocouple system was checked for accuracy with reverse osmosis water ice slurry and found to be accurate to ± 0.03°C. In total, six thermocouples were used to monitor temperature inside the chamber.

Third-instar larvae of all four species were used in the cold treatment experiment because previous work indicated this life stage to be the most cold tolerant for *B. invadens* (T. Grout, personal communication). The cold treatments in Table 1 against *A. ludens* used third instars because this stage was considered the most cold tolerant (Baker et al. 1944). In an analysis of historical cold treatment data for *C. capitata,* Powell (2003) found the third instar to be the most cold-tolerant stage. Armstrong et al. (1995) controlled all eggs and larvae of *B. dorsalis* within 12 d at 1.1 ± 0.6°C. It is not clear from their study which stage is the most cold tolerant because different techniques were used to infest fruit and evaluate survival of eggs and first instars versus those used for third instars. There is no other information on comparative cold tolerance of *B. dorsalis* stages, and the treatments for this species in Table 1 are based on Burditt and Balock (1985) who tested eggs and larvae together in fruit.

Late third instars that were still feeding were collected with 30–40 ml of diet from the rearing trays and placed into 100-mm-diameter plastic petri dishes within 1 h before placement into the cold chamber. The mean ± SEM number of larvae per dish across all treatments and replicates was 102.0 ± 6.1. Petri dishes were stacked together in random order by the date they entered the cooler, and all dishes were placed on the center shelf as it was observed that temperatures in the cooler were slightly vertically stratified. There were six replicates done at different times. Two thermocouples were placed between adjacent stacked petri dishes and the remaining four were placed around the shelf where the dishes were held.

Table 2. Minimum times to achieve 100% mortality for third instars of three tephritids by using a heating block system at four temperatures

Tephritid	Time (min) at four temp			
	44°C	46°C	48°C	50°C
A. ludens[a]	100	25	6	2
B. dorsalis[b]	150	40	5	2
C. capitata[c]	–	60	15	4
C. capitata[b] (laboratory colony)	130	25	4	1.5
C. capitata[b] (wild type)	70	15	2.5	1

[a] Hallman et al. (2005).
[b] Armstrong et al. (2009).
[c] Gazit et al. (2004).

Table 3. Time (days) at 0.94 ± 0.65°C, number (*n*) of third instars treated, and percentage that failed to pupariate after correction for control failure (Abbott 1925) for four species of tephritids in vitro

Time (d)	*A. ludens*		*B. dorsalis*		*B. invadens*		*C. capitata*	
	n	Failure to pupariate (%)	*n*	Failure to pupariate (%)	*n*	Failure to pupariate (%)	*n*	Failure to pupariate (%)
4	288	7.3	420	66.6	648	79.5	486	50.6
5	434	34.4	468	86.2	887	93.5	759	83.5
6	621	57.3	684	97.5	1075	99.6	697	92.7
7	356	56.9	430	99.3	778	99.7	684	98.8
8	146	90.1	188	100	395	100	215	100
10	57	98.2	129	100	235	100	95	100
11			142	100	104	100	59	100
12	22	95.5						

Petri dishes were removed from the cold chamber at 4, 5, 6, 7, 8, 10, 11, and 12 d. Larvae were then extracted from the diet within 24 h and held for pupariation. Control dishes were evaluated 24 h after initiation of treatment without being placed in the cold chamber. Prevention of pupariation was used to determine treatment efficacy by subtracting the number of puparia from the initial number of larvae. Failure of pupariation may not be the measure of treatment efficacy ultimately used in a cold treatment for *B. invadens*, but it suffices in this comparative study as a clearly demarcated threshold, whereas direct larval mortality may be more difficult to determine after phytosanitary treatments. For example, Hallman and Thomas (2011) found that some *A. ludens* larvae that were not detected to move for several days after fumigation with methyl bromide pupariated nevertheless.

Heat Treatments. Heat treatment experiments were conducted using a circulating hot water bath (model W45, Haake & Partner Datentechnik, Offenburg, Germany). Water temperature was monitored during each treatment with a certified mercury thermometer and found to be 44.7 ± 0.1°C. This temperature is in the range of temperatures commonly found inside fruit during heat treatments (Heather and Hallman 2008).

Third instars were used in the heat treatment research because they were the most heat-tolerant stage for *A. ludens* (Mangan et al. 1998), *B. dorsalis* (Jang 1991), and *C. capitata* (Gazit et al. 2004). Furthermore, third instars are more likely to be found deeper in fruit and thus experience slower and less total heating (Heather and Hallman 2008). For each replicate, 50 larvae of each species were removed from their diets and placed into 15-ml polypropylene graduated tubes with caps (product 430055, Corning Life Sciences, Lowell, MA) along with a small amount of fine saw dust to prevent the larvae from sticking to each other or the sides of the tubes during treatment. Tubes with larvae were held to steel rods with rubber bands to keep them submerged in the water bath during treatment. The duration of treatments ranged from 25 to 60 min, with one to four replicates of each time done on different days. The measure of treatment efficacy was prevention of pupariation, and the objective was to arrive at a time for each species that gave 100% prevention of pupariation. At the completion of each treatment, tubes were removed from the water bath, allowed to cool to room temperature, and larvae were removed from the tube and placed in 100-mm-diameter petri dishes with several grams of fine saw dust. Control tubes were held at room temperature during the duration of the heat treatments.

Statistical Analysis. Control pupariation rates were used to adjust treatment pupariation (Abbott 1925). Probit analysis was used to analyze the dose–response data (SAS version 9.2, SAS Institute, Cary, NC). Probit models using the normal and Gompertz distributions were evaluated to determine the best fit. The maximum likelihood method was used to estimate regression parameters, and likelihood ratio tests were used to make comparisons between individual treatments. The fact that sample sizes and number of replicates vary is unremarkable for most regression analyses.

Results

Cold Treatments. Percentage failure of pupariation for the four species resulting from cold treatments from 4 to 12 d are shown in Table 3. *A. ludens* was

Table 4. Probit regression parameter estimates for cold treatment (0.94 ± 0.65°C) of four fruit fly species

Species	Slope[a]	Intercept[a]	*N*	Pearson χ^2	Estimated dose (95% CI) at four levels of efficacy (d)			
					95%	99%	99.9%	99.99683%[b]
A. ludens	0.51 ± 0.12A	−3.03 ± 0.72A	1,924	<0.01	9.3 (4.4–22.7)	10.6 (5.3–25.3)	12.1 (6.3–28.1)	13.9 (7.6–31.5)
B. dorsalis	0.73 ± 0.05B	−2.50 ± 0.24B	2,461	0.87	5.7 (4.5–7.3)	6.6 (5.3–8.4)	7.7 (6.2–9.6)	8.9 (7.3–11.0)
B. invadens	0.78 ± 0.05B	−2.32 ± 0.25B	4,122	0.19	5.1 (4.7–5.4)	5.9 (4.7–8.1)	7.7 (6.2–10.3)	8.1 (6.5–10.7)
C. capitata	0.74 ± 0.06B	−2.86 ± 0.22B	2,995	0.04	6.1 (4.6–8.1)	7.0 (5.4–9.2)	8.0 (6.3–10.4)	9.3 (7.4, 11.8)

Normal distribution. df = 3.

[a] Slopes and intercepts followed by the same letters are not significantly different (α = 0.05; likelihood-ratio test).

[b] 99.99683% is probit 9, a level of efficacy historically used for fruit fly treatments in the United States (Heather and Hallman 2008).

Table 5. Time (minutes) in 44.7 ± 0.1°C water, number (*n*) of third instars treated, and percentage that failed to pupariate after correction for control failure (Abbott 1925) for four species of tephritids placed in tubes

Time (min)	*A. ludens*		*B.dorsalis*		*B. invadens*		*C. capitata*	
	n	Failure to pupariate (%)	*n*	Failure to pupariate (%)	*n*	Failure to pupariate (%)	*n*	Failure to pupariate (%)
25	50	14.0						
30	100	54.0					50	8.2
35	100	37.0	50	90.0	150	38.7	100	16.0
40	150	68.7	150	93.3	200	66.5	150	42.0
45	100	95.0	150	100	150	96.7	150	43.3
50	50	100	150	98.7	100	100	200	42.5
55			50	100			50	88.0
60							100	100

clearly the most cold tolerant because treatment durations up to 12 d did not produce 100% mortality. Complete mortality among the other three species was reached in 8 d, and *B. invadens* was no more tolerant of cold than any of the other species.

Of the models tried, probit analysis using the normal distribution fit the cold treatment data best. A significant effect of dose (days of cold treatment) ($P < 0.01$), a nonsignificant species effect ($P = 0.75$), and a marginally significant dose × species interaction ($P = 0.07$) were observed in the complete model. When the dose × species interaction term was removed, the model yielded significant effects for both dose and species ($P < 0.01$). Comparisons of regression parameter effects for the four species showed *A. ludens* to have significantly shallower slope and lower intercept than the other three species (Table 4).

Heat Treatments. Failure of pupariation of third instars of *B. invadens* and *A. ludens* was achieved after a 50-min exposure in the hot water bath, whereas *B. dorsalis* and *C. capitata* required 55 and 60 min, respectively, to achieve 100% failure of pupariation (Table 5).

Probit analysis using the Gompertz distribution provided the best overall fit for the hot water immersion data. A significant effect of dose (minutes) ($P < 0.01$) and nonsignificant effects for species ($P = 0.35$) and dose × species interaction ($P = 0.28$) were observed in the complete model. When the dose × species interaction term was removed, the model yielded significant effects for both dose and species ($P < 0.01$).

Comparisons of regression parameter effects for the four species indicated no significant differences among slopes (Table 6). Regression models were therefore calculated using the common slope. Comparisons among intercepts showed *C. capitata* to have a significantly lower intercept than the other three species (Table 5), no other difference in intercepts were observed.

Discussion

The results of this study offer evidence that cold treatment schedules that are efficacious against *A. ludens, B. dorsalis,* and *C. capitata* (Table 1) also would be efficacious against *B. invadens.* APHIS (2010) used results of this study plus an unpublished abstract of cold treatment research done in Kenya to include *B. invadens* in treatment schedule T107-k. Schedule T107-k is used to export citrus fruit from South Africa to the United States, and although *C. capitata* is not listed as a pest in the schedule, the treatment is accepted as being efficacious against this species, also. Finding that *B. invadens* was no more cold tolerant than *C. capitata* supports inclusion of the former in schedule T107-k.

This study supports the requirement for harsher cold treatment schedules for *A. ludens* compared with *B. dorsalis* and *C. capitata* (Table 1), which gives confidence in the applicability of results of this study (Table 3) to cold treatment schedules.

B. invadens was more susceptible to heat than *C. capitata.* However, it would require more supportive research compared with cold treatments to use *C. capitata* heat treatment schedules for *B. invadens,* because heat treatments tend to be more variable than other treatments, such as cold (Heather and Hallman 2008), as evidenced by the studies with *C. capitata* summarized in Table 2. Regardless, the same order of heat tolerance of fruit fly larvae observed from com-

Table 6. Probit regression (Gompertz distribution) parameter estimates for heat treatment (44.7 ± 0.1°C) of four fruit fly species

Species	Intercept[a]	*N*	df	Pearson χ^2	Estimated dose (95% CI) at four levels of efficacy (min)			
					95%	99%	99.9%	99.99683%[b]
A. ludens	−3.56 ± 0.96A	550	4	<0.01	54.8 (35.7–86.8)	62.0 (41.4–96.4)	70.0 (47.8–107.2)	79.6 (55.5–120.0)
B. dorsalis	−2.79 ± 1.06A	550	3	0.14	46.7 (28.4–77.4)	53.9 (34.1–87.0)	61.9 (40.5–97.7)	71.5 (48.2–110.6)
B. invadens	−3.60 ± 1.01A	600	2	0.44	55.2 (35.6–65.8)	62.4 (41.3–73.0)	70.4 (47.7–81.1)	80.0 (55.4–90.6)
C. capitata	−4.75 ± 1.18B	800	5	<0.01	67.3 (43.8–79.7)	74.5 (49.5–86.9)	82.5 (56.0–94.9)	92.1 (63.6–104.5)

[a] Intercepts and estimated doses were calculated using the common slope (0.095 ± 0.024) as no significant slope effect was found among the four species, intercepts followed by the same letters are not significantly different ($\alpha = 0.05$; likelihood-ratio test).

[b] 99.99683% is probit 9, a level of efficacy historically used for fruit fly treatments in the United States (Heather and Hallman 2008).

paring the literature in Table 2—*C. capitata* > *B. dorsalis* > *A. ludens* was supported in this study (Table 5)—indicating that the methodology used herein gave results within the range of expectations. We did not test whether the third instar was the most heat-tolerant stage for *B. invadens*, although it seems to be the case for tephritids in general (Heather and Hallman 2008).

Tolerance of fruit exported from *B. invadens* infested areas should be tested before using any approved treatments against the pest.

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