Determining thermotolerance of fifth-instar *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) and *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae) by three different methods

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ABSTRACT

Thermotolerances of codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), and navel orangeworm, *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae), were determined using two water-immersion methods and one dry-heat method. The two water-immersion methods were: 1) directly immersing screen tubes containing test insects in hot water (direct immersion method) and 2) immersing in hot water solid copper tubes containing insects submerged in tap water (tube immersion method). The dry heating method involved heating insects in computer-controlled heating blocks (heating block system, or HBS). Each test insect was treated at three temperature-time combinations and exposures were adjusted so that each method received the same equivalent accumulated lethal time. In five of the six tests, the HBS provided the lowest mean insect mortality among the three methods, although no statistically significant differences were observed between the direct immersion and the HBS methods. The mean insect mortality obtained with the tube immersion method was significantly higher than that from the direct immersion method and the HBS in four and three of the six temperature-time combinations, respectively. When compared with the two water-immersion methods, the HBS method yielded lower mortality data with less variation at the same mortality level, resulting in more conservative treatment recommendations.

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1. Introduction

To develop effective thermal treatments against insect pests, it is essential to determine the most heat resistant species and their life stages by experimenting with an appropriate heating method *in vitro* (Tang et al., 2000; Wang et al., 2002c). The experimental method should simulate the real heating environment for the infested insects in targeted agricultural commodities (Thomas and Shellie, 2000). Important factors that influence insect mortality in the experiments are treatment substrate, humidity, oxygen level, heating uniformity, heating rate, final temperature, and holding time. A reliable experimental method should provide a precise and controlled temperature profile for the whole population of test insects, in order to avoid the confounding effects of slow heat transfer and inconsistent heating experienced by each insect during the heating period (Jones and Waddell, 1997; Lurie et al., 2004). Insect mortality data vary with heating methods, handling processes and specific test conditions. Therefore, it is important to identify the test method most appropriate for the generation of mortality data used in developing large-scale treatments.

Several methods are commonly applied for studying thermotolerance of insects *in vitro*. One method directly exposes insects in a water bath for specific times (Sharp and Chew, 1987; Jones and Waddell, 1997; Waddell et al., 2000). Another places insects in tubes which in turn are submerged in water baths (Yokoyama et al., 1991; Thomas and Mangan, 1997; Follett and Sanxter, 2001; Lurie et al., 2004). Neven (2008) developed a glass tube immersion system for assessment of insect mortality under combination heat and controlled atmosphere treatments. Hansen and Sharp (1998) hypothesized that the direct water-immersion method restricts oxygen supply to the heated larvae and thus leads to higher thermal mortality data than the methods that provide an adequate supply of air. This has been demonstrated with codling moth larvae, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) (Hansen and

Heidt, 2006). With the direct water-immersion method, Jang (1986) and Waddell et al. (1997) reported that larvae of fruit flies were the life stage most susceptible to heat while eggs were the most heat-tolerant life stage at 48 °C. But when using air treatments, Heather et al. (1997) reported the opposite results at 47 °C. Even the type of medium that the insect is treated in can affect mortality (Hansen, 1996; Hansen and Sharp, 1997). Such contradictory results impede the development of effective thermal treatments.

Recently, an experimental heating block system (HBS) was developed for testing the response of insects to high temperatures after careful engineering analyses of heating uniformity as influenced by ramping rates (Ikediala et al., 2000; Wang et al., 2002a,b; Johnson et al., 2003). The HBS can be programmed to simulate the heating rate of the interior of fruit when subjected to different heating methods such as hot air, hot water and radio frequency heating. This method eliminates the heating non-uniformity over the test insect population and is able to generate highly repeatable results for many insect pests.

The HBS has been used to determine the thermal exposure at specific temperatures necessary to kill fruit flies and the most thermotolerant life stages and species (Gazit et al., 2004; Hallman et al., 2005; Armstrong et al., in press). Of particular interest to us are the results for *C. pomonella* and navel orangeworm, *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae) (Wang et al., 2002a,b), insects of primary phytosanitary concern for tree fruit and nut exports from California and the Pacific Northwest. The thermal death kinetic data obtained by the HBS for *C. pomonella* and *A. transitella* have been validated by efficacy tests *in situ* for certain temperature-time combinations (Wang et al., 2001, 2002c, 2006, 2007a,b; Feng et al., 2004; Mitcham et al., 2004). To develop efficacious heat treatment schedules, *in vitro* heating methods are used to exclude less heat-tolerant species and life stages in treatment efficacy testing, thus significantly reducing the overall number of tests required to establish treatment parameters.

Heating rates have been reported in the literature as an important contributing factor to thermal mortality data obtained in experiments. In particular, slow heating rates may increase the thermotolerance of insects by allowing them more time to acclimate to rising temperatures (Evans, 1986; Neven, 1998; Waddell et al., 2000). Neven (1998) stated that *C. pomonella* larvae in fruits may experience thermal conditioning and acclimation at heating rates between 0.13 and 0.20 °C/min. Thomas and Shellie (2000) reported that the exposure times needed to achieve 99% mortality of Mexican fruit fly increased from 42 to 62 min at 44 °C when the heating rate was reduced from 1.40 to 0.18 °C/min. Yin et al. (2006a,b) clearly indicated the enhanced thermotolerance of *C. pomonella* larvae after preconditioning at sub-lethal temperatures, due to the induction and synthesis of heat shock proteins. More rapid heating rates have a negligible effect on insect thermal mortality. Wang et al. (2002a) observed that the total accumulated lethal time required for 100% kill of *C. pomonella* larvae appeared to be slightly higher at 1 °C/min than at 18 °C/min, suggesting that ramp times corresponding to a heating rate at or greater than 1 °C/min did not allow insects to develop thermotolerance. Because heating rates vary widely among different experimental methods, the potential for thermotolerance developing in target insects must be considered when comparing different test methods.

The objectives of this study were to: 1) determine the equivalent holding times based on the accumulated lethal effect obtained during the ramping period for two different hot water-immersion methods and the HBS, and 2) compare the thermal mortality of fifth-instar *C. pomonella* and *A. transitella* among the three heating methods at three temperature-time combinations.

2. Materials and methods

2.1. Heating methods

Three test methods were used to compare the thermal mortality of fifth-instar *C. pomonella* and *A. transitella*: 1) directly exposing insects inside screen metal tubes to hot water (direct immersion method), 2) placing insects in copper tubes filled with water and heating in water baths (tube immersion method), and 3) heating insects in a heating block system (HBS). The screen metal tubes were made of a fine stainless steel mesh that allowed insects to be in direct contact with the hot water (Fig. 1a). The tube immersion method (Fig. 1b) used four tubes made of copper (1 mm thick) attached to a thin copper plate (1 mm thick), partially filled with water and submerged in a water bath. In both immersion methods, rubber stoppers were used to prevent insects from escaping, and each tube could handle 10 larvae at a time.

The HBS developed at Washington State University (WSU), Pullman, WA consisted of top and bottom aluminum blocks, heating pads, insect chamber, and a data acquisition/control unit (Fig. 1c). The system could treat up to 200 insects at a time. Calibrated type-T thermocouples inserted through sensor paths monitored the temperatures of the top and bottom plates. Heating rates (0.1–15 °C/min), set-point temperatures and holding times were controlled by the Visual Basic software via a solid-state relay with a PID controller. Further details of the HBS description and temperature control can be found in Wang et al. (2002b, 2005).

2.2. Test insects

Fifth-instar *C. pomonella* larvae were reared at the USDA-ARS Yakima Agricultural Research Laboratory (YARL) in Wapato, WA according to Hansen and Anderson (2006). Larvae were fed on a soy–wheat germ-starch artificial diet at 27 °C, 40–58% relative humidity (r.h.), and a photoperiod of 16:8 h (L:D). Fifth-instar *A. transitella* larvae were reared at the USDA-ARS San Joaquin Valley Agricultural Sciences Center (SJVASC), Parlier, CA, using methods described by Tebbets et al. (1978). Larvae were fed on a wheat bran diet at 27 °C, 60% r.h. and a photoperiod of 14:10 h (L:D). The insects were packed in insulated shipping cartons and shipped via overnight delivery to WSU, Pullman, WA. Previous studies showed negligible effects of shipping on insect mortality (Wang et al., 2002a,b). The insects were left at room temperature for 2 h before testing. We used only actively moving fifth-instar larvae in tests because previous studies have shown that this life stage is the most heat tolerant for both species (Wang et al., 2002b, 2004).

2.3. Determination of equivalent heating times

Previous studies showed that heat treatments with the same holding time and temperature but different heating rates did not impart the same thermal lethality to the test insects (Wang et al., 2002a, 2004). For example, treatments with slow heating rates exposed insects to lethal temperatures for longer periods than tests using faster rates. The contribution of the ramp period should be taken into account when comparing the effects of different heating methods. Using the concept of equivalent thermal lethal time at a fixed reference temperature, the cumulative thermal lethal time during the ramp-up period can be estimated based on measured temperature–time histories (Tang et al., 2000; Hansen et al., 2004). The equivalent thermal lethal time M_{ref} (min) at a reference temperature, T_{ref} (°C), can be estimated from:
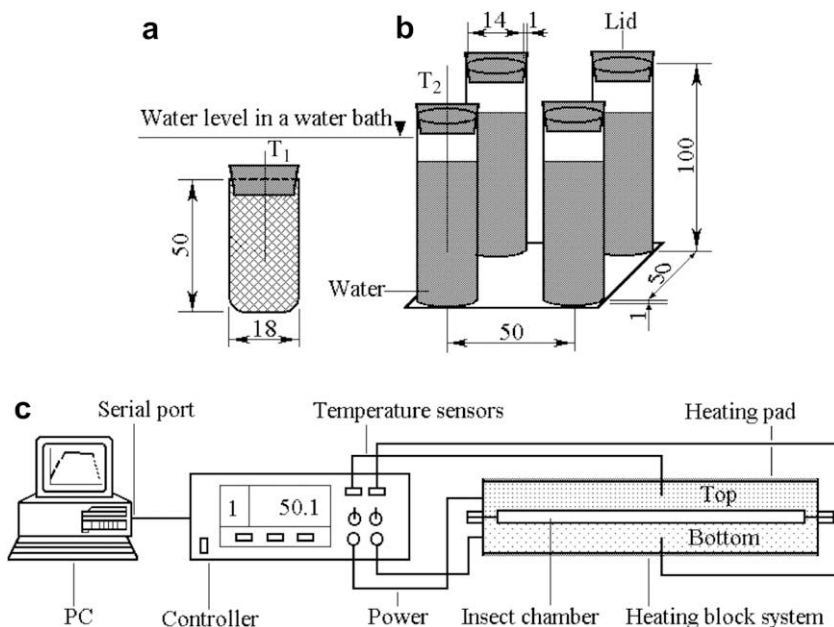
Fig. 1. Schematic view of (a) the direct immersion method, (b) the tube immersion in a water bath method (all units are in mm) and (c) the heating block system from Yin et al. (2006a). T_1 and T_2 are thermocouples for the screen metal tube and a copper tube, respectively.

$$M_{ref} = \int_0^t 10^{\frac{T(t) - T_{ref}}{z}} \, dt \qquad (1)$$

where $T(t)$ is the center temperature in tubes or HBS as a function of time t (in minutes), and z is the temperature difference required for a tenfold change in the thermal death time (TDT) for the same mortality level of insects (°C) (Tang et al., 2000). An average z value of 4 °C was used in this study based on the results of TDT curves for *C. pomonella* and *A. transitella* larvae (Wang et al., 2002a,b).

The tubes with initial temperatures of 22 °C were immersed in a water bath (Model ZD, Grant, Cambridge, UK) at 50 °C. The water temperatures in the metal screened and copper tubes were recorded at 5 s intervals using a data logger (DL2e, Delta-T Devices Ltd., Cambridge, UK) and type-T thermocouples (Fig. 1a and b). A heating rate of 5 °C/min to the desired holding temperature was used for the HBS (Fig. 1c). Eq. (1) was used to estimate from the recorded temperature data the accumulated lethal time during the ramp-up period for each of the three methods. These accumulated lethal times were then used to calculate the total heating time needed to obtain total thermal lethal times for direct and tube immersion methods equivalent to that for the HBS method (Table 1).

Table 1
Estimated total heating time in the water bath based on equivalent accumulated lethal time for ramp-up period and a selected HBS holding time (t_h).

Minutes	Heating block system (HBS, 5 °C/min)	Direct immersion	Tube immersion
Ramp-up time (t_r)	5.7	0.6	2.0
Accumulated lethal time (t_a) equivalent at 50 °C for the ramp-up period	0.42	0.24	0.68
Lethal time difference during ramp as compared to HBS ($t_l = 0.42 - t_a$)	0	0.18	−0.26
Total heating time used in the experiment to achieve equivalent lethal treatments ($t_t = t_h + t_r + t_l$)	$t_h + 5.7$	$t_h + 0.78$	$t_h + 1.74$

2.4. Treatment procedures

For both the direct and tube immersion methods, 20 tubes with 10 larvae each were treated at each temperature–time combination providing a total of 200 larvae for one replicate. As a control, the same number of tubes with insects was immersed in water at 22 °C for the longest exposure (30 min). The water level in the copper tubes was about 5 cm below the water level in the bath. Each replicate used fresh tap water to avoid reduction of the dissolved oxygen. For tests with the HBS, 200 larvae were treated at a time for each temperature–time combination. Control insects were placed in the unheated block for the longest exposure time for each temperature-time combination. The detailed handling procedures for the HBS can be found in Wang et al. (2002a,b). All the treatments were replicated three times.

To compare the thermotolerance of the insects among the three methods, three temperature-holding time combinations were selected to obtain a thermal mortality of less than 100%: 48 °C for 5 min, 50 °C for 2 min, and 52 °C for 1 min for *C. pomonella* and 48 °C for 30 min, 50 °C for 10 min, and 52 °C for 1 min for *A. transitella*. After exposure at each temperature for the determined total heating time, the larvae in the tubes were taken out and dried on a paper towel for about 2 min, and then transferred to a plastic container ($15 \times 10 \times 5$ cm). Cardboard strips were provided for pupation sites. The larvae were kept at ambient room conditions with the photoperiod similar to the rearing conditions mentioned above. The examination period started the day following heat treatments. Insects were considered dead if the body color had turned black. Moribund and surviving larvae were observed for five days.

Mortality was calculated as the percentage of dead larvae relative to the total treated larvae for each treatment. Mean values and standard deviations were calculated from three replications for each temperature–time combination. The final mortality was corrected using Abbott's (1925) formula. The mean values were compared using the SAS analysis of variance (ANOVA) procedure (SAS Institute, 2001). Where there were significant differences ($P < 0.05$), means were separated using a least significant difference (LSD) t-test (SAS Institute, 2001).

3. Results and discussion

3.1. Equivalent heating times

Typical temperature–time profiles for both immersion methods and the HBS from room temperature to a set point of 50 °C are shown in Fig. 2. The time needed to reach the set point was shortest for the screened tubes and longest for the HBS with a heating rate of 5 °C/min (Table 1). Based on the temperature–time profiles (Fig. 2), the average heating rates over the ramp-up period (the time for the center temperature to reach to within 0.5 °C of the set point) were about 5, 48, and 14 °C/min and the accumulated lethal times at 50 °C were 0.42, 0.24 and 0.68 min for the HBS, the direct immersion and tube immersion methods, respectively (Table 1). The long lethal time with the tube immersion method (T_2) was caused by the slow rise of the water temperature when approaching the set-point temperature because of the reduced temperature gradient between the medium and the tube center (Fig. 2). If the holding time for HBS treatments was 5 min (t_h) at 50 °C, the total heating times for the water bath treatments should be 5.78 and 6.74 min for direct and tube immersion methods, respectively, to provide an equivalent lethal effect to that at 5 °C/min in the HBS. Because of similar temperature–time profiles at 48 and 52 °C, the accumulated lethal times estimated by Eq. (1) for the two tube methods relative to the HBS were similar to those at 50 °C. Consequently, the lethal times in Table 1 were also used to calculate equivalent exposures at 48 and 52 °C.

3.2. Effects of heating methods on the insect mortality

Control mortality for fifth-instar C. pomonella was $11.7 \pm 4.5\%$, $11.5 \pm 2.0\%$, $4.4 \pm 2.6\%$ for direct immersion, tube immersion and HBS treatments, respectively. The control mortality for fifth-instar A. transitella was $6.5 \pm 2.7\%$, $6.8 \pm 5.5\%$, $4.2 \pm 2.8\%$ for direct immersion, tube immersion and HBS treatments, respectively. These data indicate that the effects of handling procedure on the insect mortality were minor in the HBS as compared to the direct and tube immersion methods. In particular for C. pomonella, the control mortality from the HBS was significantly less than that from the tube immersion method ($F_{(1,4)} = 15.0$; $P < 0.05$). This demonstrated one possible advantage of the HBS over the other two methods. The two immersion methods resulted in similar control mortalities for both insects.
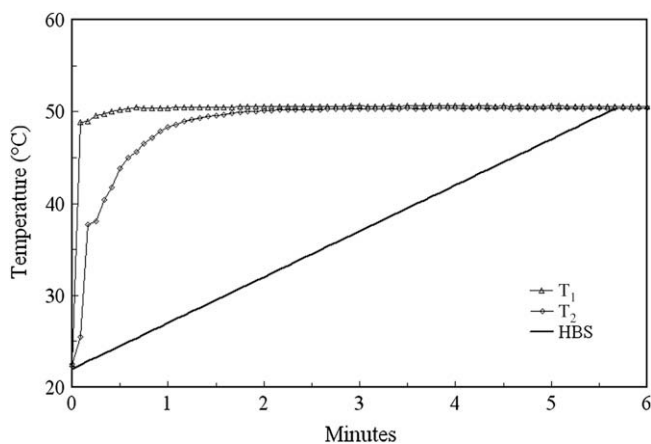
A comparison of thermal mortality of fifth-instar C. pomonella for three temperature–equivalent HBS time combination treatments obtained by the direct immersion, the tube immersion and the HBS methods is shown in Table 2. No significant difference in insect mortality was observed between the direct immersion and the HBS ($F_{(5,12)} = 0.93$; $P > 0.05$). Insect mortality in the tube immersion method was significantly higher than in the other two methods in all C. pomonella treatment combinations with the exception of the HBS at 52 °C + 1 min. The above results suggest no clear effect of heating medium, namely heat block vs. hot water, on the observed mortality data.

The C. pomonella mortality data from the HBS were comparable to those observed in previous years; these values were 71.0 ± 9.2, 92.5 ± 1.9, and $90.5 \pm 6.3\%$ (Wang et al., 2002a), or 70.2 ± 11.8, 92.3 ± 0.1, and $90.1 \pm 8.2\%$ (Wang et al., 2004) for 48 °C + 5 min, 50 °C + 2 min, and 52 °C + 1 min, respectively. No significant differences ($F_{(1,4)} = 4.3$; $P > 0.05$) were observed in thermal mortalities for A. transitella among the three tested methods except for one temperature–time combination (Table 3). Specifically, for 52 °C + 1 min, the tube immersion method produced significantly higher mortality than the other two methods ($F_{(2,6)} = 16.64$; $P < 0.05$). However, the mean mortality values for A. transitella obtained by the HBS were consistently lower than those from the two immersion methods, especially the tube immersion method (Table 3). Therefore the HBS would result in a more conservative recommended treatment. The A. transitella mortality data from the HBS were also comparable to previously reported data collected with the HBS; these values were 72.4 ± 15.0, 77.5 ± 18.5, and $31.4 \pm 14.8\%$ (Wang et al., 2002b), or 73.8 ± 9.9, 88.9 ± 5.1, and $41.1 \pm 19.3\%$ (Wang et al., 2005) for 48 °C + 30 min, 50 °C + 10 min, and 52 °C + 1 min, respectively.

The difference in the thermal mortality data between the two immersion methods may be explained by the uniformity of heating of the samples. In the direct immersion method, open water circulation inside the meshed tubes allowed fairly uniform temperature distribution. Therefore the measured temperature in the center represented the temperature experienced by all the insects. However, for the tube immersion method, the still water within the tubes created a decreasing temperature gradient from the tube surface to the center where the sample temperature was measured. Insects at different locations within the solid tube would consequently have different equivalent lethal times. The equivalent time calculated from the temperature history for the tube center may actually be less than that experienced by most individual insects in the same sample, and may explain the higher mortality rates observed with the tube immersion method. In our study, the thermal treatments developed based on the tube immersion method may underestimate the time to achieve the required security level in efficacy tests.

Our results show the difficulty in comparing mortality data from different heating methods, but do suggest that equivalent accumulated lethal times may be useful for providing comparable

Fig. 2. Temperature–time histories of water in the screen metal tube (T_1) and the copper tube (T_2) together with the block temperature of the heating block system (HBS) at the heating rate of 5 °C/min.

Table 2

Thermal mortality (mean ± SD, %) comparison of fifth-instar *Cydia pomonella* for three temperature-equivalent HBS holding time combinations obtained by the direct immersion, the tube immersion and the heating block system (HBS) at a rate of 5 °C/min (200 insects per each of 3 replicates).

Treatments	Direct immersion	Tube immersion	HBS
48 °C + 5 min	$89.1 \pm 2.3a$[a]	$99.6 \pm 0.7b$	$83.1 \pm 6.4a$
50 °C + 2 min	$87.6 \pm 4.4a$	$100.0 \pm 0.0b$	$87.1 \pm 3.0a$
52 °C + 1 min	$83.8 \pm 4.0a$	$100.0 \pm 0.0b$	$90.1 \pm 7.9ab$

[a] Different letters within a row indicate that means are significantly different ($P < 0.05$).

Table 3
Thermal mortality (mean ± SD, %) comparison of fifth-instar *Amyelois transitella* for three temperature-equivalent HBS holding time combinations obtained by the direct immersion, the tube immersion and the heating block system (HBS) at a rate of 5 °C/min (200 insects per each of 3 replicates).

Treatments	Direct immersion	Tube immersion	HBS
48 °C + 30 min	99.6 ± 0.6a[a]	99.6 ± 0.4a	63.0 ± 16.9a
50 °C + 10 min	99.8 ± 0.3a	100.0 ± 0.0a	93.0 ± 5.1a
52 °C + 1 min	69.5 ± 10.0a	99.1 ± 0.3b	40.3 ± 19.2a

[a] Different letters within a row indicate that means are significantly different (*P* < 0.05).

exposure periods across methods. The two immersion methods both rely on hot water as the heat transfer medium, differing primarily in heating rates and uniformity. The dry heat HBS used a much slower heating rate than either immersion method, and yet mortality data from this method were similar to those from the direct immersion method. Also, the rapid heating rates (≥ 5 °C/min) used by these three methods do not allow enough time for the test insects to produce heat shock proteins (Neven, 2000; Wang et al., 2005) or cause changes to the thermotolerance of the treated insects. Because the temperatures selected provided high mortality levels after relatively short exposures, the chance of reduced oxygen or increased demand for oxygen by insects at high temperatures in the immersion methods causing increased mortality is unlikely. Moreover, mortality from the tube immersion method, which required a longer exposure, was statistically similar to the direct immersion method at the temperature-combinations with the longest exposures, again suggesting that reduced oxygen levels are not a contributing factor.

In developing heat treatments the selection of laboratory heating methods should be made carefully and should reflect the conditions expected during large-scale, commercial treatments. Methods that use immersion in hot water may be more suited to the development of hot water treatments for fruits, or for insects in more aqueous environments such as fruit flies. However, as the use of water-immersion methods introduces the possibility of complicating factors such as reduced oxygen levels, particularly for longer exposures, these methods should be avoided for most terrestrial insects. The HBS may be more useful in developing rapid, dry-heat treatments such as those using radio frequency energy. Data from our study show that, while results among the three methods tested were often similar, the HBS consistently provided the lowest mortality and would likely yield more conservative treatment recommendations. In fact, the HBS was used to develop rapid, efficacious radio frequency treatments for in-shell walnuts that maintained good product quality when tested under commercial conditions (Wang et al., 2007a,b).

4. Conclusions

Equivalent lethal times accumulated through the ramp-up periods were calculated using a thermal lethality model to establish a common base for comparison of three different treatment methods, namely direct hot water immersion, water-filled tubes immersed in hot water and the HBS. Observed mortalities from the three methods were compared at three temperature–time combinations. The results showed that the HBS provided the lowest mean mortality among the three methods for all but one of the temperature–time combinations, although no significant difference in insect mortality was observed between the direct immersion method and the HBS. The mean insect mortality in the tube immersion method was significantly higher than that in the other two methods in more than half the treatments. The insect mortality

data in the HBS were consistent with those observed in previous reports using the same method.

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