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Cold Disinfestation on Orange for *Bactrocera dorsalis* (Diptera: Tephritidae)

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Abstract: Cold treatment has been extensively employed for the phytosanitary control of fruit flies for citrus cultivation worldwide. Trials with artificial infestation methods on navel and Valencia oranges at 3 °C and 2 °C against the oriental fruit fly (*Bactrocera dorsalis*) were conducted, following standard bioassay protocols and large-scale testing. The results showed that the third instar larval stage was the most tolerant stage in both cultivars. The maximum estimated cold treatment time at 3 °C required to produce 99.9968% mortality ($LT_{99.9968}$) with a 95% confidence level was 16.6 days and 16.2 days for the navel orange and Valencia orange, respectively. Meanwhile, the estimated cold treatment time at 2 °C was 14.8 days for both navel and Valencia oranges, with a 95% confidence level. Furthermore, it was also observed that no survivors came from a total of 104,420 estimated (51,396 for the navel cultivar and 53,024 for the Valencia cultivar) third instar larvae in orange fruits after being subjected to a cold treatment of 3 °C for 17 days. Meanwhile, there were also no survivors from a total of 100,556 (50,740 for the navel cultivar and 49,816 for the Valencia cultivar) third instar larvae in orange fruits after being subjected to a cold treatment of 2 °C for 15 days. The treatments at 3 °C for 17 days and 2 °C for 15 days on oranges, including navel and Valencia, against the oriental fruit fly, surpassed the required mortality assurance of 99.9968% at a 95% confidence level and also met the probit-9 mortality standard. Overall, the application of these results will provide more flexibility for the citrus industry to satisfy quarantine treatment requirements.

Keywords: oriental fruit fly; *Bactrocera dorsalis* (Hendel); orange; quarantine treatments; cold disinfestation

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1. Introduction

Bactrocera dorsalis (Hendel) (Diptera: Tephritidae), the oriental fruit fly, is recognized as one of the most destructive pests globally and is the most troublesome species within its genus *Bactrocera* [1]. The invasion and spread of *B. dorsalis* throughout Africa in the last two decades have significantly raised the pest profile of *B. dorsalis*, both as a field and market access problem [2,3]. *Citrus* spp. fruit can be infested by a range of different fruit fly (Diptera: Tephritidae) species [4]. Oriental fruit flies can be major pests of *Citrus* spp. in some production areas of China [5]. Citrus and other horticultural products, which serve as hosts for fruit flies, often receive a phytosanitary treatment to prevent the movement of exotic fruit flies through marketing channels [6] and prevent the introduction and establishment of these fruit flies in countries or areas where they do not exist [7].

Cold treatment, a non-chemical, safe, and effective method, has been employed since the early 20th century to ensure the phytosanitary control of fruit flies in various fruit cultivars cultivated worldwide [8]. Research data have demonstrated cold disinfestation as an effective approach to managing oriental fruit flies. However, the disinfestation treatments conducted previously were focused on temperatures below 2 °C [9–15]. Only

the report from Burditt and Balock [9] showed that all stages of the oriental fruit flies were killed in 12 days at 2.7 °C. The duration of cold treatment necessary varied depending on the specific experiment and the type of fruit undergoing treatment. For example, Sunkist oranges needed 14 days at 2 °C [10], navel oranges required 15 days at 1.67 °C [11], Ponkan mandarins took 12 days at 1.7–1.9 °C [12], pomelos were treated for 14 days at 1.7–1.8 °C [13], and Valencia oranges were subjected to 16 days at 0.9 °C [14]. Comparing disinfestation treatments across various commodities is challenging due to the variance in host organisms, most tolerant life stage, colony source, the identified endpoint, and the range of temperatures employed in the treatments [16]. Various cold treatment schedules have been established between trading partners throughout the world to provide controls for the large number of fruit flies, which are major economic and quarantine pests [1]. There are several cold treatment schedules for host fruit of *B. dorsalis.* However, the schedules differ depending on the importing countries and perhaps fruit species. For example, in the United States, USDA–APHIS-approved cold treatments for *B. dorsalis* in carambola, lychee, longan, and sand pear fruits during overseas transit require temperatures $\leq$ 0.99 °C for 15 days or $\leq$1.38 °C for 18 days [17] and in guava requires temperatures $\leq$ 0 °C for 12 days or $\leq$1 °C for 15 days [17]. Taiwan, Province of China, exports grapes and pomelos to Japan through a cold treatment using a schedule of $\leq$1.0 °C for 12 days, and exports Ponkans to Japan through a cold treatment involving temperatures $\leq$ 1.0 °C for 14 days [18]. China exports Ya pears to the USA through a cold treatment involving temperatures of 0 °C for 10 days, 0.56 °C for 11 days, 1.11 °C for 12 days, or 1.67 °C for 14 days [17]. China exports table grapes to New Zealand with a cold treatment protocol of 0.99 °C for 17 days or 1.38 °C for 20 days [19]. China exports apples, pears, and stone fruit to Australia using a cold treatment protocol of $\leq$3.0 °C for 18 days [20]. China exports jujubes and kiwis to Chile using a cold treatment of $\leq$3.0 °C for 18 days [21,22].

Cold treatment temperature and treatment duration are generally based on efficacy data provided by research laboratories and confirmatory or large-scale commercial tests with many thousands of insects treated during treatment development [14,23]. Based on the guidelines for the development of cold disinfestation treatments for fruit fly host commodities [24], the proposed trials were conducted in navel and Valencia cultivars of oranges (*Citrus sinensis* (L.) Osbeck), with extended periods at 3 °C and 2 °C to establish a cold treatment schedule. The two cultivars are the main international trade cultivars of oranges due to their long harvest season and lower susceptibility to postharvest decay compared to other cultivars of citrus. This schedule is designed to be acceptable under the International Plant Protection Convention (IPPC) for the development of International Standards for Phytosanitary Measures (ISPMs). It could assist authorities in importing countries to develop quarantine treatments for *B. dorsalis* and provides exporters with a wider range of suitable treatment options, thereby enhancing trade benefits.

2. Materials and Methods

2.1. Test Insects

The *B. dorsalis* population for this experiment was sourced from wide rose apples (*Syzygium jambos* (L.) Alston), which were cultivated as greenery trees in Guangzhou, Guangdong, China, during the years 2018, 2019, and 2021–2023. These insects were maintained at the Plant Quarantine Institute of Guangzhou Customs District Technology Center in Guangzhou, Guangdong, China, and were reared and reproduced for this species during different trial seasons. The detailed methods of rearing and reproduction are as follows: Late 3rd instar larvae of *B. dorsalis* that emerged out of infested fruit were transferred to moist sand for pupation in the laboratory. One day before adult eclosion, the pupae were put in an aluminum cage measuring 58 by 40 by 40 cm, with three sides covered by a mesh-type organza. Once the emergence took place, the adults were fed with a diet of 1 part solid hydrolyzed yeast and 3 parts brown sugar and water. Some slices of fresh orange were also supplied occasionally. Eggs were collected using egg-collecting bottles (0.4 L), which had their sides punctured with about 200 holes measuring 0.5 mm

in diameter all around. The egg-collecting bottle with a small amount of orange juice was placed inside cages housing gravid adults (about 2 weeks old) of oriental fruit fly for 4–8 h for egg collection. The eggs were washed out with clean water, moved to wetted filter paper, and then put on the larvae artificial diet for egg hatching. Larvae were reared using a standard larvae artificial diet modified from Liu et al. [25] for the development to late-aged third instar. The aged larvae were moved to moist sand for pupation as described above. The same methods were replicated until the number of adults was enough for testing. The colony consisted of 10,000–24,000 adults (2–4 cages each containing 5000–6000 flies, sex ratio of 1:1) according to the testing requirements and was maintained at 26 ± 2 °C and 70 ± 5% relative humidity (RH) in a dark–light cycle of 14:10 h. The fruit fly colonies had been maintained for nearly 8 months (from June to January of following year) of each trial season with an infusion of wild oriental fruit flies, for which the larvae were collected from infested guava (*Psidium guajava* L.) fruit and reared by the same method as described previously.

2.2. Test Fruits

The navel and Valencia orange cultivars were chosen for the tests due to their long availability period and their lower susceptibility to postharvest decay compared to other citrus cultivars [11,14]. Oranges with a consistent maturation grade and free of defects were sourced from Jiangxi Hongyuan Fruit Co. Ltd., Jiangxi, in China. They were kept in a cooling incubator set at temperatures ranging from 4.0 to 5.0 °C and 85 to 95% RH until they were needed for the tests. Before the cold assays, the fruits (mean weight = 215.67 ± 15.82 g for navel and 172.06 ± 10.25 g for Valencia) were treated by immersion in a Clorox chlorine solution (1 mL/10 L of water) to reduce contamination with microorganisms, rinsed with water, and then covered with insect-proof mesh and allowed to air dry at an ambient temperature.

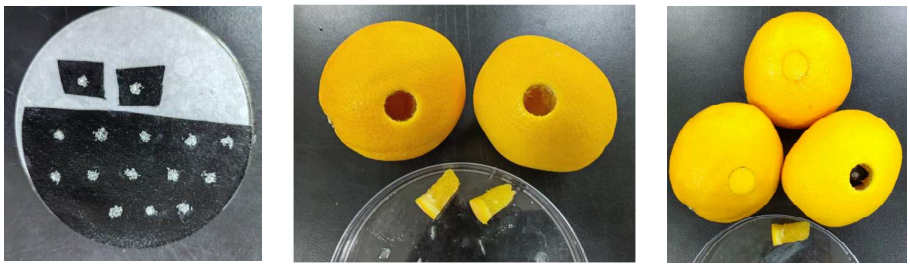
2.3. Test Facilities

Disinfestation test facilities were situated in various designated areas, including a pretreatment and post-treatment storage room, a workroom for fruit infestation and inspection, a cold chamber room for conducting treatments, and a separate colony room. The cold-treatment chamber (HLT103PB), supplied by Chongqing Well Zhenchang Technology Co. LTD, Chongqing, China, has a capacity of 3.15 m^3 (inside dimensions: 1.4 by 1.5 by 1.5 m). The cold-treatment chamber is equipped with a Siemens PLC module and a 7-inch color LCD, touch screen for time–temperature monitoring, and with a dry-wing system to prevent frost formation during low-temperature operation. It uses a high-pressure compressor to supply compressed air, which is then dried through an adsorption dryer before being introduced into the chamber to replace the humid air inside. This setup ensures that no frost forms inside the chamber during extended periods of low-temperature storage. The temperature recording system is implemented with a midi Logger GL840 (GRAPHTEC Corporation, Yokohama, Japan, ver. 1.31), which has 10 temperature sensors (Pt 100, A grade) for the chamber environment and fruit, and a sensor for chamber humidity. The two cooling incubators, CLC 222 from Climacell Bzv and CLC 707 from MMM Groups, EU, which boast a stated accuracy of ±0.5 °C were also used. The internal dimensions are 0.52 by 0.54 by 0.76 m for the CLC 222 and 0.52 by 0.94 by 1.45 m for the CLC 707, both featuring temperature and humidity controls.

2.4. Fruit Infestation

Test fruits were removed from the cooling incubator and held for 24–28 h at 26 ± 2 °C and 65 ± 5% RH to equilibrate to a temperature suitable for egg and larval development. The fruits were treated with the Clorox chlorine solution before infestation. The artificial infestation method, which covered the following steps (see Figure 1), was carried out. Eggs were collected with egg-collecting bottles for 2–4 h from the colony of 3–6-week-old adults. Egg-drops (c. 150 or 200 eggs each drop) were lined up with a pipette onto black filter

paper (90 by 90 mm) on a wetting sponge (80 by 80 by 5 mm) in a 100 mm Petri dish. The Petri dishes containing eggs were held in the cooling incubator (CLC 222) for 20–28 h at 25 °C and 65 ± 5% RH. The thicker skin of oranges compared to other hosts like stone fruits necessitated artificial infestation of the citrus fruits to acquire a sufficient number of insects at various developmental stages [11,24,26]. Inoculation was carried out using the pulp hole method described by Myers et al. [27]. The method was modified as follows: For each test fruit, a hole was made through the rind and pulp to the fruit center (c. 18 mm) using a cork borer (14 mm diameter, size 8), and the fruit plug with orange rind and its pulp were moved out. Then, the Petri dishes containing eggs were taken out from cooling incubator and the egg-drop with the black filter paper was cut with eyebrow scissors one by one (c. 10 by 10 mm). The black wet filter paper containing one egg-drop was put inside the hole, and the hole was closed with the fruit plug. The plug was sealed with paraffin wax tape strips to hold it in place. Infested fruits were arranged in a labeled plastic basket with dimensions of 40 by 30 by 12 cm, lined with a paper towel at the base, and then completely enclosed with an insect-proof mesh polyester bag to prevent any contamination from other arthropods. The plastic baskets with infested fruits were placed in the cooling incubator at 25 °C and 65 ± 5% RH for larvae development.



(**a**) (**b**) (**c**)

Figure 1. Some steps of the artificial infestation method: (**a**) egg-drop on the wetted filter paper; (**b**) pulp hole and its fruit plug; and (**c**) oranges infested by the pulp hole method.

2.5. Evaluation of Infested Fruits

The evaluation of infested fruits was carried out using the method of washing fruit pulp. The following procedures were used: The treated fruits or the fruits of the control group were put into a plastic box (35 by 28 by 22 cm). Each box was prepared with clean water containing approximately 70% of the box's volume. No more than 10 fruits were evaluated per box. The pulp hole was washed, and the fruit pulp tissues were manually broken off, collecting and recording any pupae and live larvae. The tissues were then sifted through sieves into another box, and the process of washing, breaking off, and sifting was repeated. This procedure was repeated 4–5 times; it continued until two consecutive checks yielded no live individuals. All pupae and any larvae showing movement were classified as alive. Larvae that remained motionless were considered dead. The status of any unresponsive larvae was assessed by placing them on a napkin to observe if they would exhibit any signs of movement.

2.6. Development of Immature Stages of Fruit Flies in Orange

The navel and Valencia oranges infested with oriental fruit fly eggs were exposed to 25 °C and 65 ± 5% RH for various periods to evaluate the impact on their development into various larval stages. Fruits were infested using the method of pulp hole as previously described and put in plastic baskets enclosed with an insect-proof mesh bag. These baskets were placed in a cooling chamber (CLC 222) with a stated accuracy of 0.5 °C. At days 2, 4, and 6 post-egg collection, three fruits infested with oriental fruit fly were randomly selected. The larvae were collected by washing the fruit pulp and were immediately killed with hot water (c. 80 °C). Over 100 larvae were examined during each period using a stereoscope to assess the development of the oriental fruit fly. The stages of the oriental fruit fly larvae were recorded according to the identification characters described by Zhou et al. [28].

2.7. Most-Tolerant Stage Trial

Different larval stages of the oriental fruit fly in navel and Valencia oranges were exposed to the cold-treatment chamber for various periods to examine the effect of cold disinfestation on pest mortality. Three replications were conducted for both cultivars. The schematic representation of the methods used in the trials is presented in Figure 2. In the larval trial, oranges were infested with eggs using the previously described method of pulp hole. They were then arranged on a labeled rectangular plastic basket with a paper towel lining the bottom and were fully enclosed with an insect-proof mesh bag. Following that, they were placed in the cooling incubator (CLC 222) and incubated at 25 °C and 65 ± 5% RH for 2, 4, and 6 days to allow the development of most larvae to the 1st, 2nd, and 3rd instar, respectively. Each fruit was infested with a single egg-drop, which contained approximately 150 eggs. For every replication, 80 fruits were infested for each larval stage (1st, 2nd, and 3rd instar), with 70 fruits allocated for the treatment group and 10 fruits for the control group. In the egg trial, 18–20 h old eggs were collected from the egging bottle and then pipetted onto pieces of black filter paper (90 by 90 mm). These were placed on a wetting sponge (80 by 80 by 5 mm), with a 100 mm Petri dish. Each Petri dish contained 10 egg-drops, totaling approximately 1500 eggs, along with three drops of orange juice to facilitate hatching during the experiments.

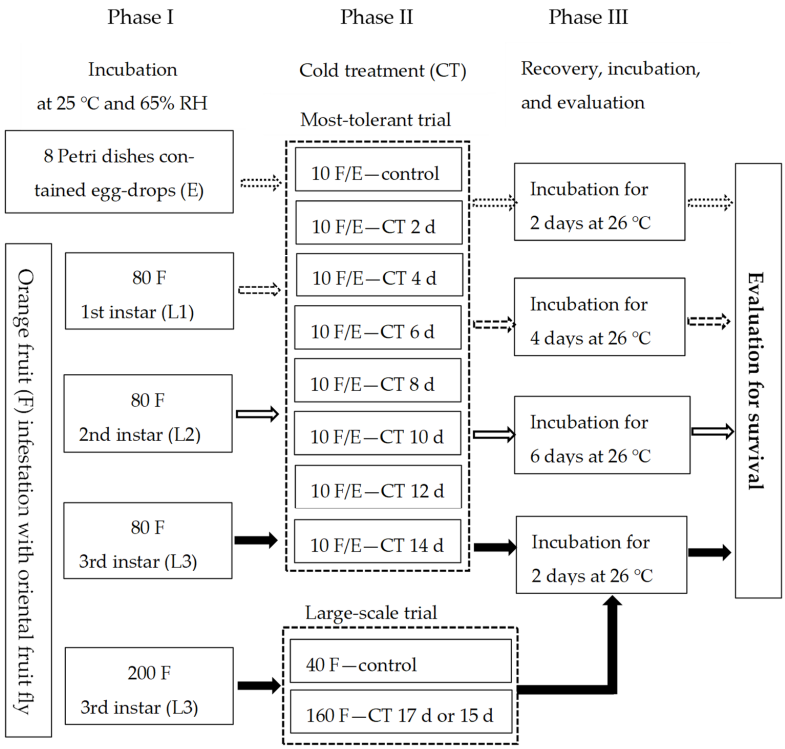


Figure 2. Schematic representation of the methods used in the cold-treatment (CT) trials. The trials were conducted with 3 replications. In each replication, 240 orange fruit (F) for the most-tolerant stage trial and 200 fruit for the large-scale trial of each orange cultivar were inoculated with oriental fruit flies. During phase I—"incubation"—inoculated orange fruits were incubated at 25 °C and 65% RH for various periods, to obtain the desired larvae stage (i.e., L1, L2, and L3). During phase II—"cold treatment at 3 °C or 2 °C"—fruit with the expected larval stage were exposed to the CT chamber for specific days (2, 4, 6, 8, 10, 12, and 14 d) in the most-tolerant stage trials and for 17 days and 15 days in the large-scale trial at 3 °C and 2 °C, respectively. In addition, a sample of 20% or more fruit was used as the control group at the start of each CT trial. Phase III—"recovery, incubation and evaluation"—involved incubating CT fruits at 26 ± 2 °C to enable any surviving individual to complete development and evaluating the effectiveness of the treatment by the method of larval endpoint.

Before being loaded into the cold-treatment chamber, ten infested fruits from the same instar were selected randomly and placed in one labeled insect-proof mesh bag. The bags which contained fruits infested with different instars of oriental fruit fly larvae at the 1st, 2nd, and 3rd instar stages were grouped together based on the treatment time (2, 4, 6, 8, 10, 12, and 14 days) for convenient removal from the treatment chamber. During the experiment, temperatures were recorded every 60 min with the midi Logger GL840. Three pulp temperature sensors which were inserted in the core of the three orange fruits (the largest one from each treatment) respectively, and placed in a diagonal pattern, and two air sensors were placed at the cold air delivery position and the air return position for checking air temperatures. In the egg treatment, the Petri dish temperatures were most consistent with the chamber air temperature, and the treatment was deemed to have started after the chamber air temperature reached the target treatment temperature. Larvae treatment was deemed to have started immediately after all pulp sensors reached the target temperature. In this study, the centers of the fruits required approximately 8 h to reach the target temperature. At days 0, 2, 4, 6, 8, 10, 12, and 14, a Petri dish with 18–20 h old eggs and one group which contained fruits infested with different instars of oriental fruit fly larvae were randomly selected from the cold chamber. They were then subjected to a recovery period in a post-treatment room at 26 ± 2 °C and 65 ± 5% RH, with mortality assessed after 2 days for eggs, 6 days for 1st instar, 4 days for 2nd instar, and 2 days for 3rd instar larvae. The number of effective larvae used in the treatment group was calculated based on the number of survivors in the control group.

2.8. Large-Scale Trial

The previous most-tolerant stage trials during our tests showed that two consecutive 100% mortality outcomes were found for oranges treated for 12 and 14 days, for both the navel and Valencia cultivars, at both 3 °C and 2 °C; therefore, the procedure of exploratory testing which was described in the guidelines for the development of cold disinfestation treatments for fruit fly host commodities [24] was skipped. The 3rd instar larvae (6 days), which were found to be more tolerant to cold based on previous trial outcomes regarding the most-tolerant stage trial, were utilized in the large-scale trial study. Artificial infestation was carried out using the pulp hole method, as previously described.

Infested fruits were placed in a labeled rectangular plastic basket lined with paper towels at the bottom and were fully enclosed in insect-proof mesh bags to prevent contamination by other arthropods. They were then stored at 25 °C and 65 ± 5% RH (CLC 707) during the development of the larvae. A total of 80% of the infested fruits were packed in cardboard boxes (36 by 25 by 22 cm) before treatment. The other 20% of infested fruits, which were enclosed with insect-proof mesh bags, were kept in the post-treatment room at 26 ± 2 °C, as the control group. Three (3) replications were performed separately and at different times. Each replication's load factor consisted of 16 boxes, each containing 10 infested fruits, supplemented with uninfested fruits of the same cultivar to fill the boxes. Additionally, 20 boxes filled with oranges of the same cultivar were included inside the cold-treatment chamber. A total of 36 fruit boxes were grouped in 12 columns and 3 rows. During the treatment process, the temperature and humidity of the chamber were monitored and recorded every 60 min using the midi Logger GL 840. Six (6) pulp sensors, which were inserted in the core of the six uninfested orange fruits (the largest one from each treatment) respectively, were strategically positioned across different rows and sites in a diagonal pattern for each replication. Additionally, two air sensors were placed at the cold air delivery position and the air return position to monitor the overall environment within the chamber. The treatment was deemed to have started when all fruit pulp sensors reached the treatment temperature (c. 18 h for 3 °C and 20 h for 2 °C). Temperature recordings were automatically logged at 60 min intervals throughout the trial. All temperature sensors were calibrated in melting ice using a certified mercury glass thermometer before each trial to verify their accuracy. At the end of the treatment, after 17 days for 3 °C or 15 days for 2 °C, the fruit boxes were moved out of the cold-treatment chamber. The infested fruits were

removed from the boxes, placed into labeled plastic buckets, fully enclosed in insect-proof mesh bags, and stored in the post-treatment room at 26 ± 2 °C for 48 h before evaluation. The control group fruits were assessed 24 h after the treatment began. The effectiveness of the treatment was gauged by calculating the number of larvae in the treatment group relative to the number of surviving larvae in the control group.

2.9. Statistical Analysis

In all trials, the number of effective individuals used in the treatment group was calculated based on the number of survivors in the control group (20%). To evaluate the cold tolerance of various life stages, time-response mortality data for *B. dorsalis* were subjected to probit and logit analysis using the PoloPlus (Version 2.0, LeOra software, Berkeley, CA, USA). This statistical analysis was applied to the data collected throughout the study to determine the predicted exposure periods needed to reach 99% (LT_{99}) and 99.9968% ($LT_{99.9968}$) mortality rates. The lethal time (LT) data according to the results of the probit and logit analysis were also analyzed with a one-way analysis of variance (ANOVA) followed by Tukey's multiple range test ($p < 0.05$) using IBM SPSS Statistics 19.0.

3. Results

3.1. Development of B. dorsalis in Oranges

The development of *B. dorsalis* larvae in navel oranges and Valencia oranges infested using the previously described pulp hole method was observed. The results showed that the development rate of the oriental fruit fly was quite synchronized. The larval development reached 100% at the 1st instar by day 2 in both navel and Valencia oranges. It further progressed to 100% at the 2nd instar by day 4 in both cultivars. By day 6, over 95% of larvae had reached the 3rd instar, with 96% in navel and 98% in Valencia oranges. The recorded temperatures (mean ± SD) were 25.2 ± 0.4 °C during the trials. Consequently, in the subsequent trials, larvae at 2 days old were identified as the 1st instar, those at 4 days old as the 2nd instar, and larvae at 6 days old as the 3rd instar.

3.2. Effect of Most-Tolerant Stage Trials

The results showed that the survival of eggs and larvae on infested oranges decreased rapidly with the increase in cold storage time both at 3 °C and 2 °C.

For the trials conducted at 3 °C (3.05 ± 0.26 °C), complete mortality of the eggs was observed in both navel and Valencia orange cultivars by day 8. The 1st instar and the 2nd instar larvae in both cultivars achieved complete mortality by day 10. By day 12, all 3rd instar larvae across both cultivars were completely killed (see Table 1). The data also demonstrated the 3rd instar larvae as the most cold-tolerant in two cultivars. The parameters from the results of the probit analysis (probit and logit model), including slope and estimated time of cold treatment required to produce 99% (LT_{99}) and 99.9968% ($LT_{99.9968}$) mortality at a 95% confidence level (CL), are presented in Table 2. As indicated by Table 2, the number of days necessary for the 3rd instar under LT_{99} or $LT_{99.9968}$ was larger than that for the other stages in both navel and Valencia cultivars, indicating that the resistance of 3rd instar was higher. Thus, the 3rd instars found in both navel and Valencia oranges were determined to be the most tolerant. Hence, the following tests were performed only with 3rd instar larvae. Furthermore, the number of days necessary of the most tolerant stage (3rd instar) under $LT_{99.9968}$ at a confidence level of 95% was 16.6 (15.1, 18.6) days and 16.2 (14.9, 17.9) days for the navel and Valencia cultivars, respectively. This result was used for selecting the treatment period in the large-scale disinfestation trials at 3 °C.

Table 1. Results of mortality (mean ± SD) of different stage trials for *B. dorsalis* on two cultivars of oranges after being subjected to cold treatment at 3 °C.

Cultivar	Stage	No. of Treated	Exposure (d)						
			2	4	6	8	10	12	14
navel	Eggs	3171	52.28 ± 4.25	95.71 ± 0.57	99.78 ± 0.04	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
	1st instar	2467	43.61 ± 11.4	93.16 ± 0.5	99.1 ± 0.16	99.92 ± 0.07	100 ± 0.0	100 ± 0.0	100 ± 0.0
	2nd instar	2511	28.91 ± 1.66	64.59 ± 10.07	92.83 ± 1.61	98.95 ± 0.24	100 ± 0.0	100 ± 0.0	100 ± 0.0
	3rd instar	2190	30.62 ± 8.28	60.18 ± 11.65	91.21 ± 2.78	97.69 ± 0.6	99.67 ± 0.14	100 ± 0.0	100 ± 0.0
Valencia	Eggs	2757	56.67 ± 7.81	80.97 ± 11.99	98.92 ± 0.76	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
	1st instar	2430	45.69 ± 12.06	86.42 ± 4.95	97.59 ± 1.1	99.56 ± 0.3	100 ± 0.0	100 ± 0.0	100 ± 0.0
	2nd instar	2128	30.02 ± 5.38	71.45 ± 0.30	90.25 ± 4.01	99.11 ± 0.39	100 ± 0.0	100 ± 0.0	100 ± 0.0
	3rd instar	2370	31.29 ± 4.73	68.48 ± 9.05	88.94 ± 4.26	98.57 ± 0.71	99.86 ± 0.14	100 ± 0.0	100 ± 0.0

Table 2. Results of probit and logit analysis of mortality of different stage trials for *B. dorsalis* on two cultivars of oranges after being subjected to cold treatment at 3 °C.

Cultivar	Analyzing Model	Stage	Slope	LT_{99} (95% CL) (days)	$LT_{99.9968}$ (95% CL) (days)
navel	probit	eggs	0.80 ± 0.02	4.8 (4.7, 5.0) c	6.9 (6.6, 7.3) c
		1st instar	0.73 ± 0.02	5.4 (4.8, 6.2) b	7.8 (7.5, 8.0) c
		2nd instar	0.49 ± 0.01	7.9 (7.5, 8.3) a	11.3 (11.1, 11.6) b
		3rd instar	0.43 ± 0.01	8.6 (8.0, 9.3) a	12.5 (11.5, 13.7) a
	logit	eggs	1.50 ± 0.04	5.0 (4.8, 5.1) b	8.8 (8.5, 9.2) b
		1st instar	1.37 ± 0.04	5.5 (5.2, 6.0) b	9.7 (8.9, 10.8) b
		2nd instar	0.88 ± 0.02	8.4 (7.9, 9.0) a	15.0 (13.9, 16.3) a
		3rd instar	0.77 ± 0.02	9.1 (8.5, 10.0) a	16.6 (15.1, 18.6) a
Valencia	probit	eggs	0.47 ± 0.01	6.7 (6.0, 7.8) b	10.3 (9.0, 12.3) ab
		1st instar	0.53 ± 0.01	6.5 (6.0, 7.2) b	9.6 (8.7, 10.9) b
		2nd instar	0.46 ± 0.01	8.0 (7.6, 8.5) a	11.6 (11.0, 12.4) a
		3rd instar	0.44 ± 0.01	8.3 (7.9, 8.9) a	12.1 (11.3, 13.1) a
	logit	eggs	0.85 ± 0.02	7.3 (6.4, 8.7) bc	14.0 (11.9, 17.6) a
		1st instar	1.00 ± 0.03	6.8 (6.2, 7.5) c	12.5 (11.2, 14.2) b
		2nd instar	0.83 ± 0.02	8.5 (8.1, 9.1) ab	15.5 (14.4, 16.8) a
		3rd instar	0.79 ± 0.02	8.8 (8.3, 9.6) a	16.2 (14.9, 17.9) a

Note: Within each column, values followed by different letters were significantly different based on lethal time of different stage trials ($p < 0.05$).

For the trials conducted at 2 °C (2.02 ± 0.21 °C), complete mortality of the eggs was observed in both navel and Valencia orange cultivars by day 8. The mortality of both the 1st and 2nd instar larvae in both cultivars reached completion by day 10. Furthermore, all 3rd instar larvae in both cultivars were completely killed by day 12 (see Table 3). The data presented also showed that the 3rd instar larvae were the most cold-tolerant in the two cultivars. The parameters from the results of the probit analysis, including slope and estimated time of cold treatment required to produce 99% (LT_{99}) and 99.9968% ($LT_{99.9968}$) mortality at a 95% confidence level, are presented in Table 4. As indicated by Table 4, the 3rd instar larvae required more days to reach the LT_{99} or $LT_{99.9968}$ mortality thresholds compared to other stages in both the navel and Valencia cultivars, indicating greater resistance at this stage. Thus, the 3rd instars found in both navel and Valencia oranges were determined to be the most tolerant. As a result, subsequent tests at 2 °C were conducted using only 3rd instar larvae. Furthermore, the number of days necessary of the most tolerant stage (3rd instar) under $LT_{99.9968}$ at a confidence level of 95% was 14.8 (14.0, 15.7) days for the navel and 14.8 (13.5, 16.3) days for the Valencia cultivars. This result was used for selecting the treatment period in the large-scale disinfestation trials at 2 °C.

Table 3. Results of mortality (mean ± SD) of different stage trials for *B. dorsalis* on two cultivars of oranges after being subjected to cold treatment at 2 °C.

Cultivar	Stage	No. of Treated	Exposure (d)						
			2	4	6	8	10	12	14
navel	Eggs	2754	54.13 ± 5.99	89.5 ± 2.22	99.6 ± 0.16	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
	1st instar	2263	47.95 ± 3.95	87.05 ± 1.78	99.24 ± 0.5	99.8 ± 0.19	100 ± 0.0	100 ± 0.0	100 ± 0.0
	2nd instar	2294	31.86 ± 7.00	70.29 ± 4.49	94.67 ± 1.84	99.12 ± 0.33	100 ± 0.0	100 ± 0.0	100 ± 0.0
	3rd instar	2195	31.72 ± 3.28	69.78 ± 1.83	93.88 ± 3.12	99.16 ± 0.54	99.96 ± 0.07	100 ± 0.0	100 ± 0.0
Valencia	Eggs	2808	56.57 ± 7.17	86.28 ± 1.81	98.31 ± 0.74	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
	1st instar	2198	51.34 ± 8.76	85.74 ± 3.03	98.33 ± 0.26	99.65 ± 0.38	100 ± 0.0	100 ± 0.0	100 ± 0.0
	2nd instar	2303	33.25 ± 8.94	70.08 ± 3.14	94.58 ± 1.27	99.83 ± 0.06	100 ± 0.0	100 ± 0.0	100 ± 0.0
	3rd instar	2487	34.19 ± 10.9	70.42 ± 5.04	94.57 ± 0.74	99.37 ± 0.33	99.93 ± 0.13	100 ± 0.0	100 ± 0.0

Table 4. Results of probit and logit analysis of mortality of different stage trials for *B. dorsalis* on two cultivars of oranges after being subjected to cold treatment at 2 °C.

Cultivar	Analyzing Model	Stage	Slope	LT_{99} (95% CL) (days)	$LT_{99.9968}$ (95% CL) (days)
navel	probit	eggs	0.60 ± 0.02	5.7 (5.5, 6.0) b	8.5 (8.1, 9.0) b
		1st instar	0.58 ± 0.02	6.1 (5.8, 6.5) b	9.0 (8.4, 9.7) b
		2nd instar	0.50 ± 0.01	7.6 (7.2, 7.9) a	10.9 (10.3, 11.5) a
		3rd instar	0.49 ± 0.01	7.7 (7.4, 8.0) a	11.1 (10.6, 11.7) a
	logit	eggs	1.10 ± 0.03	6.0 (5.7, 6.5) b	11.3 (10.4, 12.3) b
		1st instar	1.07 ± 0.03	6.4 (6.1, 6.7) b	11.8 (11.1, 12.5) b
		2nd instar	0.90 ± 0.02	8.0 (7.6, 8.5) a	14.4 (13.5, 15.5) a
		3rd instar	0.88 ± 0.02	8.2 (7.8, 8.6) a	14.8 (14.0, 15.7) a
Valencia	probit	eggs	0.49 ± 0.01	6.5 (6.1, 6.8) b	9.9 (9.3, 10.6) a
		1st instar	0.50 ± 0.01	6.5 (6.1, 7.0) b	9.9 (9.2, 10.8) a
		2nd instar	0.51 ± 0.01	7.4 (7.1, 7.8) a	10.7 (10.1, 11.4) a
		3rd instar	0.49 ± 0.01	7.6 (7.2, 8.2) a	11.1 (10.3, 12.0) a
	logit	eggs	0.91 ± 0.02	6.8 (6.4, 7.3) b	13.1 (12.1, 14.4) a
		1st instar	0.94 ± 0.03	6.9 (6.5, 7.4) b	13.0 (12.0, 14.3) a
		2nd instar	0.91 ± 0.02	7.9 (7.5, 8.5) a	14.3 (13.2, 15.6) a
		3rd instar	0.87 ± 0.02	8.1 (7.6, 8.8) a	14.8 (13.5, 16.3) a

Note: Within each column, values followed by different letters were significantly different based on lethal time of different stage trials ($p < 0.05$).

3.3. Large-Scale Trials

After obtaining two consecutive 100% mortalities in the most-tolerant stage trials (see Tables 1 and 3) and the number of days necessary of the most tolerant stage (3rd instar) under LT $_{99.9968}$ at a confidence level of 95% (see Tables 2 and 4), the treatment periods in large-scale trials were selected as 17 days at 3 °C and 15 days at 2 °C. The results found in the large-scale disinfestation trials are presented in Tables 5 and 6.

The recorded temperature was shown to be quite stable due to the cold-treatment chamber with the dry-wing system to prevent frost formation during low-temperature operation, as previously described. In the trials conducted at 3 °C, the temperatures (mean ± SD) of the three replications were 3.07 ± 0.05 °C, 3.13 ± 0.19 °C, and 3.17 ± 0.15 °C for navel oranges. For Valencia oranges, the recorded temperatures were 3.09 ± 0.11 °C, 3.11 ± 0.13 °C, and 3.16 ± 0.12 °C. The average number of treated insects per fruit of orange was 108.78 (107.08 for navel and 110.47 for Valencia), and the number of estimated larvae in the treated batch was 104,420 (51,396 for navel oranges and 53,024 for Valencia oranges). No survivors were detected in the oranges treated for 17 days at 3 °C, as indicated in Table 5. This demonstrated a 100% mortality rate for over 100,000 individuals of the most cold-tolerant stage of the oriental fruit fly on oranges. Each cultivar (navel and Valencia) treated at 3 °C exceeded the required minimum assurance of 99% mortality at the 95% confidence level. Cold treatment on oranges combining navel and Valencia cultivars at

3 °C for 17 days exceeded the required minimum assurance of 99.9968% mortality at the 95% confidence level and also passed the probit-9 level.

Table 5. Results of large-scale trials for third instar *B. dorsalis* larvae on two cultivars of oranges (navel and Valencia) after being subjected to cold treatment at 3 °C for 17 days.

Cultivar	Replications	Control		Treatment			
		No. of Fruits	Total No. of Living Insects	No. of Fruits	Total Estimated No. of Treated Insects	Total No. of Survivors	Mortality (%)
navel	1	40	3926	160	15,704	0	100
	2	40	4251	160	17,004	0	100
	3	40	4672	160	18,688	0	100
	Total	120	12,849	480	51,396	0	100
Valencia	1	40	4312	160	17,248	0	100
	2	40	4077	160	16,308	0	100
	3	40	4867	160	19,468	0	100
	Total	120	13,256	480	53,024	0	100

Table 6. Results of large-scale trials for third instar *B. dorsalis* larvae on two cultivars of oranges (navel and Valencia) after being subjected to cold treatment at 2 °C for 15 days.

Cultivar	Replications	Control fruits		Treatment			
		No. of Fruits	Total No. of Living Insects	No. of Fruits	Total Estimated No. of Treated Insects	Total No. of Survivors	Mortality (%)
navel	1	40	3844	160	15,376	0	100
	2	40	4251	160	17,004	0	100
	3	40	4590	160	18,360	0	100
	Total	120	12,685	480	50,740	0	100
Valencia	1	40	4009	160	16,036	0	100
	2	40	3957	160	15,828	0	100
	3	40	4488	160	17,952	0	100
	Total	120	12,454	480	49,816	0	100

In the trials conducted at 2 °C, the temperatures of the three (3) replications were 2.02 ± 0.05, 2.07 ± 0.09 °C, and 2.13 ± 0.11 °C for navel oranges. For Valencia oranges, the recorded temperatures were 2.05 ± 0.05 °C, 2.09 ± 0.11 °C, and 2.11 ± 0.12 °C. The average number of treated insects per fruit of orange was 104.75 (105.71 for navel and 103.78 for Valencia). The estimated total number of larvae in the treated batch was 100,556, which included 50,740 larvae from navel oranges and 49,816 larvae from Valencia oranges. After a 15-day treatment period at 2 °C, no survivors were found among the treated oranges, indicating a 100% mortality rate for over 100,000 individuals of the most cold-tolerant stage of the oriental fruit fly on oranges. Each cultivar (navel and Valencia) treated at 2 °C exceeded the required minimum assurance of 99% mortality at the 95% confidence level. Cold treatment on oranges combining navel and Valencia cultivars at 2 °C for 15 days exceeded the required minimum assurance of 99.9968% mortality at the 95% confidence level and also passed the probit-9 level.

4. Discussion

In the present study, the results showed that the third instar larvae were more cold-tolerant than the earlier stage larvae in both navel and Valencia oranges at temperatures of 3 °C and 2 °C. These results aligned with the majority of evidence in the literature, suggesting that third instar larvae were equally or more tolerant to cold treatments compared to larvae in earlier developmental stages. This was also found with *B. dorsalis* and

its synonyms, *B. invadens* [12,13,29–33], *B. zonata* [34], *B. cucurbitae*, and other tephritid species [29]. However, it was inconsistent with the research by Fang et al. [11], which showed that 2nd instar larvae appeared to be the most cold-tolerant life stage in Gannan navel oranges at 1.7 °C.

Generally, a natural infestation method and an artificial infestation method are the two main types of infection methods [6,16]. To obtain sufficient insects of the different life stages, it is necessary to artificially infest the citrus fruit. The main methods of artificial infestation in citrus have been proposed as puncturing fruits [11], injecting each fruit to a depth of 10–15 mm with 0.5 mL of eggs [23], making a 5 mm diameter hole drilled 30 mm in depth into the fruit beneath the calyx [14,16], making six holes (0.3 mm in diameter) into the cheek facing up to a depth just below the peel of each fruit through the peel with one tipped sterilized forceps [6], and using window methods [35]. In this paper, the pulp hole method of artificial infestation, as previously described, was employed. As one of the artificial methods, this method facilitated the distribution of larvae to the warmest areas within the fruits during treatment, ensuring that they had access to fresh food immediately after hatching, and promoted synchronized development among individuals. Also, this method enabled easy control of the number of larvae for each infested fruit by adjusting the number of eggs in each egg-drop. In artificially infested fruits, eggs were placed near the fruit center, whereas in naturally infested fruits, eggs were laid near the fruit surface. The fruit center is the warmest place during the treatment, and differences in the infestation methods may have an uncertain influence on the response to cold treatment [35]. However, the influence on the determination of the cold treatment schedule is negligible since cold treatment for fruit flies generally takes more than 10 days at 0.5 °C or above and some procedures should be followed for developing cold treatment schedules. These procedures include the following: (1) comparing the sensitivities of the different life stages, (2) deeming the treatment to have started after all pulp sensors reached the target temperature, and (3) ensuring that the accumulated number of treated individuals of fruit flies is normally over 30,000.

Early South African research with Mediterranean fruit flies in deciduous fruit by Nel [36] used a larval endpoint for evaluating treatments. This involved cutting open the treated fruit to ascertain the actual mortality rate of the larvae. This evaluation method was used in plenty of research studies [11,14]. However, more recently published research involving citrus utilized successful pupation as an endpoint [23,26]. The latter method is less labor-intensive as it is not necessary to dissect fruit or determine the number of larvae in the fruit. When applied to regulating the officially controlled movement of host material, such as in the international fruit trade, a larval endpoint facilitates an immediate decision when the fruits are found to contain live larvae after cold treatment. However, fruits with surviving larvae after cold treatment based on pupation need to be placed on sand at about 25 °C for several days before a decision to reject or accept the infested fruit can be made. This can result in expensive logistical delays [14]. In this paper, based on the larval endpoint for fruit fly cold treatment, the evaluation of infested fruits was carried out using the method of washing fruit pulp as previously described. Compared to the practice of cutting open the treated fruit to ascertain the mortality rate of the larvae, the evaluation method alleviated the labor intensity for researchers, as live young larvae tended to float on the water, while older larvae settled at the bottom of the container with a minimal water level, making them easily locatable and collectable. This measure is also well suited for evaluating the mortality rate of larvae in the phytosanitary treatment of table grapes, mango, and berry fruits for fruit fly, as live larvae can be easily found by washing the infested fruits. Further studies will be needed to determine how much time can be saved in the assessment of infested citrus fruit and the practicability of the measure on other types of fruits (such as melon or pomegranate).

The data reported in this paper showed that a cold treatment at 3 °C for 17 days or 2 °C for 15 days was effective against the oriental fruit fly. Both treatment durations surpassed the required minimum assurance level of 99.9968% mortality, achieved with

a 95% confidence level. The results at 2 °C for 15 days were fairly similar to the other disinfestation research results of the oriental fruit fly on citrus such as in pomelos at 1.7–1.8 °C for 14 days [13] and in Gannan navel oranges at 1.67 °C for 15 days [11]. The present results also support the point of view that the treatment schedules for *Bactrocera* spp. are quite similar and the treatment temperatures typically range from 1.0 to 3.0 °C for durations of 14 to 18 days [1,27,33,37]. Overall, the availability of temperature–time treatment schedules for *B. dorsalis* at 3 °C provides the industry more flexibility in exporting fresh fruits and disinfesting in transit in refrigerated sea containers. This research offers insights to assist the authorities of importing countries in establishing quarantine treatments for *B. dorsalis* and supports the IPPC in developing ISPMs.

5. Conclusions

The data reported in this paper showed that the 3rd instar larval stage of *Bactrocera dorsalis* was the most tolerant stage in the two cultivars of navel and Valencia oranges. Similar mortality of the most tolerant instar was observed between the two cultivars both at 3 °C and 2 °C cold treatment. It was also observed that no survivors came from a total of 104,420 estimated third instar larvae in oranges after being subjected to the cold treatment of 3 °C for 17 days. Meanwhile, there were also no survivors from a total of 100,556 third instar larvae in oranges after being subjected to the cold treatment of 2 °C for 15 days. Both treatment durations surpassed the required minimum assurance level of 99.9968% mortality with a 95% confidence level and could provide quarantine security.

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