Mortality of western cherry fruit fly (Diptera: Tephritidae) immature stages in cherries submerged in hypoxic water

Wee L. Yee[1,] and Cory J. Penca[2]*

Abstract

Determining the tolerance of immature tephritid fruit flies (Diptera: Tephritidae) to water submersion could have practical application in orchard sanitation for fly management. Herein, we determined the mortality of immature western cherry fruit fly, *Rhagoletis indifferens* Curran, submerged in water by submersing infested cherries for 4, 8, and 12 d. In 2020, 2021, and 2022, infested ripe cherries (mostly sweet cherry *Prunus avium* L.; Rosaceae) were collected from unmanaged trees outside of orchards in central Washington, U.S.A., submerged in water, and then removed from water to monitor for larval emergence as a measure of immature fly stage survival. Pre-treatment sub-samples estimated that eggs comprised 43.1–62.3% of immature stages in sweet cherries and second or third instars comprised 17.6–26.2%. Water in tests was hypoxic, containing <1–2 ppm dissolved oxygen over the majority of the 4- to 12-d tests. The 4-d submersion treatment did not prevent emergence of larvae from a tart cherry (*Prunus cerasus* L.) sample, whereas larval emergence was prevented in sweet and tart cherry samples in 8- and 12-d treatments. In the 8-d treatment, an estimated 48,689 immature fly stages did not survive treatment, for an estimated 99.99385% mortality at the 95% confidence level and a probit of 8.8400. These results demonstrate, as a proof of concept, that water submersion could be used as a method for disinfesting fruit in systems where orchard sanitation is a major method for fly management.

Key Words: *Rhagoletis indifferens*; *Prunus avium*; fly eggs; fly larvae; hypoxia

Resumen

La determinación de la tolerancia de los inmaduros de las moscas tefrítidas de la fruta (Diptera: Tephritidae) a la inmersión en agua podría tener una aplicación práctica en el saneamiento de huertos para el control de moscas. Determinamos la mortalidad de los estados inmaduros de la mosca occidental de la cereza, *Rhagoletis indifferens* Curran, sumergida en agua al sumergir cerezas infestadas durante 4, 8 y 12 días. En 2020, 2021 y 2022, se recolectaron cerezas maduras infestadas (principalmente cerezas dulces *Prunus avium* L.; Rosaceae) de árboles no manejados fuera de huertos en el centro de Washington, EE. UU., se las sumergieron en agua y luego se retiraron del agua para monitorear la aparición de larvas. como medida de sobrevivencia del estadio inmaduro. Las submuestras previas al tratamiento estimaron que los huevos comprendían entre el 43,1% y el 62,3% de los estados inmaduros en las cerezas dulces, mientras que el segundo o tercer estadio comprendía entre el 17,6% y el 26,2%. El agua en las pruebas era hipóxica y contenía <1 a 2 ppm de oxígeno disuelto durante la mayoría de las pruebas de 4 a 12 días. El tratamiento de inmersión de 4 días no impidió la aparición de larvas en una muestra de cereza ácida (*Prunus cerasus* L.), mientras que la aparición de larvas se evitó en muestras de cerezas dulces y ácidas en los tratamientos de 8 y 12 días. En el tratamiento de 8 días, se estima que 48.689 estadios inmaduros de moscas no sobrevivieron al tratamiento, para una mortalidad estimada del 99,99385% con un nivel de confianza del 95% y un probit de 8,8400. Estos resultados demuestran, como prueba de concepto, que la inmersión en agua podría usarse como un método para desinfectar la fruta en sistemas donde el saneamiento de los huertos es un método importante para el control de las moscas.

Palabras Clave: *Rhagoletis indifferens; Prunus avium*; huevos de mosca; larvas de mosca; hipoxia

Determining the effects of water submersion on mortality of immature tephritid fruit flies (Diptera: Tephritidae) infesting fruit has direct application to orchard sanitation, which is one non-chemical method for managing flies (Liquido 1993; Klungness et al. 2005; Niassy et al. 2022). Infested unpicked fruit (Rothwell et al. 2016) or fruit fallen on the ground need to be disposed of to eliminate the fruit being a source of future infestations in an orchard. Conceivably, one environmentally friendly method is water submersion of infested fruit to disinfest fruits of immature stages followed by disposal of the fruit within or around the orchard. Water that is hypoxic, defined as having < 2 ppm dissolved oxygen (Charlton 1980; Hoback et al. 2000; Hoback & Stanley 2001; Woods & Lane 2016; Harrison et al. 2018), may be lethal to terrestrial insects such as fruit flies. However, immature stages of tephritid fruit flies display a wide range of tolerances to water submersion, with larval survival in water varying from 2 to 21 d (Xie & Zhang 2007; Li et al. 2019; Bhandari et al. 2021; Yee 2021).

Western cherry fruit fly, *Rhagoletis indifferens* Curran, evolved on bitter cherry (*Prunus emarginata* [Dougl. ex Hook.] Eaton; Rosaceae) in western North America and is a major quarantine insect pest of cultivated sweet cherry (*Prunus avium* L.; Rosaceae) in the Pacific Northwest of the U.S.A. This species is well adapted to and is abundant in arid regions far from riparian zones (Frick et al. 1954; Wakie et al.

[1]USDA-ARS, Temperate Tree Fruit & Vegetable Research Unit, 5230 Konnowac Pass Road, Wapato, WA U.S.A. 98951, E-mail: wee.yee@usda.gov (W.L.Y.)
[2]USDA, APHIS, PPQ, S&T Treatment and Inspection Methods Laboratory, 13601 Old Cutler Rd, Miami, FL U.S.A. 33158, E-mail: cory.j.penca@usda.gov (C.J.P.)
*Corresponding author; E-mail: wee.yee@usda.gov (W.L.Y.)

2019). Because of this, immature stages of *R. indifferens* in cherries are probably not tolerant of being submerged in water, but how long they can tolerate submersion before 100% mortality is unknown. Determining this parameter could help develop water submersion of fruit as a viable option for cherry orchard sanitation.

The objective of this study was to determine the mortality of immature stages of *R. indifferens* in cherries submerged in hypoxic water, with a major goal of determining if water submersion could be a viable method for disinfesting cherries.

Materials and Methods

CHERRY COLLECTION SITES

Unmanaged sweet cherry trees (~4–9 m tall and wide) were sampled in 2020, 2021, and 2022, with 1 tart cherry (*Prunus cerasus* L.) sampled in 2020, at 4 sites in central Washington (WA), U.S.A. The tart cherry sample was kept separate from sweet cherry samples for analysis. Trees (unknown cultivars other than 'Bing' sweet cherry) were located in backyards or on roadsides in Kennewick (Benton county; 46.2020889 °N, 119.3030750 °W; 265 masl), Ellensburg (Kittitas county; 47.0074750 °N, 120.4599361 °W; 485 masl), Cle Elum (47.1951139 °N, 120.9228306 °W; 579 masl), and Roslyn (47.2261028 °N, 121.0016333 °W; 708 masl). Kennewick and Ellensburg trees were sampled in 2022, and Cle Elum and Roslyn trees were sampled in all 3 yrs. In 2020, 2021, and 2022, cherries were collected from 12 (including 1 tart cherry), 11, and 25 trees, respectively, with 4 trees sampled across all 3 yrs. Cherries were placed in plastic tubs (inner dimensions: 12.7 cm high × 45.1 cm long × 7.6 cm wide; Cambro Manufacturing Co., Huntington Beach, California, U.S.A.) and transported to the United States Department of Agriculture, Agricultural Research Service (USDA-ARS), Temperate Tree Fruit and Vegetable Research Unit in Wapato, Washington, U.S.A. for testing.

TESTING PROCEDURE

The water submersion treatments tested using sweet cherries over the 3 yrs are shown in Table 1, which also indicates the numbers of sampled trees, tub replicates, sample dates, and numbers of control and treated cherries per tub. In 2020, 1 tart cherry was sampled, with 442 control fruit and 450, 461, and 431 fruit for 4-, 8-, and 12-d treatments, respectively. We considered a replicate to be a tub with cherries. Cherry submersion periods were 4, 8, or 12 d. At 23–26 °C, *R. indifferens* larvae hatch from eggs in 5–8 d, and the larval stage averages 11 d (Frick et al. 1954), the rationale for the 12-d treatment. Our goal was to identify which of the 3 duration treatments can result in no survivors out of 30,000 treated individual insects, which equates to a quarantine treatment efficacy of 99.99% at the 95% confidence level (Couey & Chew 1986; Schortemeyer et al. 2011).

Before tests in all 3 yrs, subsamples of 10–20 cherries from each tree were set aside for measuring cherry diameter, weight, firmness (3-mm probe; FDP 1000, QA Supplies, Norfolk, Virginia, U.S.A.), and sugar concentration (Brix) (refractometer, Atago N1, Tokyo, Japan). In addition, subsamples of cherries from each tree were preserved in 70% ethanol to count unhatched eggs and larvae present pre-treatment (methods described in Yee 2005) (2020: 30–215 cherries/tree [1,248 total]; 2021: 14–55 cherries/tree [348 total]; 2022: 20 or 56 cherries/tree [536 total]).

Testing was conducted outdoors in the shade below roof eaves at the USDA-ARS facility. In 2020, a control and all 3 treatments were taken from each tree sample, with 1 or 2 replicate tubs for each. In 2021, each tree sample was split into 1 control and 1 treatment replicate tub. In 2022, each tree sample was split into 1 to 12 replicate tubs for the treatment and in 1 or 2 tubs for the control. The variability in tubs per tree in 2020 and 2022 was due to smaller trees having many fewer cherries than larger trees. Fruit from these larger trees were collected in high numbers and thus yielded more replicate tubs. This maximized work efficiency and helped us reach our target of 30,000 killed insects. It was not practical for us to sample more trees to reach our goal than we did, given labor and time constraints.

Control cherries were laid on a screen basket (6.4 cm high × 39.0 cm long × 30.5 cm wide) made of hardware cloth (5.6 mm square openings) placed in a plastic tub (same type as used for collection cherries, described in first paragraph of Materials and Methods). A hardware cloth screen was placed over each control tub and weighted down with a 2.1 kg (5.6 cm high × 19.2 cm long × 9.0 cm wide) concrete paver to protect against disturbance by birds and other animals.

Treatment cherries were placed on the bottom of tubs and covered with an inverted screen basket, which prevented cherries from floating to the surface. Cherries were completely submerged with ~11 liters of 19–22 °C tap water from the USDA-ARS facility. Each screen basket was weighted down with a concrete paver (inside a plastic bag to prevent possible leaching of materials) to ensure the basket and cherries remained below the water. Water that evaporated was replaced when needed, generally after 3 or 4 d.

During submersion, many larvae that had exited cherries were seen in the water. Thus, at the end of the treatment period, the water in each tub was poured through a sieve with 0.5 mm openings (U.S.A. standard testing sieve, W.S. Tyler Incorp., Ohio, U.S.A.) to catch the loose larvae. The larvae were placed on moist sand in covered Petri dishes and held for 1 d at 21–22 °C to determine if they were alive, as indicated by movement. Afterward, larvae were preserved in 70% ethanol and later measured to determine the instar under a microscope at 0.65× or 1.00× magnification.

Cherries remained whole (not decayed) after 4–12 d of water submersion and were removed from water and transferred onto a screen basket placed in another tub. A hardware cloth screen with a paver was placed over the basket to prevent disturbance of cherries by animals.

Table 1. Effects of water submersion duration on mortality of *Rhagoletis indifferens* immature stages in sweet cherry (*Prunus avium*) in 2020, 2021, and 2022. Ranges in numbers of control and water-treated cherries tested per replicate tub.

	Year 2020	Year 2021	Year 2022
Test Group	11 sampled trees[a]	11 sampled trees[b]	25 sampled trees[c]
Control - no water submersion	243–740 (13)	152–2,546 (11)	16–824 (34)
4-d water submersion	281–682 (13)	Not tested	Not tested
8-d water submersion	325–629 (13)	Not tested	47–1,476 (58)
12-d water submersion	355–708 (13)	55–4,324 (11)	Not tested

All cherries were collected in central Washington, U.S.A. Numbers of tubs equaling replicates are given inside parentheses; >one tub of cherries from some trees were set up in 2020 and 2022. [a]2020: fruit collected 13, 15, 20, 22, and 31 Jul in Cle Elum. [b]2021: fruit collected 13, 21, and 28 Jul in Cle Elum and Roslyn. [c]2022: fruit collected 21 and 23 Jun in Kennewick, 14 Jul in Ellensburg, 19, 21, and 26 Jul in Cle Elum, and 2, 5, and 9 Aug in Roslyn.

Control and treatment tubs were checked daily for larvae that exited cherries and dropped onto the bottom of tubs. All larvae or puparia present in tubs were counted until 30 d, as by 21 d most treated cherries had completely dried (most cherries dried earlier, by 14 d).

CONDITION OF WATER WITH SUBMERGED CHERRIES

To document that water for submerging cherries was hypoxic, the probe of a dissolved oxygen (DO) meter (Model D210, Extech® Instruments, Nashua, New Hampshire, U.S.A.) was inserted for 2 min into the bottom of a tub until readings stabilized. Tap water directly out of the faucet initially had 8 ppm oxygen, but within 5 min, oxygen in the stagnant water had dropped to 4 ppm. In all years, DO readings were made (outdoors) at 10:00 AM to 5:00 PM. In addition to DO readings made within 6 h of submersion, readings were made at 1–12 d after submersion (see Table 2 for more details).

In 2020, water temperatures were recorded continuously every hour using a waterproof Hobo datalogger (Onset Computer Corp., Bourne, Massachusetts, U.S.A.) placed on the bottom of 1 of each of 3 tubs with 4-, 8- and 12-d treatments. In 2021 and 2022, instantaneous temperatures were recorded at the same time that DO measurements were taken (previous paragraph). In addition, in 2022, to obtain continuous measurements as in 2020, a data logger was placed in a separate tub to record water temperatures from 21 Jun to 6 Jul to include temperature fluctuations missed using instantaneous readings.

In 2020 and 2021, sugar content in water (Brix) with submerged cherries was measured from 10 or 12 replicates per treatment and from 7 replicates, respectively. In 2022, Brix was measured from 1 sample at 8 d. The pH of the water in 1 sample at 8 and 12 d submersion was determined using pH paper (Hydrion®, Micro Essential Laboratory Inc., Brooklyn, New York, U.S.A.).

STATISTICAL ANALYSIS

A correlation between DO and water temperature (means per each of 14 replicates combining 2021 and 2022) was conducted to determine if higher temperatures of stagnant water are associated with reduced DO that could affect the results. To estimate mortality of immature stages, the number of larvae per control cherry from each tree sample was calculated, to account for differences in infestation in cherries among trees. Numbers of larvae per control cherry from a tree were multiplied by numbers of treated cherries from the same tree. The estimated total number of immature stages killed by a treatment was the sum of larvae from all tub replicates. Estimated total percent mortality with no survivors at the 95% confidence level was $(1–0.95)^{1/n}$ (Couey & Chew 1986), where n is the estimated number of treatment immature stages killed. The function 'PercentageToProbit' in the R package "ecotoxicology' (R Package Documentation 2019) was used to convert percentage mortality to a probit. Means ± SEM are presented where appropriate.

Results

CHERRY TRAITS AND EGG AND LARVAL ABUNDANCE IN CHERRIES BEFORE TESTING

Water submersion tests were conducted using ripe cherries containing eggs and all larval stages of *R. indifferens*. All tested sweet cherries were red to black and were ripe based on size, toughness, and Brix readings (Table 3). The predominant *R. indifferens* stage in sweet cherries was the egg, comprising 43.1%, 53.4%, and 62.3% of all stages in 2020, 2021, and 2022, respectively (Table 3). Third instars were the most abundant larval stage in 2020 (26.2%) and 2021 (18.8%), and second instars were most abundant in 2022 (17.6%). Cherries in the 1 tart cherry tree sample in 2020 had a mean diameter of 18.7 mm, toughness of 4.6, and Brix of 13.6%; there were 0.168 eggs, 0.032 1st instars, 0.011 2nd instars, and 0.011 3rd instars per tart cherry. Thus, the egg in the sample comprised 79.6% of all stages.

CONDITION OF WATER WITH SUBMERGED CHERRIES

At 0 h, water for submerging cherries contained ~4 ppm DO, but within 1–2 d, the DO had dropped to < 2 ppm and by days 4 and 12 it had dropped to < 1 ppm (Table 2). Thus, immature stages were in cherries submerged in hypoxic water (< 2 ppm) for nearly the entirety of the test periods. DO and water temperature readings were not correlated ($r = -0.4106$; $P = 0.1443$).

Water temperatures across years ranged from 12.2 °C at night to 30.6 °C during the day. Mean water temperatures in 2020 and 2021 were 21.8 ± 0.2 °C and 24.5 ± 0.4 °C, respectively. In 2022, mean water temperatures were 23.0 ± 1.3 °C (3 tubs) and 21.1 °C (separate tub). Slime formed on the water surface above submerged cherries in some

Table 2. Dissolved oxygen levels (ppm) in water in 4-, 8-, or 12-d treatments after cherries with *Rhagoletis indifferens* immature stages were submerged in water in 2020, 2021, and 2022 (mean ± SEM).

	Year 2020			Year 2021	Year 2022
Day after submersion	4 d	8 d	12 d	12 d	8 d
0[a]	3.41 ± 1.29	4.40 ± 0.31	4.46 ± 0.17	—	—
1	1.57 ± 0.59	1.74 ± 0.48	1.00 ± 0.19	3.73 ± 0.99	—
2	1.08 ± 0.48	1.43 ± 0.26	1.22 ± 0.27	0.96 ± 0.16	—
3	—	—	—	1.53 ± 0.38	1.70 ± 0.10
4	0.84 ± 0.32	0.84 ± 0.05	0.80 ± 0.03	1.00 ± 0.18	1.57 ± 0.09
5	—	—	—	0.60 ± 0.06	1.47 ± 0.07
6	—	0.83 ± 0.20	1.14 ± 0.44	1.03 ± 0.28	0.97 ± 0.27
7	—	0.93 ± 0.03	—	1.02 ± 0.15	0.50 ± 0.06
8	—	0.76 ± 0.05	1.61 ± 0.64	0.81 ± 0.14	—
9	—	—	—	0.92 ± 0.12	—
10	—	—	0.71 ± 0.08	0.67 ± 0.03	—
11	—	—	—	—	—
12	—	—	0.88 ± 0.13	1.22 ± 0.46	—

—no measurements taken. Means from 3 to 14 replicate tubs per day. [a]Taken within 6 h of submersion

Table 3. Pre-treatment data on sweet cherry (*Prunus avium*) traits and *Rhagoletis indifferens* eggs (unhatched) and larvae per cherry from subsamples taken from samples used for water submersion duration tests in 2020, 2021, and 2022 (mean ± SEM).

Trait	2020 (11 trees)	2021 (11 trees)	2022 (25 trees)[a]
Diameter (cm)	16.9 ± 1.1	18.4 ± 0.7	19.9 ± 0.8
Weight (g)	Not determined	3.9 ± 0.5	6.1 ± 0.5
Toughness (kg/cm^2)	5.5 ± 1.0	4.1 ± 0.2	6.8 ± 0.4
Brix (%)	20.3 ± 0.9	19.6 ± 0.9	19.6 ± 0.6
No. eggs and larvae per cherry at start of testing			
Stage	2020 (11 trees)	2021 (11 trees)	2022 (25 trees)
Egg	0.258 ± 0.056	0.533 ± 0.032	2.011 ± 0.180
1st instar (1–1.9 mm)	0.065 ± 0.015	0.123 ± 0.029	0.195 ± 0.036
2nd instar (2.0–4.9 mm)	0.119 ± 0.020	0.155 ± 0.049	0.563 ± 0.065
3rd instar (5–8 mm)	0.157 ± 0.030	0.188 ± 0.029	0.437 ± 0.071

[a]Cherries from 3 (from 5 Aug 2022) of 25 trees were not measured for Brix and toughness.

replicates beginning at 3 d during periods when air temperatures were high (~28–30 °C).

In 2020, mean Brix readings at the end of 4-, 8-, and 12-d treatments were 1.2 ± 0.2%, 2.2 ± 0.2%, and 1.7 ± 0.1%, respectively. In 2021, the mean Brix at 12 d was 2.2 ± 0.4%. In 2022, the Brix at 8 d was 1.7% (no SEM; 1 sample). The pH was 4–5 in 8- and 12-d treatments in 2022.

LARVAE RECOVERED LOOSE IN WATER AFTER TREATMENTS

Larvae found loose in water (on the bottom of tubs or water surface) at the end of 4-, 8-, and 12-d treatments were motionless. Of all larval stages recovered loose in water across the 3 yrs, 83.4–99.1%, 1.0–16.4%, and 0–0.7% were third, second, and first instars, respectively (out of 7,935 total larvae recovered), suggesting most first and second instars died in cherries without exiting the fruit. No larvae recovered from any water treatment were alive, as larvae were pale and bloated and remained motionless when placed on dry sand 1 d after removal from water, eventually turning black within a few days.

MORTALITY OF IMMATURE STAGES AFTER WATER TREATMENTS

Of the 3 treatment durations tested, only the 4-d treatment of tart cherries (450 fruit) in 2020 produced larvae, which subsequently pupariated (within 1 d) on the bottom of the tub. The control tart cherry sample produced 314 larvae from 442 cherries. None of the sweet cherry samples submerged for 4 d produced puparia (Table 4), yielding an estimated 99.92588% mortality at the 95% confidence level and a probit of 8.17813 (for discussion of confidence levels and probits, see Finney 1947; Couey & Chew 1986; Follett & Hennessey 2007). In contrast to the 4-d treatment, tart cherry samples for 8-d (461 fruit) and 12-d (431 fruit) treatments did not produce puparia. For sweet cherries, combining 2020 and 2022 data, the 8-d treatment killed an estimated 48,689 immature stages with no survivors; when combining 2020 and 2021 data, the 12-d treatment killed an estimated 16,180 individuals with no survivors (Table 4). For the 8-d treatment at the 95% confidence level, estimated percent mortality was 99.99385%, equating to a probit of 8.8400. Estimated percent mortality in the 12-d treatment was 99.98148%, yielding a probit of 8.560393. Although no larvae emerged from 8- and 12-d treatments, control cherries produced most of their larvae when corresponding treatment cherries were submerged, with 90% of total control larvae produced 7–12 d after tests started. Larval emergence from controls ended by 15–19 d.

Discussion

Results indicate water submersion of infested ripe cherries for 8 d causes 100% mortality of immature *R. indifferens*, such that a 12-d submersion is not necessary for maximum mortality. A probit 9 level results in 99.9968% efficacy, which the United States Department of Agriculture has used for approving treatments as meeting quarantine security requirements for tephritid fruit flies (Couey & Chew 1986; Follett & Neven 2006). The probit 9 standard was derived from work that used a probit transformation to describe the relationship between a treatment and the mortality of the targeted pest (Baker 1939). At the 95% confidence level, probit-9 efficacy requires no survivors in a minimum of 93,616 (or 93,613 due to rounding error) insects tested (Couey

Table 4. Numbers of *Rhagoletis indifferens* larvae emerged from sweet cherries (*Prunus avium*) after water submersion for 4, 8, or 12 d in water and estimated numbers of immature stages killed by water submersion duration treatments with no survivors.

Test year	Total no. larvae emerged	Total no. Fruit	Mean no. larvae/control cherry ± SEM	Total no. larvae emerged	Total no. fruit	Estimated no. immature stages killed with no survivors[a]
	Control			4-d water submersion		
2020	3,978	5,989	0.7242 ± 0.1147	0	5,869	4,042
	Control			8-d water submersion		
2020		Same as for 4-d treatment		0	6,309	4,246
2022	16,622	10,404	1.7711 ± 0.1399	0	32,467	44,443
	Control			12-d water submersion		
2020		Same as for 4-d treatment		0	6,268	4,191
2021	6,661	9,785	0.5732 ± 0.0978	0	17,852	11,989

[a]Based on mean no. larvae/cherry for a tree multiplied by no. treated cherries from the same tree, then summing numbers from all replicates (replicates were samples in tubs from different or the same trees). The variable numbers of cherries per replicate resulted in the means being different than those obtained by dividing total no. larvae (second column) by total no. fruit (third column).

& Chew 1986). However, according to Follett & Neven (2006), Japan, Australia, and New Zealand accept a quarantine treatment efficacy of 99.99%, which is obtained by treating 29,956 insects with no survivors (Couey & Chew 1986) and that equates to probit 8.7190 (Schortemeyer et al. 2011). Thus, our probit of 8.8400 for the 8-d treatment indicates use of the water submersion method against *R. indifferens* meets the rigorous standard of quarantine security for major export markets.

It is possible that larvae hatched from eggs when inside submerged cherries and all first instars that subsequently emerged were killed, but this is a possibility we did not examine. With respect to larval stages killed, the greater number of later instars compared with first instars at pre-treatment suggests that high mortality of larvae occurred soon after larvae hatched from eggs before cherries were submerged, or that dead first instars went undetected in our counts (Yee 2005). Data also suggest the possibility that when larval densities inside cherries are high, larvae may cannibalize others, perhaps a strategy to prevent overpopulation inside fruit (Carrol 1986).

Dissolved oxygen in water decreased from ~4 ppm down to 0.6 ppm over the course of treatments, meeting the criterion for a hypoxic environment (Charlton 1980) that is lethal to insects (Hoback 2012; Hoback et al. 2000; Hoback & Stanley 2001; Woods & Lane 2016). *Rhagoletis indifferens* third instars that were found loose in water either died inside cherries or exited cherries and died after exposure to the hypoxic water. It is unclear if the environment inside the cherries themselves was hypoxic, as pome fruit contains irregular cavities between cells to act as air pathways (Verboven et al. 2008) that may increase oxygen levels. However, an oxygen equilibrium in fruit tissue with the surroundings may be established over time that causes the outer 2 mm of flesh, where eggs are embedded, to become hypoxic, possibly suffocating embryos. Also, external breathing holes in cherries created by feeding larvae for respiration (Frick et al. 1954) that lead to cavities around the cherry seeds would result in surrounding hypoxic water entering, likely contributing to the 100% larval kill.

Brix and pH were measured to address the possibility that they affect larvae after they exit the submerged cherries into the surrounding water. Low sugar levels in water would result in low osmotic pressure, which should not harm larvae; if pH values are ~7, pH may also not harm larvae. Eliminating these 2 factors as mortality agents would suggest low oxygen level is the major mortality factor. Water temperature, Brix, pH, or other factors may play roles in speed of kill, but values of these parameters may have to be extreme (e.g., water would need to be sustained at >30 °C, the maximum recorded in this study) to have a significant effect. The Brix values were low (versus the 20% of cherries, Table 2), so osmotic pressure was unlikely harmful for the immature stages. Cherries were submersed in water that became acidic (pH 4–5), but it was probably not acidic enough to influence results.

A small percentage of immature *R. indifferens* can survive inside tart cherries submerged in water for 4 d despite hypoxic conditions. Eggs were the predominant stage in the submerged tart cherries. It is possible that larvae hatched from eggs after 4 d in water, as larvae emerged 21 d post-removal from water. The 21 d is close to the 16–19 d from *R. indifferens* oviposition to pupal formation (Frick et al. 1954). The possibility that embryos inside eggs and larvae differ in their tolerance to water submersion would need study.

The water submersion method in cherry systems would meet quarantine requirements and could be applied as a post-harvest sanitation measure after insecticide sprays have stopped and unpicked cherries remain on trees (Steigmeyer 2009; Herrick 2012; Rothwell et al. 2016). These unpicked, unprotected cherries are subject to attack by flies in both sweet cherry by *R. indifferens* in the Pacific Northwest (Messina & Smith 2010) and tart cherry by *Rhagoletis cingulata* (Loew) in Michigan, U.S.A. (Lehnert 2010; Alston & Murray 2020), acting as a source of fly infestations in orchards the following season. Cherries left on trees postharvest could be picked and submerged in water rather than sprayed with contact or systemic insecticides (Fitzgerald 2005; Lehnert 2010).

The results in this study serve as a proof of concept that water submersion of fly-infested fruit could be one viable field sanitation method that may have application in guava, mango, and citrus orchards infested by flies. Orchard or field sanitation is the major and most accessible method for managing fruit flies in Africa (Niassy et al. 2022). Field sanitation methods also are practiced or have been researched in Hawaii, U.S.A., specifically (1) sequestering culled fruit in an augmentorium (tent-like structure), (2) burying fruit 0.46 m underground, (3) placing a screen between fruit and the ground (Klungness et al. 2005; Jang et al. 2007), and (4) bagging fruit collected off the ground (Piñero et al. 2009), and unspecific treatment of fallen citrus (Yang et al. 2013). Another sanitation method, proposed for reducing populations of *Drosophila suzukii* (Matsumura) in unpicked cherries at postharvest, is to shake trees so unpicked fruit fall to the ground that are then crushed using a golf cart (Rothwell et al. 2016).

Water submersion of fruit could potentially be added to this list of methods as one of several techniques in a sanitation program. Appealing aspects of water submersion include its low cost, the low physical effort needed for implementation, i.e., no digging to bury fruit is required, and lack of negative impacts on the environment as no plastics in the form of bags are needed for fruit disposal. As orchards should be near water sources, obtaining water may only be a minor obstacle in most cases for implementing the water submersion method. In summary, water submersion may be a simple and practical method that can be applied to a wide range of fruit in support of orchard sanitation for controlling fruit flies, a possibility that warrants further testing.

Acknowledgments

We thank Chey Temple, Peter Chapman, Erik Godwin, Alexander Rose, Quintic Jacob, and Mauricio Zamora (USDA-ARS) for technical and field assistance during this study as well as various homeowners for allowing us to use their trees for cherry collections. This work was supported in part by interagency agreements between USDA-APHIS PPQ S & T and USDA-ARS (General Terms & Condition nos. 0000266 and 0000310). Mention of trade names or commercial products in this publication is solely for providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity employer. The authors declare no conflict of interest.

References Cited

Alston D, Murray M. 2020. Western cherry fruit fly in Utah orchards. Utah State University Extension. Utah pests. https://extension.usu.edu/pests/research/western-cherry-fruit-fly (accessed 19 September 2023).

Baker AC. 1939. The basis for treatment of products where fruit flies are involved as a condition for entry into the United States. U.S. Department of Agriculture Circular No. 551.

Bhandari K, Timilsina G, Gautam G, Thakur MK. 2021. Effects of desiccation and immersion on larval and pupal survival of Chinese citrus fly (*Bactrocera minax*) (Diptera: Tephritidae). Nepalese Journal of Agricultural Sciences 21: 33–43.

Carrol JF. 1986. Caribbean fruit flies, *Anastrepha suspensa* (Loew) (Diptera: Tephritidae), reared from eggs to adults on cannibalistic diet. Proceedings of the Entomological Society of Washington 88: 253–256.

Charlton MN. 1980. Oxygen depletion in Lake Erie: has there been any change? Canadian Journal of Fisheries and Aquatic Sciences 37: 72–81.

Couey HM, Chew V. 1986. Confidence limits and sample size in quarantine research. Journal of Economic Entomology 79: 887–890.

Finney DJ. 1947. Probit Analysis: a Statistical Treatment of the Sigmoid Response Curve. Cambridge University Press, Cambridge, UK.

Fitzgerald T. 2005. Spray schedule for cherries. Washington State University Spokane County Extension. https://flathead.msuextension.org/documents/ag/yard-garden/Spray%20schedule%20for%20Cherries%20-%20WSU.pdf (accessed 19 September 2023).

Follett PA, Neven LG. 2006. Current trends in quarantine entomology. Annual Review of Entomology 51: 359–385.

Follett PA, Hennessey MK. 2007. Confidence limits and sample size for determining nonhost status of fruits and vegetables to tephritid fruit flies as a quarantine measure. Journal of Economic Entomology 100: 251–257.

Frick KE, Simkover HG, Telford HS. 1954. Bionomics of the cherry fruit flies in eastern Washington. Washington Agricultural Experiment Stations Institute of Agricultural Sciences State College of Washington. Technical Bulletin 13: 1–66.

Harrison JF, Greenlee KJ, Verberk WCEP. 2018. Functional hypoxia in insects: definition, assessment, and consequences for physiology, ecology, and evolution. Annual Review of Entomology 63: 303–325.

Herrick C. 2012. Many Washington cherries left unpicked. Growing Produce. https://www.growingproduce.com/fruits/stone-fruit/many-washington-cherries-left-unpicked/ (accessed 18 September 2023).

Hoback WW. 2012. Ecological and experimental exposure of insects to anoxia reveals surprising tolerance, pp. 167–188 *In* Altenbach AV, Bernhard JM, Seckbach J (eds.), Anoxia: Evidence for Eukaryote Survival and Paleontological Strategies. Cellular Origin, Life in Extreme Habitats and Astrobiology 23. Springer Science and Business Media, Berlin, Germany.

Hoback WW, Podrabsky JE, Higley LG, Stanley DW, Hand SC. 2000. Anoxia tolerance of con-familial tiger beetle larvae is associated with differences in energy flow and anaerobiosis. Journal of Comparative Physiology B 170: 307–314.

Hoback WW, Stanley DW. 2001. Insects in hypoxia. Journal of Insect Physiology 47: 533–542.

Jang EB, Klungness LM, McQuate GT. 2007. Extension of the use of augmentoria for sanitation in a cropping system susceptible to the alien tephritid fruit flies (Diptera: Tephritidae) in Hawaii. Journal of Applied Sciences and Environmental Management 11: 239–248.

Klungness LM, Jang E, Mau RFL, Vargas R, Sugano J, Fujitani E. 2005. New sanitation techniques for controlling tephritid fruit flies (Diptera: Tephritidae) in Hawaii. Journal of Applied Sciences and Environmental Management 9: 5–14.

Lehnert R. 2010. Control cherry fruit fly after harvest. Good Fruit Grower. 1 March 2010 Issue. https://www.goodfruit.com/control-cherry-fruit-fly-after-harvest/ (accessed 18 September 2023).

Li Z, Chambi C, Du T, Huang C, Wang F, Zhang G, Li C, Kayeke MJ. 2019. Effects of water immersion and soil moisture content on larval and pupal survival of *Bactrocera minax* (Diptera: Tephritidae). Insects 10: 138. DOI: 10.3390/insects10050138

Liquido N. 1993. Reduction of oriental fruitfly (Diptera: Tephritidae) populations in papaya orchards by field sanitation. Journal of Agricultural Entomology 10: 163–170.

Messina FJ, Smith TJ. 2010. Western cherry fruit fly. *Rhagoletis indifferens* Curran (Diptera: Tephritidae). Washington State University http://treefruit.wsu.edu/crop-protection/opm/western-cherry-fruit-fly/ (accessed 18 September 2023).

Niassy S, Murithii B, Omuse ER, Kimathi E, Tonnang H, Mohamed SNS, Ekesi S. 2022. Insight on fruit fly IPM technology uptake and barriers to scaling in Africa. Sustainability 14: 2954. DOI: 10.3390/su14052954

Piñero JC, Mau RFL, Vargas RI. 2009. Managing oriental fruit fly (Diptera: Tephritidae), with spinosad-based protein bait sprays and sanitation in papaya orchards in Hawaii. Journal of Economic Entomology 102: 1123–1132.

R Package Documentation. 2019. PercentageToProbit: convert percentages to probit values. In ecotoxicology: methods for ecotoxicology. https://rdrr.io/cran/ecotoxicology/man/PercentageToProbit.html (accessed 2 September 2022).

Rothwell N, Pochubay E, Powers K. 2016. How to manage unharvested or diverted cherries. Michigan State University Extension https://www.canr.msu.edu/news/how_to_manage_unharvested_or_diverted_cherries (accessed 18 September 2023).

Schortemeyer M, Thomas K, Haack RA, Uzunovic A, Hoover K, Simpson JA, Grgurinovic CA. 2011. Appropriateness of probit-9 in the development of quarantine treatments for timber and timber commodities. Journal of Economic Entomology 104: 717–731.

Steigmeyer R. 2009. Too many cherries at once causes trees to go unpicked. The Wenatchee World. https://www.wenatcheeworld.com/news/local/too-many-cherries-at-once-causes-trees-to-go-unpicked/article_ae44ae09-d4f0-53a0-9329-d4ae4580b447.html (accessed 4 November 2022).

Verboven P, Kerckhofs G, Mebatsion HK, Ho QT, Temst K, Wevers M, Cloetens P, Nicolaï BM. 2008. Three-dimensional gas exchange pathways in pome fruit characterized by synchrotron X-ray computed tomography. Plant Physiology 147: 518–527.

Wakie TT, Yee WL, Neven LG, Kumar S. 2019. Modeling the abundance of two *Rhagoletis* fly (Diptera: Tephritidae) pests in Washington State, U.S.A. PLoS ONE 14: e0217071 DOI: 10.1371/journal.pone.0217071

Woods HA, Lane SJ. 2016. Metabolic recovery from drowning by insect pupae. Journal of Experimental Biology 219: 3126–3136.

Xie Q, Zhang R. 2007. Responses of oriental fruit fly (Diptera: Tephritidae) third instars to desiccation and immersion. Journal of Agricultural and Urban Entomology 24: 1–11.

Yang WS, Li CR, Lan J, An HL. 2013. The spread mode and dispersal history of Chinese citrus fly by literature research. Journal of Southern Yangtze University (Natural Science Edition) 10: 8–11.

Yee WL. 2005. Seasonal distributions of eggs and larvae of *Rhagoletis indifferens* (Diptera: Tephritidae) in cherries. Journal of Entomological Science 40: 158–166.

Yee WL. 2021. Tolerances of apple maggot (Diptera: Tephritidae) larvae and different age puparia to water flotation and immersion. Environmental Entomology 50: 1407–1415.