

Hot Water Immersion to Ensure Quarantine Security for *Cryptophlebia* spp. (Lepidoptera: Tortricidae) in Lychee and Longan Exported from Hawaii

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J. Econ. Entomol. 94(5): 1292-1295 (2001)

ABSTRACT We determined whether immersion in 49°C water for 20 min, a quarantine treatment developed for disinfestation of fruit flies in lychee, *Litchi chinensis* Sonn., and longan, *Dimocarpus longan* (Lour.) Steud., exported from Hawaii, would also disinfest fruit of two species of *Cryptophlebia*. The pattern of tolerance to heat in *Cryptophlebia illepidata* (Butler) was generally eggs < neonates < early instars = late instars < pupae. No *C. illepidata* survived immersion for 16 or 20 min. Late fourth and fifth instars were determined to be the most tolerant stage that occurs in harvested fruit. Late instars of *Cryptophlebia ombrodelta* (Lower) were more tolerant of hot-water immersion than those of *C. illepidata*, but no *C. ombrodelta* late instars survived immersion for 16 or 20 min. The hot water immersion quarantine treatment for fruit flies should effectively disinfest lychees and longans of any *Cryptophlebia*.

KEY WORDS *Cryptophlebia*, quarantine pest, heat treatment, disinfestations

HOT WATER IMMERSION at 49°C for 20 min is approved by the U.S. Department of Agriculture, Animal Plant Health Inspection Service, as a quarantine treatment for Hawaii's tephritid fruit fly pests attacking lychee, *Litchi chinensis* Sonn., and longan, *Dimocarpus longan* (Lour.) Steud. (Federal Register 1997). In addition to fruit flies, two species of *Cryptophlebia* are also pests of quarantine concern in sapindaceous fruits (lychee, longan, and rambutan) exported from Hawaii. *Cryptophlebia illepidata* (Butler), the koa seedworm, is endemic to Hawaii, and *C. ombrodelta* (Lower), the litchi fruit moth, is native to Australia. *Cryptophlebia* spp. are the most commonly intercepted insect pests in sapindaceous fruits from Hawaii carried in passenger baggage and arriving in the mail on the U.S. mainland (USDA-APHIS-PPQ 1997).

Cryptophlebia spp. lay eggs singly on the fruit surface, and neonates bore through the skin and feed at the skin-pulp interface. Although many eggs may be laid on the fruit surface, typically only one larva is found feeding in a fruit. *Cryptophlebia* spp. are potentially multivoltine in Hawaii (Jones et al. 1997). Laboratory tests and field surveys in Hawaii indicate that lychee and longan are poor hosts for *Cryptophlebia* (McQuate et al. 2000; P.A.F., unpublished data). Actual damage from larval feeding is often minimal, and larval survival to pupation is rare when fruit are on the tree. However, when a neonate enters the fruit at the calyx or stem end, it may bore through woody tissue to the seed and feed there, improving the chance for survival to pupation. Successful larval development in fruit also may improve once fruit are harvested (P.A.F., unpublished data). In a study of >36,000 mature fruits of six varieties of lychee and 9,700 fruit of

four varieties of longan harvested from orchards in Hawaii over a two-year period, *Cryptophlebia* infestation rates were 1.5 and 0.14%, respectively (G. T. McQuate, USDA, Hilo, HI, personal communication).

Current regulations for hot-water immersion quarantine treatment of Hawaii-grown lychee stipulate that exported fruits must be found free of *Cryptophlebia* spp. (Federal Register 1997). Thus, the presence of *Cryptophlebia* can prevent export of lychee from Hawaii. The quarantine treatment used for lychee has been proposed for longan also exported from Hawaii (P.A.F., unpublished data). If the accepted hot-water immersion quarantine treatment for pest fruit flies is also effective against *C. illepidata* and *C. ombrodelta*, these species could be added to the regulation, preventing potential interruption of shipments because of the presence of these insects. The objectives of the studies with *Cryptophlebia* reported here were to examine the effectiveness of the hot-water immersion treatment against various stages of *Cryptophlebia*, and thereby determine if the approved quarantine treatment provides quarantine security against these pests.

Materials and Methods

Insect Rearing. A colony of *C. illepidata* was started using larvae ($\approx 1,000$) collected from macadamia nuts from an orchard in Pahala, HI. Because of difficulties collecting *C. ombrodelta* in the field, *C. ombrodelta* pupae (108) were obtained from a laboratory colony maintained at the Maroochy Experiment Station in Queensland, Australia, to start a colony; the Maroochy colony had been maintained in the laboratory for 3 yr and wild moths from infested macadamia were intro-

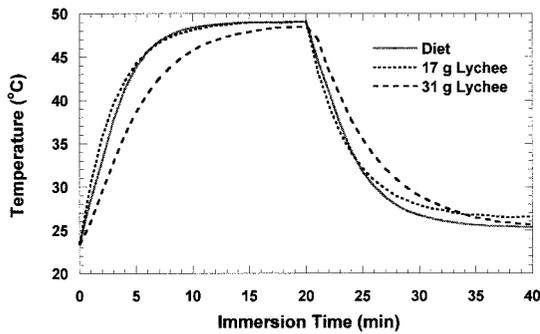


Fig. 1. Temperature profiles of diet in vials and of lychee fruit during hot water immersion at 49°C for 20 min followed by immersion in ambient temperature water for 20 min.

duced annually to maintain genetic variation. For both species, adults were placed in 3.8-liter plastic jars with ventilated lids for mating and provided with a 5% honey water solution through a wick. Eggs were laid on the inside surface of the jar. Emerging neonates were transferred daily to 29-ml diet cups with laboratory diet. The laboratory diet was modified from Sinclair (1974) as described in Follett and Lower (2000). As larvae approached pupation, diet cups (with larvae) with lids removed were placed in 3.8-liter plastic tubs (Rubbermaid, Wooster, OH) with 30 g of sand covering the bottoms to a depth of 0.25 cm. *C. illepidata* larvae typically leave the diet trays to pupate in the sand, whereas *C. ombrodelta* typically pupate in the diet. Emerging moths were cooled for handling and transferred to the plastic mating jars. Rearing conditions were 25 ± 2°C and a photoperiod of 10:14 (L:D) h for the duration of the experiments. For stage-specific tests, larvae were placed into categories (L1, L2/3, and L4/5 corresponding to instars) based on size and known developmental rates (unpublished data).

Hot Water Immersion Treatment. *Cryptophlebia* are difficult to rear on fresh lychee and longan; therefore, all heat treatments were applied to *Cryptophlebia* in 29-ml plastic cups (eggs) or 25-ml glass vials containing 10 g of artificial diet (larvae and pupae). Direct exposure to hot water of eggs attached to the inner surface of cups is similar to exposure on the surface of fruit. The amount of diet required in vials was determined by comparing thermal diffusion profiles of different amounts of diet with that of lychee fruit. Vial

cups had Teflon-silicone septa to permit insertion of temperature probes. All hot-water immersion treatments were done in a 75-liter circulating bath heated by two electric heaters (immersion circulator model 73, PolyScience, Preston Industries, Niles, IL) to 49 ± 0.2°C. Bath temperature was verified before and after each run using a mercury thermometer with 0.1°C gradations. During treatment, temperature was monitored in eight vials at the surface and center of the diet, and at the inside wall of the glass vial above the diet, at 30-s intervals using temperature probes and a data logger (model 5160-64-A, Omni International, Logan, UT) calibrated to a mercury thermometer. Vials with individual larvae or pairs of pupae were placed in wire baskets (1 cm mesh) and submersed to the bottom of the tank for the desired treatment time. For larval treatment, baskets with vials were tapped on a table to dislodge insects from the wall of the glass vial onto the diet surface before submersion. For egg treatment, gravid females were confined to 29-ml plastic cups overnight, and open cups with eggs were placed in wire cages and treated as above (i.e., eggs were in direct contact with the water.) Regardless of treatment time, the basket with vials or cups and insects was transferred to a 75-liter ambient-temperature (~22–24°C) cooling bath for 20 min after heat treatment. (Although not part of the official treatment, a 20-min cooling bath is recommended after hot water immersion of lychee fruits.) Larval mortality was measured after 24 h by prodding larvae with a camels-hair brush for signs of any movement. Pupae were held after treatment in 29-ml plastic cups at 25°C for 2 wk for adult emergence. Treated eggs attached to plastic cups were held for 1 wk at 25°C for neonate emergence.

The two-year survey mentioned earlier that examined the fruit pests in lychee and longan in Hawaii showed mean fruit weights of 17.2 g (SD = 4.8, n = 36,351) and 9.4 g (SD = 2.3, n = 9,700), respectively (G. T. McQuate, personal communication). In preliminary studies, the heating rate at the seed surface of lychee fruit of typical size (17–19 g) ranged from 3.1 to 3.3°C min⁻¹ during the first 7 min of the treatment (the time period when the rate of heating is the greatest). Diet was heated so that the thermal profile at the surface of the diet in vials mimicked the profile for the seed surface of a typical lychee fruit (Fig. 1). The heating rate at the diet surface in our tests averaged 3.3°C min⁻¹ during the first 7 min after immersion. The

Table 1. Mean ± SEM percentage mortality of *C. illepidata* after immersion in water at 49°C

Immersion time, min	Mortality ^a											
	Eggs (1 day old)		Eggs (3 d old)		L1		L2/3		L4/5		Pupae	
0	586	13.7 ± 3.8	231	19.8 ± 9.1	97	13.8 ± 5.2	94	0.0 ± 0.0	111	1.0 ± 1.0	118	5.5 ± 3.1
4	585	100.0 ± 0.0	569	100.0 ± 0.0	157	68.2 ± 4.8	116	16.2 ± 5.7	123	4.2 ± 1.8	162	17.4 ± 2.3
8	624	100.0 ± 0.0	466	100.0 ± 0.0	152	97.4 ± 2.0	114	51.4 ± 12.5	124	35.6 ± 6.2	158	16.1 ± 4.5
12	686	100.0 ± 0.0	452	100.0 ± 0.0	137	100.0 ± 0.0	115	96.7 ± 3.3	121	98.3 ± 1.7	160	95.3 ± 3.0
16	522	100.0 ± 0.0	426	100.0 ± 0.0	121	100.0 ± 0.0	101	100.0 ± 0.0	106	100.0 ± 0.0	155	100.0 ± 0.0
20	698	100.0 ± 0.0	378	100.0 ± 0.0	114	100.0 ± 0.0	78	100.0 ± 0.0	99	100.0 ± 0.0	149	100.0 ± 0.0

^a Mortality of each life stage is preceded by the number of test subjects. Larval stages: L1, neonates; L2/3, early instars; L4/5, late instars.

Table 2. Linear regressions of percentage mortality against immersion time for *C. illepidia* larvae immersed in 49°C water

Stage ^a	n	Intercept ± SE	Slope ± SE	R ²	Predicted time (95% CL) for 100% mortality, min
L1	446	49.8 ± 19.2	4.6 ± 2.2	0.81	10.9 ^b
L2/3	446	-8.1 ± 17.1	7.4 ± 1.6	0.92	14.6 (10.5 - 59.8)
L4/5	474	-28.0 ± 22.0	8.8 ± 2.0	0.90	14.6 (10.2 - 400.1)
Pupae	635	-31.9 ± 31.9	8.7 ± 2.9	0.82	15.2 ^b

Linear regression, $y = b + mx$, where b is the intercept, m is the slope, x is immersion time (min), and y is percentage mortality.

^a Larval stages are L1 = neonates, L2/3 = early instars, L4/5 = late instars.

^b 95% CL not calculable because of a poor fit.

heating rate of a particularly large (31 g) lychee fruit was 2.7°C min⁻¹ during the first 7 min of the treatment (Fig. 1).

All hot water immersion tests used laboratory-reared *Cryptophlebia*. *C. illepidia* eggs (1 d old, 3 d old), larvae (neonates, second-third instars, and fourth-fifth instars), and pupae (7–10 d old) were treated at 49°C for 0, 4, 8, 12, 16, or 20 min in a factorial design, and percentage mortality was determined. Although the pupal stage of *Cryptophlebia* is not found in fruits, we tested the effects of heat against this stage for the purpose of comparison. Pupal development to the adult stage is 11 d under our rearing conditions (25 ± 2°C). For each egg, larval, or pupal age-stage, four to six replicates were treated on different dates, and a control group in each replicate was handled in the same way as treated groups except they were immersed in ambient rather than heated water. The number of insects per replicate was ≈25 larvae and pupae and 100 eggs.

To determine whether *C. illepidia* or *C. ombrodelta* was more heat tolerant, late (fourth and fifth) instars of each species were treated as above for 0, 8, 10, 12, 16, and 20 min at 49°C, and percentage mortality was determined. Each treatment was replicated four to seven times, and a control group in each replicate was handled in the same way as treated groups except they were immersed only in ambient rather than heated water. Extensive confirmatory testing of late instars in glass vials treated at 49°C for 20 min as above was conducted for both species.

Data Analysis. Simple linear regressions were done on untransformed data from studies of life stage and species-specific tolerance of hot water immersion (SAS Institute 2000). For each life stage, data used in the linear regression model included any hot-water immersion dose causing mortality between 0 and 100%, and the lowest dose causing 100% mortality. Mortality values <100% were adjusted for control mortality using Abbott's formula (Abbott 1925). After comparison with probit and logit models, linear re-

gression was used because of the limited number of doses tested and the better fit to the data (Jandel Scientific 1994, Robertson et al. 1994). Untransformed data from the comparative study between *C. illepidia* and *C. ombrodelta* were subjected to analysis of variance (ANOVA), and means were separated using a *t*-test (SAS Institute 2000). Data from the comparative study were fitted to a logistic rather than linear model because of the better fit to the data (Jandel Scientific 1994). For all models, predicted time to 100% mortality was calculated to estimate the time required at 49°C to completely eliminate live insects from fruit.

Results

None of the 1-d-old or 3-d-old *C. illepidia* eggs treated at 49°C for ≥4 min survived (Table 1). Control mortality was 13.7 ± 3.8% (mean + SEM) and 19.8 ± 9.1% in 1-d-old and 3-d-old eggs, respectively. Neonate mortality was 97.4 ± 2.0% after 8 min, and no individuals survived immersion for ≥12 min. Mortality of early instars (L2/3) was 96.7 ± 3.3% after 12 min, and no individuals survived immersion for 16 or 20 min. Mortality of late instars (L4/5) was lower for the 4 and 8 min treatments than for L2/3, but similar at 12 min; none survived treatment for 16 or 20 min (Table 1). The pupal stage had the lowest mortality at 8 min. (16.1 ± 4.5%) among the life stages. However, pupal mortality was high at 12 min (95.3 ± 3.0%), and no pupae survived immersion for 16 or 20 min. Control mortality for L1, L2/3, L4/5, and pupae was 13.8, 0.0, 1.0, and 5.5%, respectively. The predicted immersion time at 49°C to achieve 100% mortality ranged from 10.9 min for L1 to 15.2 min for pupae (Table 2).

Fourth- and fifth-instar larvae were chosen for the species tolerance comparison tests because of their higher survival at 4 and 8 min, relative to L2/3s, in the dose-response tests. *C. ombrodelta* fourth-fifth instars were more tolerant of hot water immersion compared with those of *C. illepidia* (Table 3). Mean percentage mortality was higher for *C. illepidia* compared with

Table 3. Mean ± SEM percentage mortality of late instar *C. illepidia* and *C. ombrodelta* immersed in 49°C water

Species	Immersion time, min					
	0	8	10	12	16	20
<i>C. illepidia</i>	0.2 + 0.2a	35.4 + 8.2a	49.1 + 8.5a	99.5 + 0.3a	100.0 + 0.0a	100.0 + 0.0a
<i>C. ombrodelta</i>	0.5 + 0.3a	28.6 + 6.7a	25.0 + 4.4a	74.0 + 7.9b	100.0 + 0.0a	100.0 + 0.0a

Means ± SEM within the same column followed by the same letter are not significantly different ($P = 0.05$) by *t*-test.

Table 4. Logistic analysis of percentage mortality against immersion time of late-instar *C. illepipa* and *C. ombrodelta* immersed in 49°C water

Species	n	Model parameters (\pm SE)			R^2	Predicted time (95% CL) for 100% mortality, min
		a	b	c		
<i>C. illepipa</i>	1,539	109.9 \pm 32.0	9.6 \pm 1.5	-5.8 \pm 4.8	0.91	14.7 (11.8–232.2)
<i>C. ombrodelta</i>	1,510	130.2 \pm 109.8	12.0 \pm 5.7	-4.4 \pm 4.9	0.99	15.7 (14.8–16.9)

Logistic dose response model, $y = a/[1 + (x/b)^c]$.

C. ombrodelta after immersion for 8, 10, and 12 min at 49°C, but differences were significant only for 12 min ($t = 3.2$, $df = 10$, $P = 0.01$); no individuals of either species survived immersion for 16 or 20 min. The predicted time to 100% mortality was 14.7 min for *C. illepipa* and 15.7 min for *C. ombrodelta* using the logistic model (Table 4).

Extensive testing of late instars confirmed the efficacy of the approved fruit fly quarantine treatment against *Cryptophlebia*. None of the 4,434 late-instar *C. ombrodelta* and 4,546 *C. illepipa* survived immersion in 49°C water for 20 min.

Discussion

No *C. illepipa* and *C. ombrodelta* immersed for 16 or 20 min in 49°C water survived in any of our tests. Therefore, the hot-water immersion quarantine treatment for Hawaii's lychees and longans should effectively disinfest fruit of any *Cryptophlebia*.

Other heat treatments (heated air or water) have been approved by USDA-APHIS for internal and external pests on various commodities exported to the mainland United States (USDA-APHIS-PPQ 1998). The only other hot water immersion treatment approved for internal pests is for fruit flies in mangoes. The hot-water immersion treatment for lychee is unique in that it does not require measurement of fruit temperature during treatment (unlike all heated air treatments), and does not specify any treatment adjustments for different fruit sizes (unlike the hot-water immersion treatment for mangoes). In our study, treating larvae in vials with diet was not a perfect representation of a natural situation with insects in fruit, but was necessary because of the poor host status of lychee for *Cryptophlebia*. Mortality of larvae in lychee fruit will be a function of the size of the fruit and the location of the larvae in the fruit. Survival of larvae may be higher than predicted when the heating rate during treatment is slower, as would be the case with larvae feeding near the seed in large fruit. However, treatment at 49°C for 20 min provides a substantial level of overkill and therefore should be effective over a wide range of lychee fruit sizes. Longan is a smaller fruit than lychee, so we assume that heat penetration will be more rapid and larvae will be more susceptible to heat treatment in longan than in lychee.

Acknowledgments

We thank Geoffrey Waite at the Maroochy Experiment Station in Queensland, Australia, for graciously providing *C. ombrodelta* to start our colony; Alan Yamaguchi of Kau Agribusiness for pointing us to a heavily infested macadamia orchard in Pahala, HI, to collect *C. illepipa*; John Brown, USDA Systematic Entomology Laboratory, Beltsville, MD, for confirming *Cryptophlebia* species identifications; and Bob Lower, Zona Gabbard, Shannon DeLuz, Camille Mehau, Chris Johnson, and Malia Laber for assisting with maintenance of the *Cryptophlebia* colonies. Permission to import *C. ombrodelta* was granted by the Hawaii Department of Agriculture (Permit No. 99-11-h-123) and USDA-APHIS (Permit No. 9398878).

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Received for publication 14 December 2000; accepted 24 March 2001.